The Erythrocyte Ghost Is a Perfect Osmometer

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ABSTRACT The osmotic swelling of intact erythrocytes in hypotonic solutions was measured using microhematocrit tubes, Van Allen tubes, and a calibrated Coulter counter. In agreement with earlier workers the intact cells did not behave as perfect osmometers, the cells swelling less than predicted by the Boyle-van't Hoff law. Erythrocyte ghosts were prepared from fresh intact erythrocytes by one-step hemolysis in 0.25% NaCl at an extremely dilute concentration of cells and the membranes were sealed at 37°. The ghosts were mixed with NaCl solutions of different osmolarities and the MCV (mean cell volume) of the shrunken cells immediately monitored by a calibrated Coulter counter. It was found that the MCV values of the shrunken ghosts were accurately predicted by the Boyle-van't Hoff law. These results indicate that these erythrocyte ghosts behaved as perfect osmometers.

INTRODUCTION

The work presented in this paper shows that erythrocyte ghosts, freshly prepared and with membranes sealed at 37°C, behave as perfect osmometers with semipermeable membranes. Many reports have confirmed the finding that intact erythrocytes do not behave as perfect osmometers (1–7). The change in cell volume in response to variations in the solute concentration of the suspending medium is consistently smaller than that which would be expected from the Boyle-van't Hoff law. In an attempt to explain this discrepancy or deviation various authors have considered the following factors. (*a*) Leakage of ions from the cells to the hypotonic medium or from the hypertonic medium into the cells (1). (*b*) A rigidity of the cell membrane resisting changes in volume (1). (*c*) The concentration dependency of the osmotic coefficient of hemoglobin (8). (*d*) Relative immobilization of water of solvation to the intracellular proteins (5). (*e*) Changes in the net charge of the hemoglobin molecule as a function of its concentration (6).

Since the first two possibilities localize the cause for the anomalous behavior in the membrane, it is of interest to know to what extent the erythrocyte ghost behaves as an osmometer.

The results of earlier studies on the osmotic behavior of the erythrocyte ghost have been inconclusive, if not conflicting. Teorell (9) found that it was

only possible to predict the osmotic shrinking of the ghosts if he assumed that there was a fixed pressure inside the ghosts of about 1600 cm H_2O (equivalent to a 32 mM NaCl solution).

The ghosts used by Weed et al. (10) were also far from perfect osmometers; from their data it can be calculated (see Methods in this present paper) that it is only possible to predict the shrinking of Weed's ghosts if one assumes an osmotically inactive volume of approximately 11 μ^3. For volumes larger than 80 μ^3, this value becomes even larger. Apparently the ghosts used by Weed et al. either lost ions or the membrane offered considerable resistance to bending.

Struve (11) showed that it was possible to get a straight line relationship between volume and the reciprocal of the osmotic pressure with ghosts shown to be permeable to the osmotically active solute. He did not, however, correlate quantitatively the leakiness and the remaining osmotic responsiveness; that is, no attempt was made to predict precisely the amount of shrinking that should occur in solutions of given osmolarities, aside from the fact that linear shrinking was observed.

The studies of Teorell and of Weed et al., therefore, indicate that the erythrocyte ghost shrinks predictably only if one accepts a high intracellular or a high inactive volume. There are two types of procedural difficulties that these workers had to deal with. The first was that there was a considerable time interval between the time of addition of the hypertonic solution and the time of measurement of the shrunken cells; considerable leakage of ions could have taken place across the membrane during this interval. The second difficulty is that the intracellular ionic contents were not known or measured; the ghosts had been washed repeatedly with different solutions and there was no reason to believe that the ghosts had the same intracellular composition as the stock medium in which they were suspended.

These two difficulties are minimized in the series of experiments to be reported in this paper. The time interval between shrinkage and measurement of cell size has been reduced down to fractions of a minute, and, second, the cells were hemolyzed in one step without any washing of the ghosts. The ghosts, therefore, were assumed to have the intracellular composition of the hemolyzing medium.

The results in this paper show that erythrocyte ghosts, freshly prepared by one-step hemolysis and with membranes resealed at 37°C, behave quantitatively as perfect osmometers. Intracellular pressures, of the size postulated by Teorell, could be excluded. The results indicate that the perfect osmotic or semipermeable properties of the ghost membrane cannot account for the imperfect osmotic responses of the intact cell. This conclusion is compatible with the work of Gary-Bobo and Solomon (6) who have attributed the ap-

parent anomalous osmotic behavior of intact erythrocytes to cooperative interactions between hemoglobin molecules.

METHODS

Preparation of the Stock Erythrocyte Suspension

Venous blood from a fasting volunteer was heparinized with 50 units per ml of whole blood and centrifuged at 1500 × *g* for 15 min. The plasma and buffy coat were removed and the cells were resuspended in a 0.9% NaCl solution in 10 mM sodium phosphate buffer, pH 7, to a final hematocrit of approximately 40%. With the use of the same 0.9% NaCl solution the cells were then diluted 1:1250 (v/v).

Preparation of the Stock Ghost Suspension

Ghosts were prepared by one-step hemolysis as follows. An aliquot of 0.5 ml of the suspension of intact erythrocytes was added to 10 ml of 0.222% NaCl in 10 mM sodium phosphate buffer, pH 7; the final concentration of NaCl, therefore, was 0.25%. Larger quantities of ghosts were obtained by adding 7.5 ml of the suspension of intact erythrocytes to a volume of 150 ml of 0.222% NaCl which was being stirred vigorously on a magnetic stirrer. The ghosts were allowed to seal for at least 2 hr at 37°C (12). This time is sufficient for the cells to reswell to perfect spheres (13–16).

Procedure to Determine the Osmotic Response of Ghosts

Sodium chloride solutions of different concentrations were prepared in 10 mM sodium phosphate buffer and were kept at 37°C. Equal volumes (5 ml) of the ghost suspension and these NaCl solutions were mixed in disposable Coulter counter vials and the mean cell volume of this cell population was determined immediately, using a Coulter counter (Model F) with mean cell volume computer (Coulter Electronics, Hialeah, Fla.). The Coulter probe had an aperture of 100 μ in diameter, the aperture setting was 16, the attenuation was 0.707, the threshold setting on both the counter and computer was 10 units, and the sample volume was 0.5 ml. The time taken by the Coulter counter to pull 0.5 ml through the 100 μ aperture was 14 sec; the MCV computer required only 7 sec for one determination. Approximately 20,000 cells were averaged during these 7 sec while 40,000 cells were counted over the entire 14 sec period. Further details of this recording system, as well as the calibration of the MCV computer by microhematocrits and phase contrast microscopy, are presented elsewhere (14–18). The brief pulses of electrical current do not affect the shape, size distribution, or fragility of intact erythrocytes (19).

In order to maintain the Coulter probe and the ghost suspension in the vial at 37°C a special Lucite jacket was made with side holes for the aperture-monitoring light path and with inlet and outlet pipes for circulating water through the jacket. This Lucite jacket remained clamped on the Coulter counter sample platform throughout the experiment and the vials were placed in it without any difficulty. Further details may be found elsewhere (16).

The effect of each NaCl solution on the ghosts was obtained in triplicate at least (i.e. three mixtures of NaCl and ghosts), and each mixture was monitored five to six

times in the Coulter counter and MCV computer with a replicate reproducibility of better than 0.6% (20); each determination, therefore, plotted in the graphs in this paper, in effect was derived from an average of about 15 readings at least. The mean cell volumes were constant for at least 5 min after mixing.

In each experiment the vials contained the same concentration of ghosts; this diminished the possibility of coincidence variations which might affect the measured mean cell volumes. Before each MCV determination the Coulter counter probe was rinsed with a solution of electrolytes precisely equal in composition to that present in the vial after the addition of the ghosts. The rinsing solutions were also kept at 37°C.

When the ghosts had shrunk too much, the automatic threshold setting of the MCV computer caused the smaller ghosts of the cell population to be excluded from the MCV computations. This led to values for the MCV which were too high. The necessary correction for this cutoff could be obtained, however, as follows. Since the number of cells present in the vial was known, any decrease in this number consisted of cells too small for sizing (since the thresholds of the Coulter Counter and the MCV computer were both the same and both were set at 10 units). At 50 μ^3, for example, 6% of the cells were found to be below threshold; the maximum correction that could then be applied was 3 μ^3, resulting in a true volume between 47 and 50 μ^3. At 80 μ^3 no cutoff of cell count was observed. Most of the mean cell volumes measured were such that no correction was necessary (see Table II).

Procedure to Determine the Osmotic Response of Intact Erythrocytes

Erythrocytes were suspended in media of varying osmolarities and the swelling or shrinking of the cells was monitored by means of a Coulter counter or microhematocrit tubes or Van Allen tubes. A description of these methods has been presented previously (17). It has also been shown previously that good quantitative agreement exists between the different methods (17).

Method to Determine Perfect Osmotic Behavior for Intact Cells and Ghosts

In the intact erythrocyte a large part of the total volume is occupied by solids, mainly hemoglobin. The solid volume, V_{solid}, will not take part in the osmotic process. The total cell volume, V, consists, therefore, of an osmotically sensitive water volume, V_w, and the solid volume. The expected osmotic behavior of a cell with known water and solid volume can be described by equation 1.

$$V = \frac{k}{C} + V_{\text{solid}} \tag{1}$$

where V is the volume of the cell, k is a constant, and C is the solute concentration in the medium. The constant k can be determined from the water volume of the cell, V_w, in a given reference medium concentration, C_{ref}, according to equation 2.

$$V_w \times C_{\text{ref}} = k \tag{2}$$

where $V_w = V - V_{\text{solid}}$. It is assumed that the red cell suspension is so dilute that

water movements across the cell membranes will only have a negligible effect on the solute concentration in the extracellular medium. For an ideal osmometer the constant k in equation 1 should be independent of the volume of the cell and also independent of C and V_{solid}. The results presented in this paper show that for the intact erythrocyte k is in fact dependent on these parameters while for the ghost it is independent of V and C.

The solid volume of the intact cell, V_{solid}, was obtained by dividing the dry weight per cell, DW_{cell}, by 1.17 which is the density of hemoglobin (21). There is very little error in assuming that hemoglobin accounts for virtually all the cell solids; if the fraction of cell water for intact erythrocytes is 60%, then using a value of 1.17 for the density of hemoglobin, it can be readily shown that the density of the intact cell is around 1.07, which is the value usually found (22). DW_{cell} was measured by drying an aliquot of erythrocytes (packed at 1500 × g for 15 min) at 80°C to constant weight; the number of erythrocytes in the aliquot was determined by counting a dilution of the cells in a Coulter counter. The amount of medium trapped between the erythrocytes packed at 1500 × g for 15–20 min is about 6% (23) and this 6% correction was made for all packed cell volumes, including the Van Allen hematocrits. For microhematocrit determinations, wherein the capillary tubes were centrifuged at 15,500 × g for 3 min (in order to calibrate the Coulter counter; see references 14–18), a correction of 2% was made for the trapped medium (24).

V_{solid} for the ghost cells was obtained as follows. A known number of washed erythrocyte ghosts (counted by the Coulter counter) was dried at 80°C to constant weight; the weight of a single ghost determined in this way worked out to be between 10^{-12} and 2×10^{-12} with most of the values being about 1.3×10^{-12} g. This value agrees with that obtained for washed ghost membranes previously reported by Dodge et al. (25). Since the dry membrane represents about 70% of the wet membrane of the ghost at 20°C (26), the weight of the wet ghost membrane is about 1.9×10^{-12} g. Since the membrane density is of the order of 1.17 (27, 28), the volume of the wet ghost membrane is around 1.5 μ^3.

The expected osmotic behavior in the presence of a constant intracellular pressure was determined from Teorell's equation (9).

$$(C + a) \cdot (V + b) = k_1 \tag{3}$$

where C is the solute concentration, V the cell volume, k_1 a constant, and a and b two correction factors. The constant internal pressure is represented by the factor a, while b represents the solid volume of the ghost.

RESULTS

The Volumes of Intact Erythrocytes at Different Osmolarities of the Medium

As has been reported by many investigators (see Introduction), the osmotic response of intact erythrocytes does not quantitatively agree with theoretical predictions. The volume change observed is always less than that predicted by the van't Hoff law. This is shown in Fig. 1 for the intact erythrocytes of one person.

In an "isotonic" medium of 333 milliosmols/liter the MCV of the intact cells was 82.5 μ^3, 50 μ^3 of which was water while the remaining 33 μ^3 consisted of solids. The water content was lower than usual (5) and was apparently a result of the hypertonicity of the medium of 333 milliosmols/liter. In Fig. 1 the solid line for the perfect osmometer was drawn in accordance with equations 1 and 2, where k was $\frac{16.4\ \mu^3 \times \text{osmol}}{\text{liter}}$ at the reference osmolarity of 333 milliosmols/liter. It can be seen that the experimental points do not fall on the line expected for a perfect osmometer.

The data in Table I and Fig. 3 give the value of k at each osmolarity for several experiments on intact cells. In all experiments the value of k fell steeply when the volume of the cell was increased. These results merely con-

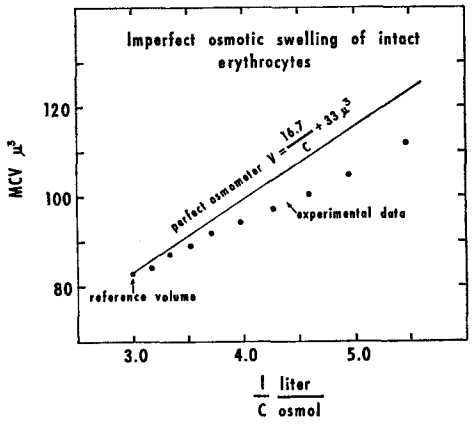

FIGURE 1. The osmotic swelling of intact erythrocytes in hypotonic solutions is less than that predicted by the Boyle-van't Hoff law.

firm the well-known fact that the intact erythrocyte is not a perfect osmometer.

It is possible to calculate Ponder's R value (1) from the data presented in Fig. 1 and Table I; these R values come out to be between 0.64 and 0.82. The R value is usually slightly higher than this (see Kwant and van Steveninck (23) for example).

The Volumes of Erythrocyte Ghosts at Different Osmolarities of the Medium

The osmotic responses of ghosts are presented in Fig. 2 and Table II. The solid lines in Fig. 2 represent the predicted ideal osmotic behavior according to equations 1 and 2 outlined in the Methods.

Since the experimental points for the mean cell volumes fall on the predicted line of the perfect osmotic response, the erythrocyte ghost behaves as a perfect osmometer. The results also indicate that the molarity of the hemolyzing solution, the size of the freshly prepared ghosts, and the choice of

the donor did not make any difference to the perfect osmotic response of the ghosts. The results in Table II and Fig. 3 indicate that the Boyle-vant' Hoff constant (k) for ghosts is independent of the cell volume or the osmolarity of

TABLE I

INTACT ERYTHROCYTES. DEPENDENCE OF THE BOYLE-VAN'T HOFF CONSTANT ON THE VOLUME OF THE INTACT ERYTHROCYTE

Method	Mean cell volume of intact cells V^*	Osmolarity of suspending medium C	$V - V_{solids}$	Boyle-van't Hoff constant k
	μ^3	*osmols/liter*	μ^3	μ^3-*osmols/liter*
Van Allen hematocrit	72.0‡	0.430	42.2§	18.2
	81.9‡	0.303	52.1§	15.8
	94.8‡	0.233	65.0§	15.1
	101.0‡	0.209	71.2§	14.9
Van Allen hematocrit	69.0‡	0.430	24.0‖	17.4
	81.0‡	0.303	52.5‖	15.9
	94.8‡	0.233	66.3‖	15.4
	101.0‡	0.209	72.5‖	15.1
Van Allen hematocrit	62.0‡	0.572	30.6¶	17.5
	86.0‡	0.288	54.6¶	15.7
	104.0‡	0.203	72.6¶	14.7
Coulter counter	82.5±0.7**	0.333	49.5±0.7**, ‡‡	16.4
	83.7±0.8	0.312	50.7±0.8	15.8
	87.0±0.8	0.300	54.0±0.8	16.2
	89.0±1.0	0.281	56.0±1.0	15.8
	91.5±0.8	0.270	58.5±0.8	15.8
	93.1±0.6	0.254	60.1±0.6	15.3
	97.0±0.8	0.233	64.0±0.8	14.9
	100.2±0.8	0.215	67.2±0.8	14.4
	104.5±1.1	0.202	71.5±1.1	14.4
	111.5±1.2	0.184	78.5±1.2	14.4

* Mean of quadruplicate determinations.
‡ Volume of erythrocytes in isotonic medium (0.3 osmolar) = 83.0 μ^3.
§ V_{solids} = 29.8 μ^3.
‖ V_{solids} = 28.5 μ^3.
¶ V_{solids} = 31.4 μ^3.
** Mean ± standard deviation for 15 determinations.
‡‡ V_{solids} = 33.0 μ^3.

the medium. The Ponder R value for ghosts, therefore, under the conditions of these experiments is $R = 1$.

The Absence of High Intracellular Pressures in the Ghost

The dashed line in Fig. 2 represents the expected ghost volumes if an internal pressure of 200 cm H_2O (equivalent to 7.9 milliosmols/liter) existed. This

line was drawn according to equation 3, with $a = 7.9$ milliosmols/liter and $b = 1.5\ \mu^3$; the k_1 value was 19.42 $\frac{\mu^3 \times \text{osmols}}{\text{liter}}$ when $V = 151\ \mu^3$ at $C =$ 122 milliosmols/liter.

Since the experimental points deviate from the dashed line, it can be concluded that a constant intracellular pressure larger than 200 cm H_2O does not exist in the fresh erythrocyte ghost. This method, however, is not sensitive enough to exclude internal pressures smaller than 200 cm H_2O.

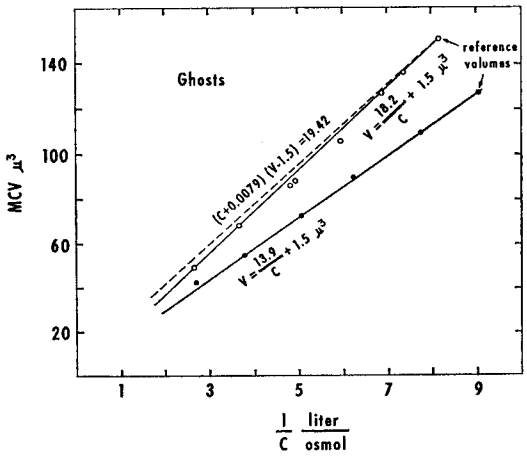

FIGURE 2. This figure shows that the erythrocyte ghost is a perfect osmometer. The circles are the experimental mean cell volumes. The solid lines represent the values predicted by the Boyle-van't Hoff law. The interrupted line plots the values predicted by Teorell's equation with an intraghost pressure of 200 cm H_2O. The open circles represent ghosts obtained from one donor, while the solid circles represent those of another donor.

DISCUSSION

Osmotic Properties of Erythrocyte Membranes

The fact that erythrocyte ghosts show no detectable discrepancy from perfect osmotic behavior implies the following two points.

1. The erythrocyte membrane does not offer any bending resistance large enough to account for the osmotic anomalies that occur in the intact cell. This is compatible with the work of Rand and Burton (29) who found that the erythrocyte membrane "stiffness" (or resistance to deformation because of membrane rigidity and tension) was very low (around 0.02 dyne/cm).
2. Net ion movements across the ghost membrane were negligible during the short time interval between mixing and sizing of the ghosts.

TABLE II

ERYTHROCYTE GHOSTS. INDEPENDENCE OF THE BOYLE-VAN'T HOFF CONSTANT OF THE VOLUME OF THE ERYTHROCYTE GHOST

Osmolarity of hemolyzing medium	Mean cell volume of ghosts V^*	Osmolarity of suspending medium C_{final}	V-V_{solid} / V-1.5	Boyle-van't Hoff constant k
Osmolar	μ^3	*osmols/liter*	μ^3	μ^3-*osmols/liter*
0.121	124.4±1.8	0.144	122.9±1.8	17.8
	105.3±1.7	0.168	103.8±1.7	17.3
	88.4±0.8	0.201	86.9±0.8	17.6
	68.1±0.5	0.269	66.6±0.5	17.9
	52.9±0.5	0.371	49.0±0.5‡	18.2‡
0.110	133.6±1.2	0.110	132.1±1.2	14.5
	109.7±1.7	0.129	108.2±1.7	13.9
	89.6±0.4	0.160	88.1±0.4	14.1
	72.6±1.2	0.199	71.1±1.2	14.2
	56.9±0.7	0.260	53.7±0.7‡	14.0‡
0.121	151.5±1.0	0.121	150.0±1.0	18.2
	137.5±1.0	0.136	136.0±1.0	18.5
	127.5±0.8	0.144	126.0±0.8	18.4
	106.5±0.8	0.168	105.0±0.8	17.8
0.110	112.0±0.5	0.129	110.5±0.5	14.2
	88.4±1.8	0.160	86.9±1.8	13.9
	70.8±0.8	0.199	69.3±0.8	13.8
	56.8±0.7	0.260	53.6±0.7‡	13.9‡
0.126	162.0±1.8	0.126	160.5±1.8	20.2
	154.0±1.5	0.132	152.5±1.5	20.2
	142.0±1.0	0.143	140.5±1.0	20.1
	124.0±0.8	0.163	122.5±0.8	20.0
	109.0±1.0	0.184	107.5±1.0	19.8
	95.2±0.9	0.211	93.7±0.9	19.7
0.120	128.2±1.0	0.141	126.7±1.0	17.8
	106.8±0.8	0.165	105.3±0.8	17.4
	86.5±0.8	0.201	85.0±0.8	17.2
	64.4±0.6	0.269	62.9±0.6	17.0
	50.4±0.8	0.371	46.0±0.8‡	17.1‡

* Mean ± standard deviation for 12 to 15 determinations of each MCV (mean cell volume).
‡ Corrected for threshold effect of Coulter counter.

A Comparison of Membrane Rigidities of the Ghost and the Intact Cell

It might be argued that the apparently perfect semipermeable properties of the ghost membrane do not exist when the membrane is in the native state in the intact cell. This is conceivable since certain structurally important molecules may be lost during hemolysis, thereby changing the properties of

the membrane. It is possible that the formation and sealing of membrane holes, large enough for hemoglobin release (30), may have irreversibly altered membrane structure.

Although it is difficult or impossible to prove, it is unlikely that the loss of membrane components occurs during one-step hemolysis and the subsequent sealing of ghosts. The reasons for believing this are as follows.

1. Ca^{++} ions, considered important for membrane integrity and structure (see references in Seeman et al. [14]), do not desorb from the membrane during one-step hemolysis (31).

2. Extensive washing of intact erythrocytes, as has been done by many investigators (5), does not alter the anomalous osmotic properties. It is

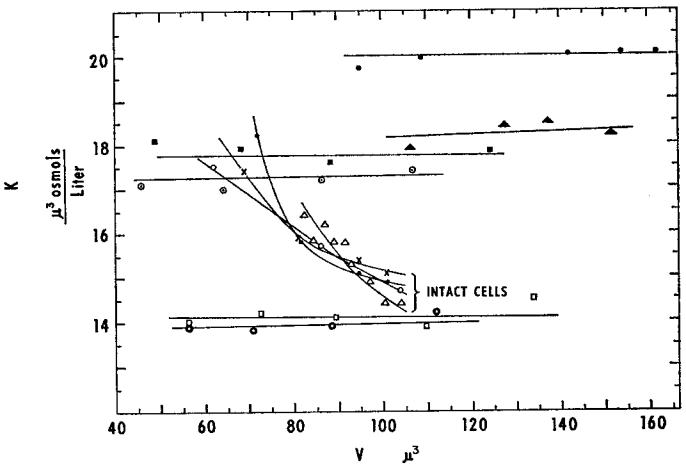

FIGURE 3. The Boyle-van't Hoff constant, k, is independent of the volume of the erythrocyte ghost but is inversely proportional to the size of the intact cell.

difficult to say how effective repeated washing is in removing hypothetical components as compared to one-step hemolysis.

A change in membrane structure due to hole formation and subsequent resealing cannot be excluded. The work of Weed and of Hanahan (10, 13, 25), however, shows that membrane enzymatic activities are not altered.

Passive Ion Permeability of the Erythrocyte Membrane

One of the differences between the ghost and the intact erythrocyte membrane is the long-term permeability to ions. The 2 hr reswelling phenomenon of these fresh ghosts to spherical shape (14) is probably due to a colloid osmotic pressure across the membrane as a result of some residual hemoglobin (13). This indicates that the ghost membrane has a higher passive permeability to ions than the native membrane in the intact erythrocyte. In the short-term interval of 30–60 sec, however, the net ion movement across the

ghost membrane must be negligible, otherwise the ghost would not behave as a perfect osmometer.

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