Biophysical Chemistry 12 (1980) 57–61

A NOTE ON OSMOTIC PRESSURE MEASURED WITH IONOPHORE TREATED RED BLOOD CELLS

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Received 26 June 1979
Revised manuscript received 3 November 1979

A method is described by which the osmotic pressure of macromolecules or many low molecular weight substances can be measured relative to the known osmotic pressure of a reference substance. Measurements can also be made in the presence of univalent electrolytes. The method involves the use of ionophore treated mammalian red blood cells as osmometers. Details are given for the establishment of the isosmotic identity line for dextran $\bar{M}_w$ = 9 400, $\bar{M}_n$ = 5 500 and sucrose using nystatin treated human red blood cells. The sucrose concentrations used were from 20 to 33 mOsm (50–80 kPa).

1. Introduction

The aim of the work described here was to determine the concentration of a particular dextran fraction that just balanced osmotically the hemoglobin in human red blood cells. As it may have a much wider interest, it is presented here as a method for the measurement of osmotic pressure of substances which do not penetrate red blood cells treated with the ionophore nystatin; red cells treated in this way are very permeable to univalent ions [1].

The use of biological material as osmometers has a long history. De Vries [2] used plant cells. Mammalian red blood cells do not have nuclei or other apparently separate compartments and for this reason are more suitable than other animal cells for the use of osmometry. Their osmometric properties have been described extensively previously [3,4,5,6,7] and recently also in the presence of nystatin [8].

2. Principle

The osmotic balance over the normal red blood cell membrane is a complex function involving charged protein molecules, energy dependent uphill Na^+ and K^+ transport and leakages for different ions. The osmotic pressure in the cells is normally about 300 mOsm. This state can be simplified by both the two following procedures.

1) Proteins are titrated to the iso-electric pH of the dominating species, hemoglobin, by a procedure described previously [1,7].

2) The membranes are made very permeable to all univalent small ions by treatment with the ionophore nystatin as described in [1].

In this way the effective osmotic pressure of cells at equilibrium is reduced about a ten-fold and is due essentially to hemoglobin. Under these conditions the cells will swell so that hemoglobin and other components escape, unless there is a sufficient concentration outside of a non-penetrant solute. The limit to which untreated human cells can swell without losing hemoglobin varies from 1.54 to 1.74 times the normal volume [9]. Nystatin treated cells, in the absence of ions with valencies higher than one, will thus function as osmometers towards non-permeable external solutes. When the concentration of a non-permeant solute in the medium is altered, the establishment of the new osmotic equilibrium involving flow through the membrane takes some time; with a nystatin concentration of 75 μg/ml this took approximately 10 min at about

0°C. Freedman and Hoffman [8] used a nystatin concentration of 50 μg/ml and also found the cells equilibrated within 10 min at 2°C.

Cell volume or a variable related to cell volume (e.g. water fraction) is determined as a function of the concentrations of both reference and test media. The osmolarity of the non-permeable molecule in the test medium will be the same as that in the solution of the non-permeable reference solute if the cell volume is the same in both solutions.

The cell-medium volume ratio can be kept low so that concentrations in the medium are practically unaltered by cell volume alterations and are besides affected similarly in reference and test solutions if the cell concentration is the same in both.

3. Materials

Dextran T 10 Lot 4202 $\bar{M}_w = 9\,400$, $\bar{M}_n = 5\,500$ (Pharmacia Fine Chemicals, Uppsala, Sweden), ^{51}Cr-EDTA (NEN Chemicals GmbH, Dreieichenhain, German Federal Republic) Nystatin = Mycostatin® for laboratory use only (Squibb & Sons, Inc., New York, N.Y.).

4. Solutions

Washing solution. An isotonic solution (309 mOsm from the freezing point depression (Knauer, Berlin, G.F.R.) was used for washing the cells free from plasma constituents. The solution used contained 18 mM NaCl, 133 mM KCl and 27 mM sucrose. The Na^+/K^+ ratios are the same as in normal human red cells and were chosen simply in order to minimize the time necessary for electrolyte equilibration of the cells after adding nystatin. Other ratios can be used without great disadvantage.

Test solutions. The 8 different test solutions were prepared on the day of the experiment by the addition of a fresh solution of nystatin in methanol to solutions containing the other constituents. The final concentrations were 1.5 per cent (V/V) methanol. 75 μg/ml nystatin, 18 mM NaCl, 133 mM KCl, 1 mM Na-EDTA and x sucrose or dextran. x varied between 21 and 32.5 mM for sucrose and between 90 and 120 g/ℓ for dextran.

5. Methods

Dextran concentrations were determined by polarimetry using a Kreispolarimeter 0.05° (Karl Zeiss, Oberkochen, G.F.R.) with a 2 dm cuvette. For the particular low molecular weight fraction of dextran used in this investigation a specific rotation value of $[\alpha]_{Na} = 188.7$, as given by the manufacturer, was used.

Determination of wet and dry weights. Packed red cells were obtained by centrifuging about 0.6 ml of a cell suspension (hematocrit about 50%) in a nylon tube (internal diameter 3.15 mm) [10] for 20 min at 44 000 × g (0°C). The tubes were cut with a razor blade below the interface and the supernatant and the top portion of the cells were discarded. The tube bottom was cut off, the contents were emptied with the aid of a plunger on to an aluminium pl. nchet and weighed as quickly as possible. The cells were then dried at 98°C for about 12 hrs. Dried cells are somewhat hygroscopic and precautions were taken to minimize reentry of water before weighing.

Trapped medium was calculated as

$$\frac{\text{cpm} \times (\mu\ell \text{ cell sample})^{-1}}{\text{cpm} \times (\mu\ell \text{ supernatant})^{-1}}$$

from the radioactivity of an extracellular space marker. ^{51}Cr-EDTA was used in this case. Other substances such as sucrose, proteins or polymers can be used.

^{51}Cr activities in 0.1 ml samples diluted with 1 ml of distilled water were counted on a scintillation counter. The statistical counting error was kept below 0.7 per cent. The cell and supernatant samples were pipetted with disposable capillary pipettes (Microcaps, Drummond Sci. Co., Broomall, Penn. USA) taking great care to wash out the contents with the diluent water (1 ml).

Water ratios [(water weight) × (dry weight)$^{-1}$] in samples of packed red cells were calculated from the measured wet and dry weights. As the packed cell mass contained some trapped medium, the water fraction of the red cells alone was calculated using the values of fractional trapped medium determined (V/V) and the measured values of the densities of the different media and an assumed density for the dry matter of red cells equal to that of Hb, i.e. 1.355 [11]. This calculation will be simpler if the fractional trapped medium is measured on a W/W basis.

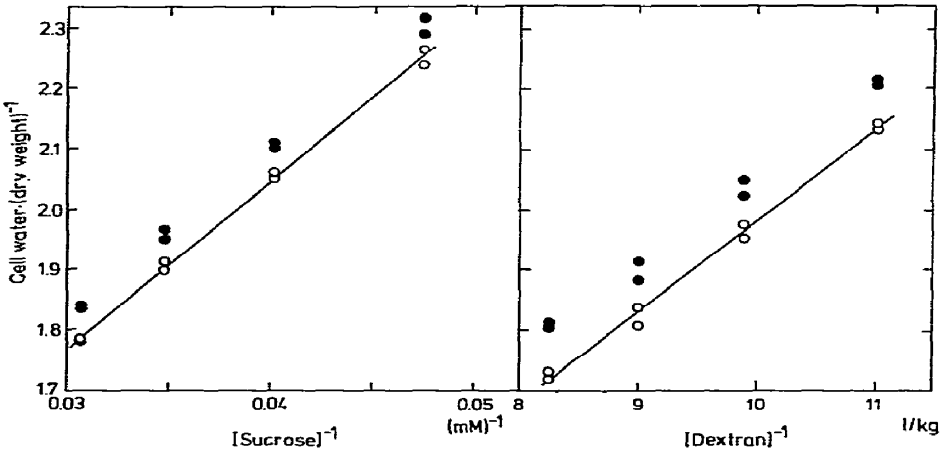

Fig. 1. Cellular water in nystatin treated red cells as a function of sucrose and dextran concentrations. Red cells were titrated with CO_2 to the isoelectric point of Hb. They were then made permeable to univalent ions by the addition of the ionophore nystatin to the suspending media, which contained sucrose or dextran and univalent electrolytes; the concentrations of the latter were always the same. The cellular water ratio is shown before (filled circles) and after (open circles) correction for medium trapped in the cell column after centrifugation with 44 000 × g during 20 min. The abscissae show the reciprocal concentrations for sucrose (mM) and dextran (kg/ℓ). The straight lines were calculated by linear regression analysis, which for sucrose and dextran gave correlation coefficients of 0.999 and 0.996, respectively.

6. Procedure

40 ml blood was collected in heparinized test tubes (Vacutainer) from apparently healthy humans. The blood was stirred in a beaker with a stirring magnet and a mixture of air and CO_2 was blown over the surface until the pH as measured (PHM 26, Radiometer, Copenhagen, Denmark) with a combined glass-reference electrode fell to 7.4 at 20°C. The blood was then centrifuged one min at 17 000 × g and the supernatant replaced by washing solution. The pH was titrated by CO_2 to pH 6.89 at 20°C. The cells were then packed for 4 min at 27 000 × g, the supernatant discarded and the buffy coat of white cells carefully sucked off. The cells were washed 3 more times, on each occasion with 25 ml washing solution, during which procedure the pH of the cell suspension remained constant. The following procedure was done between 0 and 5°C by keeping the test tubes on crushed ice in water and by centrifuging at about 0°C. The cells were packed for 5 min at 27 000 × g, after which 1.5 to 2 ml of cells were added to 38 ml of each test solution and allowed to stand for 20 min before recentrifugation, after which the supernatants were replaced by fresh test solutions of the same kind, shaken, allowed to stand for 10 min and then centrifuged again, the supernatant again being discarded and replaced by 2 ml of new test solutions now also containing the extracellular marker ^{51}Cr-EDTA. After stirring the suspension on a vortex agitator, nylon tubes were filled with cell suspensions, 4 tubes from each batch. These tubes were then centrifuged for 20 min at 44 000 × g; two of the tubes were used for determinations of wet and dry weights and two for determination of ^{51}Cr activity in the cell pellets and in the supernatants.

7. Results

The fraction of trapped medium may be expected to vary (with a constant centrifuging technique) depending both on the density of the red cells and the density and the viscosity of the medium. The viscosity and elasticity of the red cells certainly also play a role, as the cells will have to change shape during packing, if the relative amount of trapped medium is to be as small as it is. The extreme percentage values for trapped

medium within the sucrose group were 1.79 and 2.20 and in the dextran group 3.39 and 4.70. The corresponding measured values for the densities of the medium were 1.005, 1.007 (sucrose), 1.039 and 1.050 (dextran) and for the cells calculated as 1.083 and 1.097, respectively. Neglect of trapped medium in the calculation of dextran osmotic pressure from the presented data would result in a systematic error of 2 per cent. In fig. 1 the values for cell water ratios are given both with and without correction for trapped medium.

By combining the equations describing the two lines in fig. 1, a new expression is obtained which relates dextran and sucrose concentrations of the same osmotic activity. The isosmotic identity line was:

$$\frac{1}{D} = \frac{185.1}{S} + 3.024, \qquad (1)$$

where D = dextran concentration in kg/ℓ and S = sucrose concentration in mM.

Values for water ratio and water fraction in "normal" cells were, for untreated cells in their own plasma 1.863 [(water) × (dry weight)$^{-1}$] or 0.651 [(water) × (wet weight)$^{-1}$] (uncorrected for trapped plasma) and in CO_2-titrated washed cells (no nystatin) 1.862 and 0.651, respectively, (corrected for trapped medium). From fig. 1 it can be found that 29.9 mM sucrose corresponds to these values on cells treated with nystatin.

Fig. 1 further shows that the cells functioned as osmometers, i.e. the cell water varied as an inverse linear function of the concentration of the non-permeant compound in the medium. The slope of the line relating relative cell volume [(volume) × (normal volume)$^{-1}$] to the inverse relative concentration in the medium was, however, only 0.358 ± 0.007 (S.D.) for sucrose and 0.534 ± 0.017 for dextran. This is an interesting observation — which, however, does not affect the method presented.

8. Discussion

Precision measurements of osmotic pressure at and near 25°C in concentrated dextran solutions were made by Vink [12] who used fractions with $\bar{M}_n$ values of 28 700 and 374 000. From the molecular weight dependent second and third virial coefficients given by Vink the osmotic pressure of the dextran solution used in this investigation was calculated from linear extrapolation. The osmotic pressure obtained in this way and from eq. (1), assuming that for sucrose 1 mM = 1 mOsm, differed by no more than 0.7 per cent. This agreement is surprisingly good with regard to experimental errors, the difference in temperatures used (Vink 25°C, this investigation 0 to 5°C, although the osmotic pressures of dextran solutions are nearly athermal [12]), and considering that calculations from Vinks data were based on an extrapolation from $\bar{M}_n$ values of 374 000 and 28 700 to a $\bar{M}_n$ value of 5 500. Even if the good agreement must to some extent be fortuitous, it seems reasonable to conclude that the method appears to have a high reliability. No measurements were made, however, to test the reproducibility.

The method described here is laborious. It can, however, be very much simplified if a somewhat lower precision is acceptable, such as is obtained when the interstitial medium is neglected. The magnitude of this error will also depend on the use of an efficient centrifuging technique and on the densities of the test media.

The method is limited, of course, to measurements of water soluable substances which do not interfere with the red cell membrane or penetrate it. No experiments were made to elucidate the range of useable concentrations of test substances, but Freedman and Hoffman [8] studied the osmotic properties of nystatin treated red cells in media with sucrose concentrations varying from 15 to 260 mM. Thus this concentration range should be useful also in the present connection. A further limitation of the method is the density of the test solution since the method requires that red cells can be separated from the medium.

The method offers the advantage to the biologist of observing the effective osmotic pressure using a biological instead of an artificial membrane and this also in the presence of electrolytes. It must, however, be pointed out that the method as presented here is based on the comparison of red cells from one batch in test and reference media which are similar in salt composition and differ only with regard to their content of test and reference substance. Further, the salt concentration was about normal for the cells. From the knowledge that red cells in nystatin have a constant volume with a constant sucrose concentration in the suspending medium independently of whether

the salt concentration of the latter varied from 1 mM to 1 M [1] or from 11 to 600 mM [7] it is reasonable to conclude that at a constant pH the cell volume depends only on the external concentration of non-permeants. The method should thus work also if the concentration of univalent ions in test and reference media are not the same. However, if the electrolyte concentrations are markedly different from those normal to the cells, they may not tolerate an abrupt change in osmolarity, whereas slow and progressive changes of concentration successfully have been performed [1,7]. The question of the validity of the method under greatly varying salt concentrations will require further study.

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