CELL VOLUMES AND WATER CONTENTS OF FROG MUSCLES IN SOLUTIONS OF PERMEANT SUGARS AND SUGAR ALCOHOLS

GILBERT N. LING
Department of Molecular Biology, Pennsylvania Hospital, Eighth and Spruce Streets, Philadelphia, PA 19107.

1. Previous work has suggested that living cells may acquire and then maintain different water contents and hence volume, in solutions containing different concentrations of solutes that are permeant to the cell membrane. Toward better understanding of this phenomenon, two hypotheses were introduced: one hypothesis is based on the membrane-pump theory; another represents an extension of the polarized multilayer theory of cell water, a part of the association-induction (AI) hypothesis. To test the different predictions of these hypotheses, the water contents of frog muscle equilibrated at 25° C in solutions of different concentrations of seven pentoses, seven hexoses, seven dissacharides, two trisaccharides, and six sugar alcohols were determined.

2. The earlier finding of sustained shrinkage of muscle cells in concentrated solutions of permeant solutes was confirmed once more.

3. In equimolal solutions of sugars and sugar alcohols with different steric conformations but the same or closely similar molecular weight(s), muscles had the same or closely similar water content(s).

4. In equimolal solutions of different sugars and sugar alcohols, the equilibrium water contents of the muscles increased with decreasing molecular weights of these solutes.

5. The water contents of muscles, equilibrated in 0.4 M solutions of different sugars and sugar alcohols, are positively correlated with the equilibrium distribution coefficients (or q-values) of the sugar and sugar alcohols in the muscle cell water with a linear correlation coefficient of +0.973.

6. The relationships between the equilibrium water contents of muscles (in solutions containing different concentrations of different sugars and sugar alcohols) and the concentrations of these sugars and sugar alcohols agree in general contours with that predicted by an equation derived on the basis of the polarized multilayer theory of cell water.

7. The experimental findings described above do not agree with the prediction based on the membrane-pump hypothesis; they do agree with all four predictions of the hypothesis based on the polarized multilayer theory of cell water.

INTRODUCTION

Volume changes of living cells were of central interest to cell physiologists. Studies of the swelling and shrinkage of plant cells provided the foundation for Pfeffer's membrane theory (Pfeffer, 1877). According to the original version of this theory, a concentrated solution that causes sustained shrinkage of living cells was regarded as bona fide evidence that the cell membrane is impermeable to the solute in the solution. Immersion of living cells in a concentrated solution of a permeant solute causes a shrinkage followed by unrestrained swelling. Yet even as early as the 30's, there was evidence that a concentrated solution of a permeant solute in fact also causes *sustained shrinkage* of living cells (see

below). This communication reports investigations aimed at further understanding of this phenomenon. To achieve this aim, I shall first introduce two hypotheses. One hypothesis is based on the conventional membrane-pump theory; it represents an extension of the published theory of volume regulation by ions to volume regulation by solutions of nonelectrolytes. The other hypothesis represents an extension of the polarized multilayer theory of cell water, a subsidiary hypothesis of the association-induction hypothesis. Results of experimental testing of the divergent predictions of the two theories comprise the remainder of this report.

THEORY

1. Hypotheses Based on the *Membrane*-pump Theory. In the 30's Nasonov and Aizenberg (1937) demonstrated maintained shrinkage of living cells in solutions of nonelectrolytes permeant to the cell membrane; radioactive tracer and related studies soon established the same for solutions containing ions (Cohn and Cohn, 1939; Heppel, 1939; Steinbach, 1940). To explain the sustained maintenance of normal cell volume in a solution containing mostly NaCl, the "pump leak hypothesis" was suggested. In this model it was argued that to maintain a normal cell volume, the cell membrane does not need to be absolutely impermeable to the major ion in the bathing solution but effectively so. This "effective impermeability" to Na^+ could be achieved by the Na pump (Wilson, 1954; Leaf, 1959; Tosteson and Hoffman, 1960; Post and Jolly, 1975). Like the Na pump, sugar pumps have also been proposed, notably by Cohen and Monod (1957), and given the names "permeases". Yet to the best of my knowledge, a similar pump-leak model had not yet been proposed to explain the sustained volume maintenance in solution containing sugars and other demonstrably permeant nonelectrolytes. Perhaps one of the reasons for this apparent lack of interest so far might have arisen from the divergent nature of the sugar permeases already proposed and the sugar pumps needed to explain the phenomenon described by Nasonov and Aizenberg (1937). The sugar permeases represent largely inward pumps; the sugar pumps required here are outward pumps. Nevertheless, it seems appropriate and necessary to extend the pump-leak model to this phenomenon and this is what I have done (see below).

The extensive studies of E. coli permeases led to the recognition that a Y-gene, as part of a segment of the E. coli genome called the Lac operon, specifies the lactose permease. Following the general principles of genetic control of protein synthesis, one may anticipate that the maintained levels of sugars and other permeant nonelectrolytes by the enzyme — like permeases, must be highly sterospecific. That is, the lactose permease does not act or acts differently on other disaccharides like sucrose, maltose, and trehalose. Similarly one would anticipate that a permease or sugar pump maintaining at a certain steady level in the cell, a specific pentose or hexose will not act or act differently on their sterically different isomers. Since in the pump-leak model, the maintained steady level of, say, Na^+ determines the sustained volume of the cell equilibrated in the ~a*-containing solution; by analogy, one can predict that living cells equilibrated in solutions containing the same concentration of different isomers will maintain different steady levels of the isomers and different cell volumes. I shall refer to this relationship as the "rule of stereospecificity".

2. The Association-Induction (AI) Hypothesis. In contrast to the membrane-pump theory, the AI hypothesis attributes little significance to the cell membrane and none to its postulated pumping activities in cell volume regulation. Instead, it emphasizes interaction of the bulk of cell water with cer-

tain proteins called "matrix proteins" postulated to exist throughout the cell. The polypeptide NHCO groups of the "matrix proteins" and/or other proteins closely associated with the "matrix proteins" are directly exposed to and polarize in multilayers the bulk of cell water. It is this multilayer polarization, that lowers the activity of cell water thereby providing what is commonly expressed as "osmotic activity" of the cells. K^+, the major osmotically active intracellular ion in the membrane theory, plays a minor role here because extensive evidence now exist indicating that most of the cell K^+ is in an adsorbed, and hence osmotically inactive, state (see Ling, 1984, Chap. 8).

Free solutes in the (polarized) cell water, however, do contribute to the intracellular osmotic activity; their maintained levels vary from solute to solute. In general, the equilibrium distribution coefficients of the solutes, or the q-values, follow the "size rule". That is, the larger the solute molecule, the greater the loss of entropy and/or enthalpy on being transferred from the external medium containing normal liquid water to the cell water in the state of polarized multilayers and the lower the q-value.

In 1929, De Boer and Zwikker presented a quantitative theory of polarized multilayers of gaseous molecules condensed on solid polar surfaces. Bradley (1936a) also derived an isotherm for the multilayer polarization and adsorption of gaseous molecules with a permanent dipole moment like water. The Bradley isotherm relates the quantity of adsorption, a, to the reciprocal of the partial vapor pressure of the gaseous molecule (p/p_o) as follows:

$$\log\left(\frac{p_o}{p}\right) = K_1 K_3^{a} + K_4 , \qquad (1)$$

where p is the vapor pressure in question, p_o is the vapor pressure at full saturation; K_1, K_3, and K_4 are constants all at the same temperature and other physical conditions.

In 1970, Ling and Negendank showed that about 5% of the water in living frog muscle is adsorbed very firmly and retained down to very low p/p_o. This fraction is described by a monolayer adsorption isotherm. The remaining 95% of the cell water follows eqn. (1), demonstrating a rectilinear relation between $\log\left[\log\left(\frac{p_o}{p}\right) - K_4\right]$ and a.

Next I shall make the following postulations:

1. the lowering of vapor pressure produced by a specific solute at a specific concentration is roughly the same in water existing in the normal liquid state as in the state of polarized multilayers;
2. for cells which, at equilibrium, maintain a permeant solute at intracellular concentration C_{in} when immersed in a solution containing this solute at (molar) concentration C_{ex}, where $\frac{C_{in}}{C_{ex}} = q$, the water content of the cell is related to the difference in the concentration of the solute in the cell water and that in the external aqueous solution;
3. the vapor pressure of water in its normal liquid state or in the state of polarized multilayers follows Raoult's law:

$$\frac{p_o}{p} = \frac{n_1 + n_2}{n_1} , \qquad (2)$$

where n_1 and n_2 are the number of moles of the solvent and solute respectively in the system.

With the aid of these basic postulations and eqn. (1), one can write out in a straightforward manner the following equation:

$$\log\left[1 + \frac{(1-q)\, n_2}{n_1}\right] = K_1 K_3^{a} + K_4 , \qquad (3)$$

or

$$a = \frac{1}{\log K_3} \left[\log [\log (1 + \frac{(1-q)\, n_2}{n_1} - K_4] - \log K_1\right]. \quad (4)$$

n_2 is the molal concentration of the solute and n_1 is 55.51, the number of moles of water in which n_2 moles of the solute are dissolved.

Figure 1 is a theoretical plot of the water content of living cells, a, (or a-value) expressed in units of grams of water per 100 grams of dry cell weight in solutions of the permeant sugars, sugar alcohols, or other nonelectrolytes at different concentrations, expressed in molality, where the equilibrium distribution coefficients or q-values of the solutes vary from 0 to 0.98. The details of these computations and numerical values used are given in the legend of the figure.

The following predictions can be drawn from this theory:

***Prediction* I.** Solutes with larger sizes and molecular weights will tend to have lower q-values (size rule) and lower a's. Conversely solutes with smaller sizes and molecular weights will tend to have higher q- and higher a-values.

Prediction 2. Solutes of similar size will tend to have similar q- and a-values.

Prediction 3. A positive linear correlation should exist between the q-values of a variety of solutes of basically similar structures and the a-values of muscles equilibrated in equimolal solutions of these solutes.

***Prediction* 4.** The cell water contents and hence volumes should vary with the concentrations and nature of the major solutes of the external solution in accordance with the general contours of the theoretical curves shown in Figure 1.

Predictions 1 and 2 are the opposite of the prediction of the membrane-pump theory

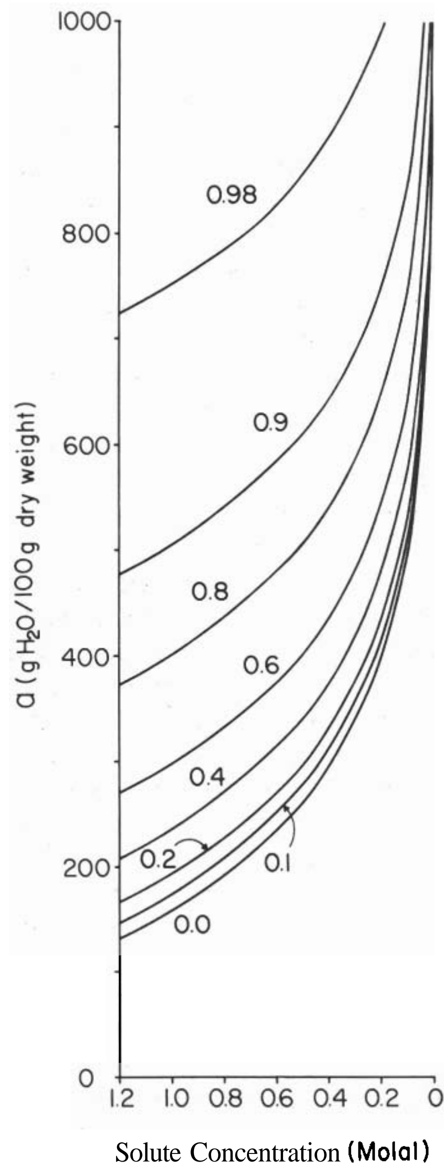


FIGURE 1. Theoretical curves of the water contents of living cells equilibrated in solutions of different concentrations containing solutes which distribute between the cell water and the external medium with different equilibrium distribution ratios, or q-values varying between O.D. and 0.98. Theoretical curves were calculated according to eqn. 3 (or 4) with $1/\log K_3$ equal to –550, K_4 = 0, and $\log K_1$ = 580. a is given in units of grams of water per 100 grams of dry cell weight. Concentrations of solutes is in molality.

(i.e., the rule of stereospecificity). Predictions 3 and 4 are also not predicted by the membrane-pump theory. Agreement or disagreement with these predictions offer means of testing the alternative hypotheses.

MATERIALS AND METHODS

Materials. Small muscles isolated from small and medium sized Vermont leopard frogs (Rana pipiens pipiens, Schreber) were used: sartorius, semitendinosus, tibialis anticus longus, ileofibularis, and peroneus. Semitendinosus and tibialis anticus longus were split into two and peroneus muscles split and trimmed into slender bundles before use.

All chemicals used were of CP grade. Sources of sugars and sugar alcohols were as follows: D-arabinose (Nutritional Biochemicals, Chagrin Falls, Ohio, Lot 78C0440); L-arabinose (Sigma Chemical Co., St. Louis, Missouri, Lot 24C-0400); D-xylose (Sigma, Lot 109C); L-xylose (Sigma, Lot 95C-0197); D-lyxose (Sigma, Lot 666C-0305); L-lyxose (Sigma, Lot 58C-0241); D-ribose (Sigma, Lot 57C-0405); D-glucose (J. T. Baker, Phillipsburg, New Jersey, Lot 931129); L-glucose (Sigma, Lot 44C-1000); β-D(−)fructose (Sigma, Lot 114F-0352); D(+)galactose (Sigma, Lot 23F-0330); α-D-fucose (Sigma, Lot 80C-2580); D-mannose (Pfanstiehl, Waukegan, Illinois, Lot 7458A); tagatose (Sigma, Lot 79B-0720); turanose (Nutritional Biochemicals); cellobiose (Pfanstiehl, Lot 7224); milibiose (Pfanstiehl); sucrose (Fisher Scientific, Fair Lawn, New Jersey, Lot 851322); trehalose (Sigma, Lot 57C-3875); maltose (Pfanstiehl, Lot 6846); lactose (J. T. Baker, Lot 30452); melezitose (Sigma, Lot 34F-0128); D(+)raffinose (Sigma, Lot 52C-2790); ethylene glycol (J. T. Baker, Lot 018341); glycerol (J. T. Baker, Lot 223603); i-erythritol (Sigma, Lot 57C-0058); xylitol (Sigma, Lot 59C-00401); sorbitol (Sigma, Lot 34F-0016); D-mannitol (Sigma, Lot 81F0517; Pfanstiehl, Lot 10954).

Incubation Solutions. All incubation solutions contained NaCl (12 mM), KCl (2.5 mM), $MgCl_2$ (0.9 mM), $CaCl_2$ (0.42 mM), Tris-buffer at pH 7.2 (0.19 mM), and varying concentrations of the different sugars and sugar alcohols investigated. For convenience, sugar and sugar alcohols were all prepared in molar concentrations. The equivalent molal concentrations were obtained from the molar concentrations by the equation:

$$G = \frac{1000\,\mathrm{M}}{1000\,\mathrm{R} - (\mathrm{M} \times \mathrm{E})}, \qquad (5)$$

where G and M are respectively the molal and molar concentrations. R is the density of the solution and E, the molecular weight of the solute. The densities of sucrose solutions are available from handbooks (Hodgman et al., 1961); the densities of other sugar and sugar alcohol solutions were determined by careful weighing of a measured volume of different solutions, from which a calibration curve plotting G against M for each solution was obtained and used to convert molar concentrations of all sugar and sugar alcohol solutions to the molal concentrations.

Assay of Equilibrium Water Contents of Frog Muscles. To determine the equilibrium weights of muscles in a specific sugar or sugar alcohol solution I began with time-course studies. Ling and Negendank (1970) showed that the weights of frog sartorius muscles reached equilibrium values much more rapidly in a hypertonic solution (30 min.) than in a hypotonic solution (150 min.). Nasonov and Aizenberg (1937) showed that weight changes of frog muscle in hypertonic solution of sugars, sugar alcohols, solutes, etc., reached equilibrium in 2 to 2½ hours. Results of my studies with mixed small muscles agreed in general with Nasonov and Aizenberg's. I chose a uniform incubation time of 4 hours. The muscles were incubated while being shaken in sugar or sugar-alcohol containing

solutions approximately 10 times the volume of the muscles. The rate of shaking was 60 cycles/min. and the radius of rotation 1 cm, on a New Brunswick rotating shaker, kept in a constant temperature at 25° ± 1°C. The choice of a 4 hour incubation time, while quite adequate for most studies, allowed the attainment to 90% of the equilibrium volume for larger muscle in glycerol and ethylene glycol; however, still longer incubation frequently led to cell deterioration especially in the more dilute solutions. Therefore for glycol and glycerol studies, muscles from smaller frogs were used as a rule.

Each muscle was blotted between wetted filter paper by the standardized procedure described by Ling and Bohr, 1969, and weighed on a torsion balance before and after 4 hours of incubation to yield the initial and final fresh weight. After the second weighing, the muscles were placed on a polypropylene drying rack and dried first in a warm room (50°C) overnight and then either in a 90°C degree oven (sugars) or a 80°C vacuum oven (glycerol), or more prolonged drying in the warm room (ethylene glycol) until constant weights were reached. Ethylene glycol evaporates rapidly at 80°C in vacuo and slowly but significantly at 40°C (1 10 gram sample in a Petri dish loses on the average 0.7 grams per day). For this reason we compared results obtained by warm room drying with those obtained by radioactive THO labeling method but found no significant difference. The differences between final fresh weights and the dry weights yielded the water contents. The water contents in grams was then divided by 20% of the fresh weights of each muscle and multiplied by 100 to yield a, in grams of water per 100 grams of the dry weight of the muscle.

The q-values of various sugars and sugar alcohols were determined some time ago but largely unpublished. Almost all studies utilized ^{14}C or ^{3}H labeled sugars and sugar alcohols and were carried out at O°C to avoid metabolic interferences. At 0°C, frog muscle does not even measurably metabolize D-glucose (Ling et al., 1969b). For q-value determinations two different methods were used:

Method I. A large number of isolated small frog muscles, totalling in weight of approximately 5 grams, was shaken in 10 ml of an incubation solution containing the labeled sugar or sugar alcohol in a screw capped test tube lying horizontally. From time to time, 0.5 ml aliquots of the external solutions were taken out and analyzed for their labeled sugar or sugar alcohol contents. From the final equilibrium levels reached, the concentrations of the labeled materials in the cell water were calculated after corrections for the labeled materials in extracellular space (8.9%) (Ling and Kromash, 1967).

Method II. Details of this method have been repeatedly described (Ling, et al., 1969a). Incubation of muscles in a Ringer solution (O°C) containing labeled sugar or sugar alcohols at different concentration for 18 hours, which was longer than needed for equilibration. The muscles were then taken out, blotted dry and extracted in 3 ml of 0.3 M TCA overnight. The radioactivity of the TCA extracts were assayed in Bray's scintillation fluid in a TriCarb β-Scintillation Counter. The final concentration of the sugar or sugar alcohol in the cell water, [S],.,., was then plotted against the final external labeled sugar concentration, [S],,. When the best-fitting lines are rectilinear, one way to present the data was to divide each experimental point of intracellular concentration by the corresponding external equilibrium concentration of the solute. A q-value was then obtained from each point and an average of all the q-values obtained. This method will be referred to as Method IIa. An alternative way was to determine the best fitting straight line, the slope of which yields a single q-value. This method will be referred to as Method IIb. Where the best fitting curve is not a straight line, Troshin's limiting slope method was used

to determine the q-value (Troshin, 1966; Ling, 1984, pps. 340-344): $q = \frac{[S]_{c.w.}}{[S]_{ex}}$ when $[S]_{c.w.}/[S]_{ex}$ reaches a constant value at high $[S]_{ex}$

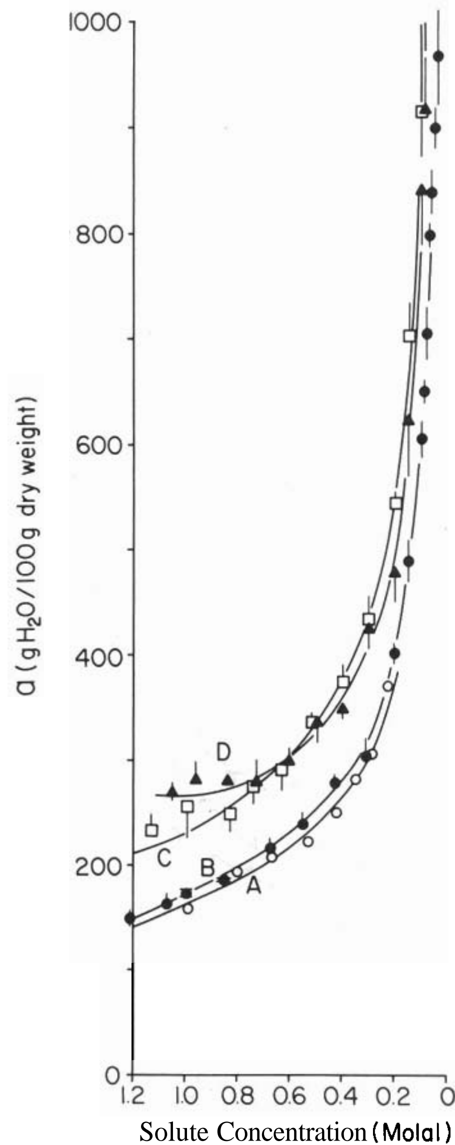
RESULTS

Water Contents of Frog Muscles in Solutions Containing Different Concentrations of Various Sugars and Sugar Alcohols. Figure *2* shows the water content (a) of frog muscle in units of grams of water per 100 gm. of dry weight after 4 hours of incubation in solutions containing different concentrations of sucrose (B), D-glucose (C) and D-xylose (D).

The data of curve (A) of Figure 2 were from Ling and Negendank **(1970),** and obtained after small frog muscle strips had been equilibrated in a vapor phase of different vapor pressures equal to those of the sucrose solutions at molal concentrations given at the abscissa. Note that in these studies, the air phase separating the muscle tissues from the solutions provided a perfect semipermeable barrier allowing equilibrium of only water (as vapor) but not that of the NaCl or H_2SO_4 in the solutions. Curve A therefore corresponds to the theoretical curve of Figure 1 with q = 0. The curve immediately above (Curve B), however, was obtained by immersing the muscles in sucrose solutions of different concentrations. Here both sucrose and water are in equilibrium with the cells. When compared with theoretical curves of Figure 1, the relative position of this curve (Curve B) in relation with Curve **A** corresponding to a q = 0, suggests a very low but nonzero q-value for sucrose in the cell water (see below).

Curves C and D were obtained from muscles immersed in solution of D-glucose and D-xylose respectively. Obviously, their positions suggest higher q-values for these two sugars than for sucrose.

Figure 3 shows similar plots of a, from muscles equilibrated in solutions containing various concentrations of D-mannitol (B), i-erythritol (C), glycerol (D), and ethylene glycol (E). Like the data in Figure 2, the general contours, if not details, agree with the **theore-**



Solute Concentration (Molal)

FIGURE 2. Water contents of frog muscles equilibrated at 25°C in solutions of various sugars with the exception of Curve A. Curve A (taken from Ling and Negendank, 1970) was from vapor equilibrium studies. The equilibrium water uptake at different partial vapor pressure (p/p_o) of Curve A is plotted here against different sucrose concentration giving the same p/p_o. Curve B: sucrose; Curve C: D-glucose; Curve D: D-xylose.

tical curves shown in Figure 1. In addition, the relative positions of the curves suggest increasing q-values in the rank order: D-mannitol < erythritol < glycerol < ethylene glycol in agreement with q-values determined (see Table 3 below). Again the bottom curve (A) is the same as that shown in Figure 2 obtained from vapor equilibrium studies (q = 0).

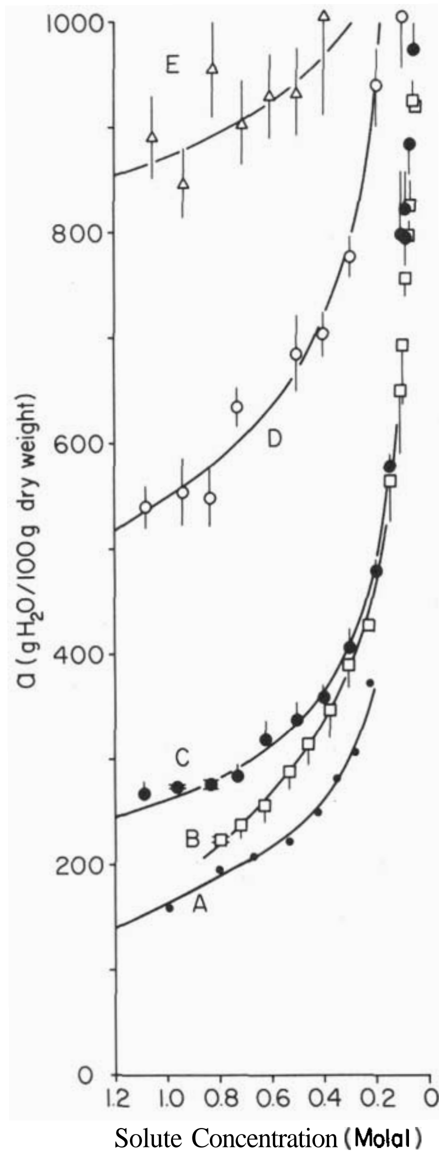


FIGURE 3. Water content of frog muscles equilibrated at **25°C** in solution of various sugar alcohols with the exception of Curve A. Curve **A** (taken from Ling and Negendank, 1970) and introduced for comparison was from vapor equilibrium studies. The equilibrium water uptake at different partial vapor pressure **(p/p_o)** of Curve A is plotted here against different sucrose concentration giving the same **p/p_o**. Curve B: mannitol; Curve C: erythritol; **Curve** D: glycerol; **Curve** E: ethylene glycol.

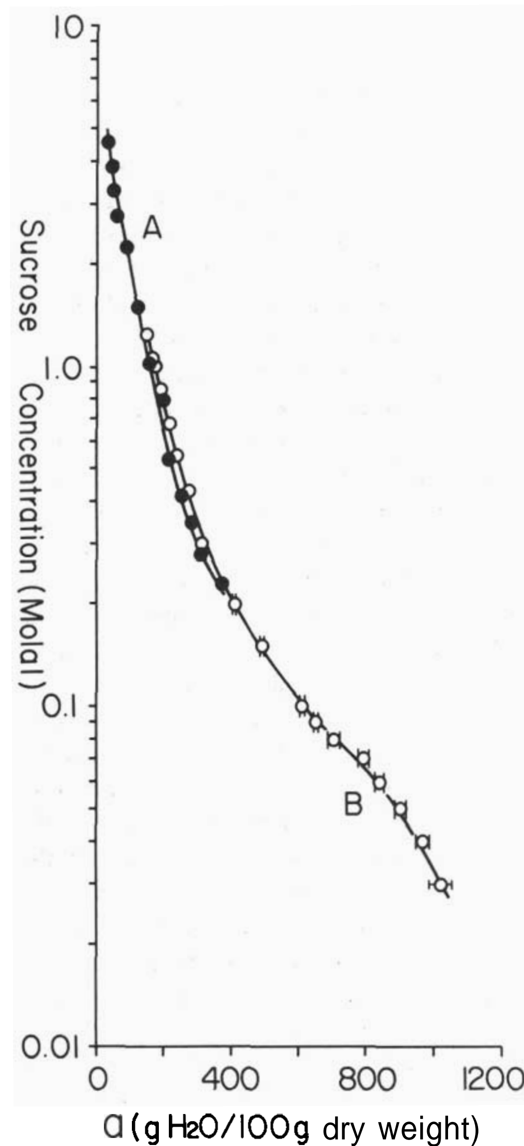


FIGURE 4. A semilogarithmic display of the water contents of frog muscles immersed in solution containing different concentrations of sucrose (Curve B). Curve **A,** taken from Ling and Negnedank (1970) for comparison, was obtained in vapor equilibrium studies at different partial vapor pressures corresponding to the sucrose concentrations presented here on the ordinate.

Figure 4 presents the complete set of sucrose data (Curve B) giving the details at the low sucrose concentrations, again side by side with part of the data from vapor equilibrium studies (Curve A) mentioned above.

In summary, studies of a-values of frog muscles immersed in solutions containing different concentrations of sugars and sugar alcohols reveal relationships between **a**-values, solute q-values, and solute molal concentrations which in general contour, if not all details, agree with the theoretical curves calculated from eqn. (3), thereby confirming Prediction 4.

Do Sugars of Similar Molecular Weights but Different Steric Conformations Yield Similar or Dissimilar a-Values? According to the AI hypothesis, the q-value of a solute in cell water existing in the state of polarized multilayers should follow the "size rule", i.e., lower q-values for larger solutes.

In Table 1, I presented the water contents of muscles equilibrated in a 0.4 M or 0.1 M solutions of various pentoses, hexoses, disaccharide~and trisaccharides. Members in each group of sugars with the same number of carbon atoms have identical or closely similar molecular weights. In agreement with Prediction 1 based on the AI hypothesis, the **a**-values are more closely similar to different members of each group, but less similar between members of different groups with different molecular weights. These data do not agree with the membrane-pump theory, which predicts different a-values for sugars with the same molecular weights but different steric conformations.

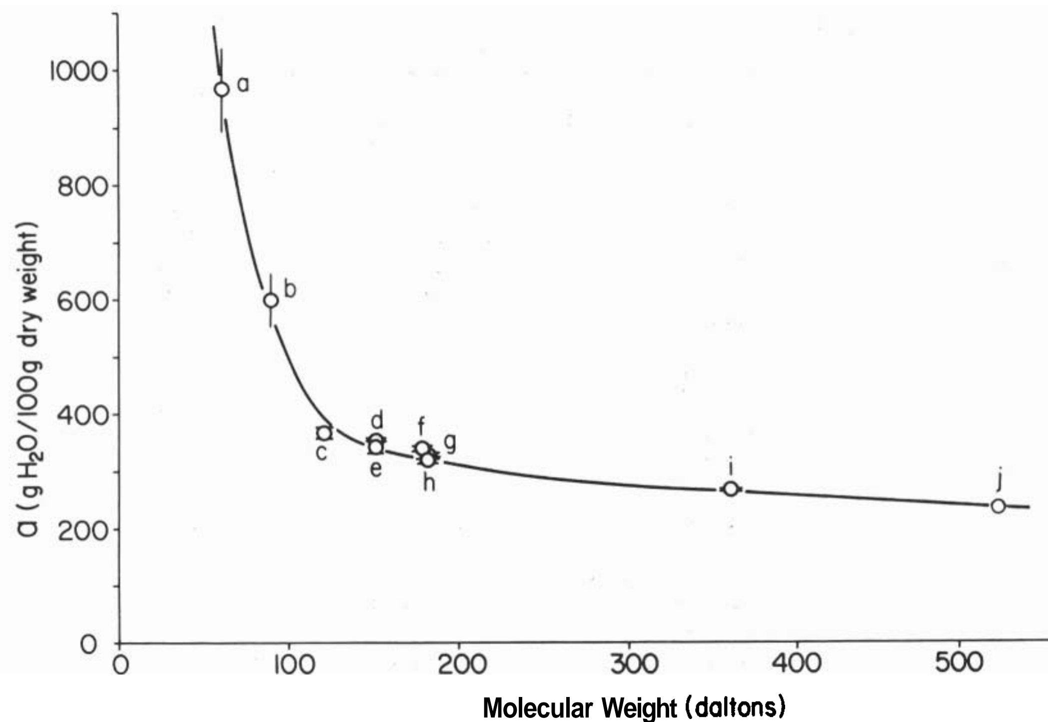


FIGURE 5. Water contents of frog muscles after 4 hours incubation (25°C) in 0.4 M sugar and sugar alcohols plotted against their molecular weights. a, ethylene glycol; b, glycerol; c, erythritol; d, 7 pentoses (344 ± 5) (m.w., 150.13); e, xylitol; f, 7 hexoses (334 ± 6) (m.w. 180.16); g, sorbitol; h, mannitol; i, 7 disaccharides (263 ± 2) (m.w., 347.4); j, melezitose. Note: a-values (and molecular weights (m.w.) when necessary) for sugars are averages of data given in Tables 1 and 2.

Table 2 tabulates results of similar studies on various sugar alcohols. The averages and standard errors of the a-value data given in Tables 1 and 2 are plotted in Figure 5 against the molecular weights of the sugars or sugar alcohols. All points fall on or near a single continuous line. Worth noticing is the fact that the dependence of a-values on molecular weights transcends the boundaries between sugar and sugar alcohols.

To emphasize the quantitative dependence of a-value on the molecular weights of the solutes, the data represented by points c to j in Figure 5 are replotted in Figure 6 with a more expanded vertical axis and shrunken horizontal axis. This plot further emphasizes the general agreement of the data with Prediction 1 of the AI hypothesis. The water content of frog muscle equilibrated in the same concentration of sugars or sugar alcohols varies with the sizes of sugar and sugar alcohol molecules measured as their molecular weights. This relationship seen was not predicted by the membrane-pump theory.

The Relationship Between the q-values of Sugar and Sugar Alcohols in Cell Water and the Water Contents of Muscle Equilibrated in a Solution Containing Different Sugar or Sugar Alcohols at the Same Concentrations. Table 3 summarizes the q-values of different sugars and sugar alcohols in frog muscles.

TABLE 1. Water contents, in gm H_2O/100 gm dry cell weight, of frog muscles equilibrated in 0.1 M and 0.4 M pentoses, hexoses, disaccharides, and one trisaccharide. Incubation time was 4 hours at 25°C. The first number in brackets indicates the number of sets of experiments, the second number represents number of individual assays.

		0.1 M	0.4 M
Pentoses	D-arabinose	735 ± 31 [2, 8]	324 ± 9 [2, 8]
	L-arabinose	894 ± 42 [2, 8]	355 ± 35 [2, 8]
	D-xylose	836 ± 56 [1, 4]	340 ± 9 [1, 4]
	L-xylose	861 ± 28 [2, 8]	340 ± 7 [2, 8]
	D-lyxose	927 ± 26 [1, 4]	336 ± 10 [1, 4]
	L-lyxose	709 ± 34 [1, 4]	347 ± 5 [1, 4]
	D-ribose		368 ± 12 [1, 4]
He.oses	D-glucose	691 ± 22 [1, 4]	326 ± 5 [1, 4]
	fructose	639f 10 [1, 4]	339 ± 5 [1, 4]
	L-glucose	713 ± 41 [2, 8]	318 ± 13 [2, 8]
	galactose	705 ± 39 [1, 4]	336f 8 [1, 4]
	fucose	728 ± 24 [1, 4]	367 ± 4 [1, 4]
	mannose	648 ± 21 [2, 8]	342 ± 7 [2, 8]
	tagatose	659 ± 30 [1, 4]	311 ± 4 [1, 4]
Disa charides	turanose	552 ± 12 [1, 4]	254 ± 23 [1, 4]
	cellobiose	553 ± 11 [2, 8]	267 ± 4 [2, 8]
	melibiose	578 ± 7 [1, 4]	268 ± 8 [2, 8]
	sucrose	566 ± 16 [1, 4]	255 ± 5 [1, 4]
	trehalose	580 ± 9 [1, 4]	262f 6 [1, 4]
	maltose	599 ± 4 [2, 8]	265 ± 6 [1, 4]
	lactose	588 ± 8 [2, 8]	267 ± 3 [1, 4]
Tri-saccharid s	melezitose	545 ± 10 [1, 4]	234 ± 5 [1, 4]
	raffinose	539 ± 12 [1, 4]	

Very high q-value was observed for ethylene glycol followed by glycerol, erythritol, etc. In Figure 7 the a-values of 10 sugars and sugar alcohols at 0.4 M concentration given in Tables 1 and 2 are plotted against their respective q-values. A linear coefficient of +0.973 was obtained. Thus Prediction 3 based on the AI hypothesis was also confirmed.

DISCUSSION

Quantitative cell physiology began with the studies by De Vries (1884) and Pfeffer (1877) of volume changes of plant cells when placed in solutions of different concentrations. This work in turn provided the foundation for Pfeffer's membrane theory. One of the most important earlier findings (Höfler, 1918, 1930, 1931) was a constancy of the product of cell volume (V) and the concentration of the solution (C) in which the cells were immersed in agreement with the Boyle van't Hoff law:

$$VC = \text{constant}. \quad (6)$$

Subsequent studies of Höfler (1932) of the change of the volume of the cytoplasm and of the central vacuole, and by Chambers and Hofler (1931) of isolated central vacuoles revealed that this exact osmotic behavior reflected the properties of the central vacuole enclosed in a tonoplast rather than that of the plasma-membrane enclosed cytoplasm. Indeed, later workers like Plowe (1931), Strugger (1932), and Ullrich (1939) showed that in strong solutions of electrolytes the cytoplasm swelled while the central vacuole shrank, confirming the earlier conclusion of Höfler and his coworkers. Rubenstein (1939, p. 3), a strong supporter of Pfeffer's membrane theory, made the following com-

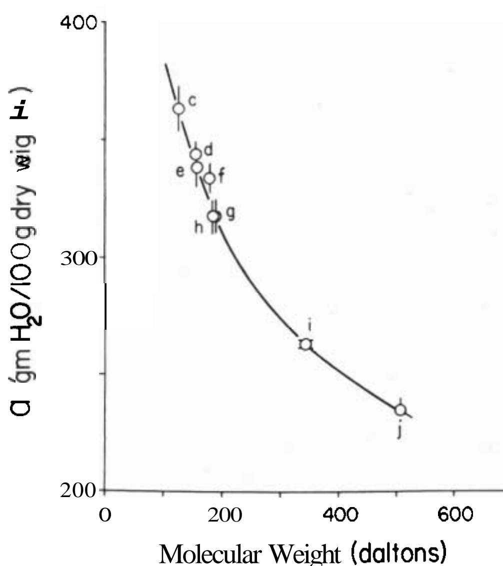


FIGURE 6. Water contents of frog muscles after 4 hours incubation (25°C) in 0.4 M sugars and sugar alcohols plotted against their molecular weights. Data on ethylene glycol and glycerol excluded and the vertical and horizontal scales expanded and condensed respectively. Data same as shown in Figure 5.

TABLE 2. Water contents of frog muscles equilibrated in 0.4 M sugar alcohols. Incubation time was 4 hours at 25°C. The first number in brackets indicates the number of sets of experiments, the second number represents number of individual assays.

	m.w.	a (gm H_2O/100 gm dry cell weight)
Ethylene glycol	62.07	967 ± 74 [3, 8]
Glycerol	92.09	652 ± 40 [3, 8]
Erythritol	122.12	363 ± 10 [3, 8]
Xylitol	152.1	339 ± 12 [1, 4]
Mannitol	182.2	318 ± 10 [1, 4]
Sorbitol	182.2	322 ± 9 [1, 5]

ments, ". . . we are compelled to disregard completely in our calculations all results obtained by the osmotic method in the study of plant cells, results which historically, as is well known, were the basis of all contemporary teaching on the semipermeable plasma envelope . . ." (English translation from Troshin, 1966, p. 32). Rubenstein then suggests more studies on animal cells which possess no central vacuoles.

It turned out that animal cells do not behave like perfect osmometers either. Thus Overton (1902) found that quantitatively the volume of frog muscles did not follow the prediction of eqn. (6). To explain, Overton postulated that 35% of the muscle cell water was "osmotically inactive". The need to postulate "osmotically inactive" water also arose from studies of red blood cells (Ponder, 1934), eggs of marine animals (Lucké, 1940), and other cell types.

A. V. Hill (1930) suggested that deviation of frog muscle from ideal osmotic behavior was due to the presence of a substantial percentage of dead cells. This explanation is no longer acceptable for the simple reason that swelling in hypotonic solution of carefully dissected frog muscles is fully reversible and reproducible. But even the acceptance of a fraction of osmotically inactive water does not solve the problem, because the size of this fraction needed varies with both the nature and concentration of the solute used (Schiodt, 1931).

The conventional way of osmotically manipulating cell volume is limited in the range of high osmotic activity by the solubility of the solutes used (e.g., sucrose, NaCl) and by the concentration (C) of the solute that can be tolerated by the cells. In the vapor sorption method, described by Ling and Negendank, this limitation was removed. In that case, one

TABLE 3. Equilibrium distribution coefficients or q-values of various sugars and sugar alcohols in frog muscles. Data from unpublished results from experiments carried out over a period of many years. Whenever similar methods were used, all the data are pooled and analyzed statistically (e.g., as in the case of ethylene glycol). When different methods were used, different groups of data are separately presented (e.g., mannitol) and an overall average given. First number in brackets under q refers to number of sets of experiments performed. The second number refers to number of individual assays. I, IIa, and IIb in brackets under "Methods Used" refers to methods as described in text; the number following refers to number of sets of experiments performed. *Assays on single muscle fibers, taken from Ling, et al. 1969a.

	q (mean ± S.E.)		Methods Used
Ethylene glycol	1.10 ± 0.046 [6, many]		[I, 2; IIa, 4]
Glycerol	0.877 ± 0.024 [8, many]		[I, 4; IIa, 4]
Erythritol	0.331	0.332 [1, 15]	[IIb, 1]
		0.330 [1, many]	[IIb, 1]
Xylitol	0.185 ± 0.012 [1, 14]		[IIa, 1]
Mannitol	0.156	0.179 ± .005 [1, 30]	[IIa, 1]
		0.164 ± 0.010 [1, 30]	[IIa, 1]
		0.125*	[IIb, 1]
Sorbitol	0.179 ± 0.013 [1, 15]		[IIa, 1]
D-ribose	0.25		[IIb, 1]
D-xylose	0.30 ± 0.034 [7, many]		[IIa, 7]
D-glucose	0.24		[IIb, 1]
Sucrose	0.21	0.295	[IIb, 1]
		0.125*	[IIb, 1]

could produce near zero p/p_o by equilibrating muscles in an atmosphere whose vapor pressure was controlled by concentrated sulfuric acid in direct contact with the vapor phase but not the muscle tissues. The results clearly showed that the C vs. V plot is rectilinear (and thus in agreement with eqn. (6)) only over a limited range of C. At lower (p/p_o), produced by high concentrations of sulfuric acid, the C vs. V plot was no longer rectilinear but curves downward to converge on the origin of zero volume at zero (p/p_o). As a whole, the data show that eqn. (6) is not applicable and that the practice of extrapolating from the rectilinear portion of the V vs. $1/C$ plot to zero $1/C$ to yield an "osmtically inactive" fraction of cell water was incorrect and misleading.

However, long before the publication of Ling and Negendank, other scientists had reached the conclusion that osmotic behavior of living cells might originate from the colloidal properties of the living cells (Vasil'yev, 1922).

Nasonov and His Coworkers' Findings on Sustained Shrinkage of Living Cells in Solutions of Permeant Solutes. In the Introduction, it was pointed out how studies using radioactive tracers in the 40's had shown that Na^+ salt fully capable of causing sustained cell shrinkage, is, contrary to one widely-held belief, quite permeant to the cell membrane. This finding eventually led to the theory of Na pump-maintained cell volume. However, in the 30's Nasonov and his coworkers had

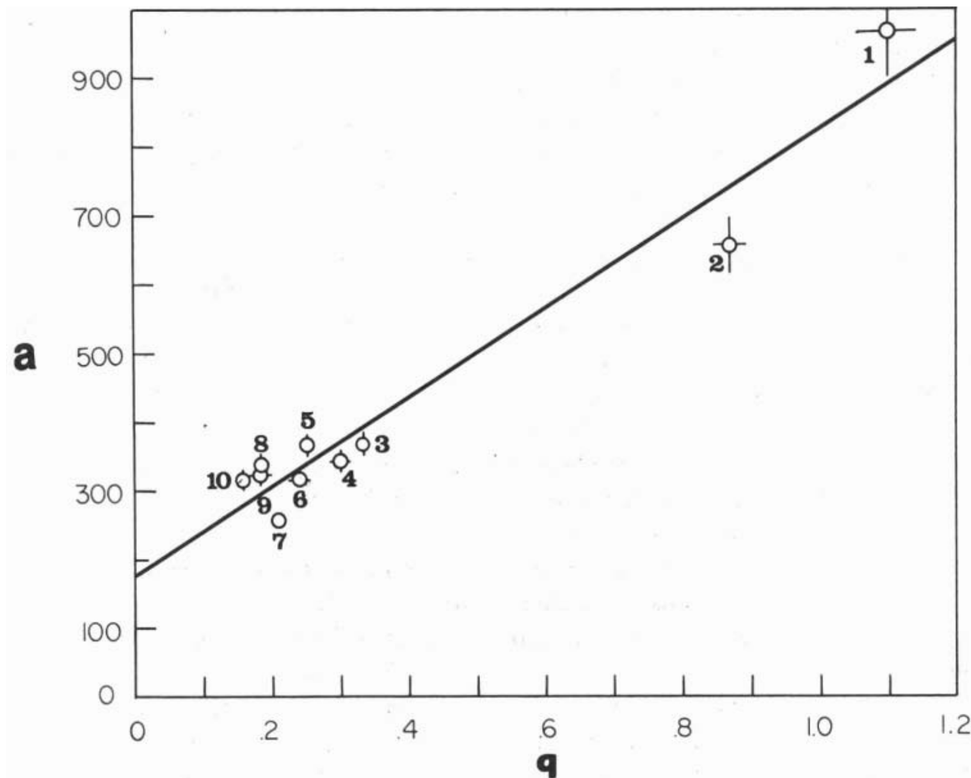


FIGURE 7. The relationship between the water contents (a) of frog muscles when immersed in a 0.4 M solution of various sugars and sugar alcohols with different equilibrium distribution coefficients (q) in the muscle water. Solid line obtained by the method of least square. The linear correlation coefficient is +0.973. 1, ethylene glycol; 2, glycerol; 3, erythritol; 4, D-xylose; 5, D-ribose; 6, D-glucose; 7, sucrose; 8, xylitol; 9, sorbitol; 10, mannitol.

clearly shown that penetrating noneectrolytes can cause sustained shrinkage of frog muscle (Nasonov and Aizenberg, 1937). They analyzed by chemical methods the concentrations of various nonelectrolytes that had entered the frog muscle cells after immersion of the tissues in solutions of the nonelectrolytes.

Water Uptake of Living Cells as a Multilayer Adsorption Phenomena. In 1923 Walters compared the water uptake of living cells with water sorption of gelatin, starch, casein, and nucleic acid (for reproduction of Walter's key figure, see Ling, 1984, p. 286). Ling proposed the polarized multilayer theory of cell water in 1965. As mentioned above, the predicted obedience of 95% of muscle cell water to Bradley's adsorption isotherm, shown earlier in eqn. (1), was published in 1970 (Ling and Negendank, 1970). In 1967, Cope was able to extend the polarized multilayer theory of cell water to shown that eqn. (1) can be rewritten in such a way that a logarithmic relation between solute concentration ([S],,) and cell volume (V) can be predicted:

$$V = E \log_{10} [S]_{ex} + F , \qquad (7)$$

where E and F are constants. Data shown in Figure 4 in general affirms this relationship as well as its limitations. This equation, however, does not take into account the entry of solute into the cells and is therefore applicable only to vapor sorption data where solute of the solution used to provide a specific vapor pressure does not come into contact with and therefore cannot enter the cells or to cases where the solute in question has near zero q-values.

The derivation of Bradley's multilayer adsorption isotherm was a major accomplishment, if one considers how complex and difficult the problem is. Nevertheless it has limitations. Thus the three constants K_1, K_3, and K_4 are not explicitly given but are obtained only by data fitting. In the frog muscle study of Ling and Negendank, a chosen set of K_1, K_3, and K_4 were able to fit the data obtained from partial vapor pressure ranging from 0.043 to 0.996, which, by the usual standards, represent the full range of vapor pressure.

In the present study, we extended the upper range of partial vapor pressure considerably beyond 0.996. To fit the data from this high range of vapor pressure, K_4 must be set to zero. By doing so, eqn. (2) takes on the form of the original multilayer adsorption isotherm of De Boer and Zwikker (1929),

$$\log \frac{p}{K_3 p_o} = K_2 K_1^{a} , \qquad (8)$$

or of Bradley's first multilayer adsorption isotherm (Bradley, 1936):

$$T \log \left(\frac{p_o}{p}\right) = K_1 K_3^{a} . \qquad (9)$$

Indeed Cope's derivation of Equation 7 was also based on Equation 9 at constant temperature (T) or Equation 1 with K_4 set to zero.

Another limitation is from the Raoult's law. However, here the limit is surprisingly lenient. Thus from the extremely accurate data of Frazer et al. (1920), Raoult's law held to a mannitol solution of up to and including 0.9908 molal. This is equivalent to a molar concentration of 0.907 and not very far from the upper limit of the concentration range studied (1.0 M).

The Relationship Between Water Content and Cell Volume. Normal frog muscle contains by weight 80% water. Of the 20% dry weight, salt ions, lipids and carbohydrates may account for not much more than 1% of the cell weight. Thus the primary contributions of the cell weight are the 80% water and 19% proteins. Most proteins have a partial specific volume of 0.70 to 0.75 cc/g. (Edsall, 1953). Assuming an average of 0.73 cc/g., the volume of the 19% protein would amount to

0.19 X 0.73 = 0.139 cc. Assuming cell water to have the same density as normal liquid water, the total volume of one gram of fresh muscle is then 0.80 + 0.139 = 0.939 cc, corresponding to a density of 110.939 = 1.06 in agreement with the value of 1.066 measured for guinea pig muscle (Morales, Rathbun, Smith, and Pace, 1945).

For muscle cells equilibrated in a solution containing a solute, with a q-value in the cell equal to zero, the volume of the muscle originally weighing 1 gram is then

$$\text{volume} = a \times \frac{0.2}{100} + 0.19 \times 0.73$$

$$+ 0.002a + 0.139. \qquad (10)$$

However, for muscles equilibrated in a solution containing a solute at concentration C and with a q-value in the cell higher than zero, then the cell volume must include this solute present in the cell. Hence

$$\text{volume} = a \times \frac{0.2}{100} + 0.19 \times 0.73 =$$

$$= qC \times a \times \frac{0.2}{100} \times \frac{E}{d}, \qquad (11)$$

where d is the density of the solute in question; E, its molecular weight.

Equation 11 can give us a rough estimate of the contribution of the volume of solute that has penetrated the cells to the total cell volume. For illustration, one chooses two extreme cases:

(1) sucrose at 1.0 molal concentration. From Figure 4, one obtains an a-value of 170 gms. H_2O/100 gm. dry weight. The q-value for sucrose is 0.21 (Table 4); its density at 1.0 molal concentration is 1.588 (Hodgman, et al., 1961). Thus

$$\text{volume} = 170 \times \frac{0.2}{100} + 0.19 \times 0.73$$

$$+ 0.21 \times \frac{1}{1000} \times 170 \times \frac{0.2}{100} \times \frac{342.3}{1.588}$$

$$= 0.340 + 0.139 + 0.015 = 0.494 \text{ cc.}$$

with cell sucrose making up 0.015/0.494 = 0.031 or 3.1% of the total cell volume.

(2) Ethylene glycol at 1 molal concentration gives an a = 840 gm. H_2O/100 gm. dry weight. The q-value for ethylene glycol is 1.10; its density at 1.0 molal concentration 1.116.

$$\text{volume} = 840 \times \frac{0.2}{100} + 0.19 \times 0.73$$

$$+ 1.10 \times \frac{170}{1000} \times \frac{0.2}{100} \times \frac{62.07}{1.116}$$

$$= 1.68 + 0.139 + 0.021$$

$$= 1.84 \text{ cc.}$$

The ethylene glycol in the cell makes up $\frac{0.021}{1.84}$ = 0.0114 or 11.4% of the cell volume.

The Relation Between *q*- and a-values. A correlation coefficient of +0.973 was obtained between the q-values for sugars and sugar alcohols obtained earlier and the a-values obtained here. One must now consider two assumptions made for this comparison of q- and a-values: (1) that the q-values obtained mostly in the sugar and sugar alcohol concentration range of 100 mM or lower are valid at concentration 4 times higher; (2) that the q-values or at least the quantitative relationships among the different q-values are essentially similar at O°C (at which temperature q-values were determined) and at 25°C (at which temperature, the a-values were obtained). While the validity of these assumptions can only be fully tested in future studies, it is important to point out that they are by and large correct. Firstly, the correlation between a-values and q-values obtained at the solute concentration of 0.1 M is poorer (r = +0.848) (see Materials and Methods for possible cause) but not drastically different

from the correlation obtained at the solute concentration of 0.4 M (+0.973). The concentration of 0.1 M corresponds to the upper range of solute concentrations used to obtain the q-values shown in Table 3. Secondly, in cases where q-values of probe molecules in frog muscles were studied at two temperatures, the differences were minor. Thus Ling, Walton, and Ling (1979) showed that the q-value for Mg^{++} in frog muscle is 0.281 at 5°C and 0.264 at 25°C, differing by only $\frac{.281-.264}{.281}$ = .06 or 6%.

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