Changes in ionic selectivity with changes in density of water in gels and cells

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ABSTRACT Gels equilibrated with aqueous solutions of impermeant solutes reached a steady state in which, in the absence of a pressure difference, the activity of water in the pores of the gel was higher than that of water in the external solution. The chemical potential of water in the gel/polymer solution slurry was higher than that in the supernatant polymer solution removed from the gel. Water in the pores of the gel decreased in density to 0.96 as increasing osmotic stress was applied. It is argued that at constant temperature and pressure water can equilibrate between two compartments of unequal osmolality only by adjusting its molar volume. Experiments showed that when gel water had a higher activity than external water it was K^+ selective; when it had a lower activity it was Na^+ selective. It is proposed that a continuous spectrum of water structures can exist in these two compartment systems from dense, reactive, weakly-bonded water which selects highly hydrated ions, to expanded, stretched, unreactive, viscous water which is strongly hydrogen bonded and selects K^+ and univalent anions. These findings are related to the state and properties of cytoplasmic water which is probably held under osmotic stress by the activity of the sodium pump.

INTRODUCTION

The long-running debate concerning the structure and properties of intracellular water has focused almost exclusively upon the effects of surfaces upon water–water interactions (Ling, 1962; Clegg, 1984*b*; Parsegian and Rau, 1984). The problem has always been to decide how far from a surface might a perturbation of water–water interactions be propagated, estimates varying at different times from one water molecule to 1 μm. Experiments on nonbiological systems have suggested that changes that might be mechanistically significant for reactions in aqueous solutions and for distribution of solutes are unlikely to extend beyond a few nanometers (Wiggins and van Ryn, 1986). It is therefore somewhat surprising to find nonuniform distributions of solutes between nucleus and cytoplasm, as Horowitz and co-workers have consistently reported (Horowitz and Paine, 1979; Horowitz et al., 1979; Miller et al., 1984), a decrease in the self-diffusion coefficient of water in cells, measured both by nuclear magnetic resonance (Seitz et al., 1981) and by quasielastic scattering of neutrons (Trantham et al., 1984), and a decrease in its density (Clegg, 1984*a*). Moreover, physiologists have had to postulate the existence of "osmotically inactive water" to explain the immediate responses of many cells to changes in extracellular osmolarity (Dupre and Hempling, 1978).

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In the course of investigating the properties of water in the small rather hydrophobic pores of dense films of cellulose acetate, Wiggins and van Ryn (1986) found that the increased hydrogen bond strength of this water and its modified solvent properties were both amplified by solutes which were excluded from pore water, and abolished by solutes which were accumulated into pore water. It was suggested that these effects were due to osmotic pressure gradients across the imaginary interface between the two aqueous phases. For example, the osmolality of NaCl in such a pore was lower than that in the external solution at equilibrium, because NaCl has a stretched water/water partition coefficient less than unity. The activity of water in the pore was therefore higher than that of the external solution. To equalize its chemical potential in the two phases it apparently expanded outward, decreasing its density and increasing its hydrogen bond strength still more. As a result the partition coefficient of NaCl decreased with increasing concentration. The osmolality of KCl in such a pore, on the other hand, was higher than that of the external solution at equilibrium. The activity of the water was lower than that of the external solution and pore water equilibrated by increasing its density and decreasing its hydrogen-bond strength. The partition coefficient of KCl became essentially unity at quite low concentrations.

If this interpretation was correct it should be possible under appropriate conditions to dispense with the surface-water interaction, and using osmotic pressure gradients alone, change the density of water up or down, generating

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both water which selects Na^+ over K^+ as well as water which selects K^+ over Na^+. The experiments to be described have tested this hypothesis in simple gels, which are more amenable experimentally than complex biological systems. The significance of the results, however, is in their relevance to osmotic balance across biological membranes, volume regulation of cells, and the properties of water in organelles. They offer an explanation for those unusual properties of intracellular water that seem unlikely to result from surface-water interactions.

MATERIALS AND METHODS

Materials

Sephadex G-100 (Sigma Chemical Co., St. Louis, MO) and Biogels P-2, P-4, P-6, P-30, and P-100 (Bio-Rad Laboratories, Richmond, CA) were washed exhaustively and dried at 110°C before use. Agarose (type VII, low gelling temperature, was obtained from Sigma Chemical Co.). The dialysis tubing (Spectrum Medical Industries Inc., 60916 Terminal Annex, Los Angeles, CA) was 1 cm wide and had a molecular weight cut off of 8,000–15,000. Polyethylene glycol (PEG) 20 M was obtained from BDH Ltd., Poole, UK, and PEG 6000 from Merck-Schuchardt, Munich, FRG. Dextran of average molecular weight 264,000 and dextran sulphate of average molecular weight 500,000 were obtained from Sigma Chemical Co. Solutions were buffered with either Na^+ or K^+ phosphate (NaP_i or KP_i), 5 mM in phosphate, with the pH adjusted to 7 with NaOH or KOH, respectively.

Methods

Osmolality was measured using either a Wescor Inc. 5100C vapor pressure osmometer (Logan, UT), or a freezing point osmometer (Advanced Instruments Inc., Needham Heights, MA). Whereas these two instruments gave the same readings of osmolality for simple solutions, they gave different values for PEG solutions. This reflects the complex interactions of PEG with water; its osmotic coefficients are highly abnormal and strongly dependent on temperature. The vapor-pressure measurements made at room temperature are a better measurement of the chemical potential of water in PEG solutions at room temperature than are the depression of freezing point measurements. For convenience, however, the latter were used as a method of determination of PEG concentrations from a standard curve of concentration against osmolality. Standard curves were constructed using a constant concentration (5 mm) of either KP_i or NaP_i and increasing concentrations of PEG, or just increasing concentrations of PEG in water. The appropriate standard curve was used for each experiment.

The density of water in gels was measured in several steps, using 10-ml density bottles.

1. Calibration of density bottles

Small reagent bottles were filled with MilliQ water which had been deaerated by suction, and left, stoppered, overnight beside the balance in an air-conditioned balance room. One bottle was used to measure the temperature of the water. Empty density bottles, which had also been left beside the balance overnight, so that they would be in equilibrium with the ambient humidity and temperature, were weighed, together with their stoppers. Forceps were used throughout to handle the bottles so that the hydration of their external surfaces and the temperature of their contents did not change during weighing. It also proved important to weigh as quickly as possible, to avoid changes in temperature due to the proximity of the operator. Deaerated water was dispensed by Pasteur pipette from one reagent bottle to one density bottle, which was filled to such a level (found by trial and error) that, with insertion of the stopper, the water level rose to within 1–2 mm of the top of its capillary. The bottle was then placed on the pan of a model H 54 5-figure balance (Mettler Instrument Co., Highstown, NJ), and the final small volume of water added from a syringe needle, when the balance had reached its equilibrium position. These weighings were reproducible to the fourth decimal place. The fifth place was less reliable because the density bottle lost water slowly through its capillary during weighing.

The capacity of the bottle was calculated from the weight of deaerated water that it contained and the density of deaerated water at the ambient temperature obtained from standard tables.

2. Density of the dry gel

The effective volume of the dry gel material was obtained by equilibrating low weights (0.03–0.08 g) of the gel in preweighed and calibrated density bottles, which were filled to the top of the stopper with fully aerated MilliQ water. The tops of the stoppers were covered with parafilm and the bottles were left for 1 wk to equilibrate beside the balance. At the end of the week they were finally filled to the top and weighed as described in the previous section. Great care was needed to exclude all air bubbles. On the same day, the density of the fully-aerated water used in this measurement was also determined by weighing in calibrated weighing bottles.

The rationale of this procedure was that the dry gel contained air, which was displaced by water during equilibration. If water had been initially air-free, some of the displaced air would have dissolved in the water, changing its density by an unknown amount. If, however, the water used was initially fully aerated, then displaced water formed a bubble which rose to the top and was lost to the atmosphere. The problem of retention of air by the gel was also corrected for by this method, because such air would be a constant volume per unit weight of gel. It turned out that the difference in density between fully aerated and deaerated water at the same temperature amounted only to one or two in the fifth decimal place, and this precaution of using deaerated water to calibrate the density bottles, and fully aerated water to measure the effective density of the gel was probably unnecessary.

The apparent density of a gel decreased in the presence of other solvents in the order water > methanol > ethanol > hexane. Presumably there were micropockets in the gel matrices which were accessible to water molecules but became increasingly inaccessible as the molecular weight of the solvent increased. It was essential, therefore, to derive the volume inaccessible to water from equilibrations with water.

3. Average density of water equilibrated with gel

The procedures used were the same as before, except that larger amounts of gel were used. The ranges of weights were: 0.1–1.6 g for Biogel P-2, 0.1–0.8 for Biogel P-30, and 0.1–0.5 for Biogel P-100.

4. Density of water in gels equilibrated with solutions of PEG

Various weights (from 0.1–1.1 g) of Biogel P-100 were equilibrated in density bottles with a solution of PEG 20M, 12% wt/wt. On the same day as the final weighing, the densities of a range of PEG solutions from 12 to 20% wt/wt were measured, and a standard curve of density against concentration constructed. After the final weighing samples of the supernatant solution were removed, and their osmolality measured. This was then converted into a concentration from a standard curve of osmolality against concentration (wt/wt).

Calculations

1. Effective density of dry gel (d_g g ml^{-1})

The apparent density of dry gel, calculated assuming that all water in the density bottle had the same density (d_w g ml^{-1}) as bulk water at the same temperature, is given by:

$$d_g(\text{app}) = W_g/(V_b - W_t/d_w),$$

where V_b is the volume of the density bottle in milliliters, W_t the total weight of water, and W_g the weight of gel in grams. The true density of the gel is:

$$d_g = W_g/(V_b - W_i/d_i - W_e/d_e),$$

where the subscripts i and e refer to internal and external water, respectively. If

$$d_e = d_w, \text{ then } 1/d_g - 1/d_g(\text{app}) = W_i(1/d_w - 1/d_i)/W_g,$$

W_i/W_g is the water regain in grams per gram dry weight of gel, and is therefore constant for each gel. Therefore,

$$1/d_g - 1/d_g(\text{app}) = k(1/d_w - 1/d_i).$$

In other words, the apparent density of the gel is larger or smaller than its true density by a constant unknown amount. Therefore, the true density must be derived indirectly.

The average density of water in contact with the gel is:

$$\bar{d} = (W_i + W_e)/(W_i/d_i + W_e/d_e).$$

If water inside and outside the gel has the same density, d_w, then $\bar{d} = d_w$, and is independent of both the weight and the density of gel. If d_i is not equal to d_e, $\bar{d}$ depends both on the weight and the density of gel, because it is calculated as

$$\bar{d} = W_t/(V_b - W_g/d_g).$$

It is thus a nonlinear function of W_g, which must extrapolate to d_w at zero gel weight. The best estimate of d_g was therefore obtained by assigning it values, and determining that value for which $\bar{d}$ extrapolated to d_w over a range of low gel weights (0.03–0.08 g). That value was used in further calculations. This was probably the greatest source of error in the density measurements, but it was a systematic error, and negligible in the experiments in which gel was equilibrated with PEG solutions.

2. Density of water in gels equilibrated with PEG solutions

The initial concentration of PEG was 12% (wt/wt), so that the total weight of PEG was W_t (12/112) g, where W_t is now the total weight of solution in the density bottle. It was assumed that when this solution had equilibrated with gel, all the PEG remained outside the gel, but some water entered the pores. If the final concentration of PEG in the external solution was x% (wt/wt), then the weight of solution external to the gel was given by:

$$W_e = W_t(12/112)/(100 + x)/x \text{ g},$$

and the weight of internal water by:

$$W_i = W_t - W_e.$$

The volume of internal water is

$$V_i = V_b - W_e/d_x$$

where d_x is the density of a solution of PEG at a concentration of x% (wt/wt), and the density,

$$d_i = W_i/V_i \text{ g ml}^{-1}$$

Equilibration experiments

Dry Sephadex or Biogel was weighed into 4 ml polyamide tubes (internal diameter 1 cm, height 7 cm), rotamixed with solution and left stoppered at room temperature for 6 d with daily rotamixing. Solutions of PEG were usually made up wt/wt, because weighing was more accurate than pipetting these viscous solutions, and calculations of the water contents of gels were more accurate. When it was necessary to keep concentrations of other solutes constant, however, they were made up wt/vol.

Agarose gels were made by bringing 1.5 g agarose in 7.5 ml water to the boil. In preliminary experiments the solution was injected directly into (dialysis) tubing, but in subsequent experiments it was poured on to a glass plate and spread with a glass rod, to a uniform thickness of ~1 mm. The gel was cooled at 4°C overnight, and then cut into strips just thinner than the tubing, which was 1 cm across. Using forceps, strips were inserted into wet tubing, which had been boiled in excess distilled water, and the tubing tied at both ends. Preliminary experiments showed that gels of this thickness lost water to a PEG solution over a period of 2 d, and exchange of ions took considerably longer. Gels were equilibrated in 10 ml solution in 15-ml plastic tubes on a Matburn Mixer (Smith Biolab Ltd., Auckland, New Zealand) for 5 d. The strip of gel was removed from the dialysis bag, weighed, dried at 110°C, and reweighed to obtain wet weight, dry weight, and water content.

The dialysis bag was removed, and its contents weighed, dried at 110°C for 2 h, and reweighed to obtain wet and dry weights of the gel. The dry gel was extracted overnight in 3 ml 10% trichloracetic acid (TCA) and the extract analyzed for Na^+ and K^+ using a model 400 flame photometer (Corning Glass Works, NY) with external standards made up in 10% TCA. In some experiments HNO_3 (0.1 M) was used both to extract ions and to make external standards. The results were the same, indicating that all the ions were extracted under these conditions. It has been established previously that an overnight extraction is sufficient. Phosphate was determined by the method of Baginsky et al. (1967).

Infrared spectral measurements

After equilibration, the gel was spun at 5,000 rpm for 5 min in a bench-top centrifuge, and the supernatant removed quantitatively. Infrared spectra were recorded from 16 scans using an FTS-60 FTIR spectrometer (Digilab Division, Biorad, Cambridge, MA) with an air-cooled deutero triglycine sulphonate detector. Resolution was 4 cm^{-1}. The gel sample was placed between silicon windows with a spacer of 0.015 mm in a presslock cell.

RESULTS

A microporous bead imbibes water to a characteristic swelling volume, which depends upon the degree of cross-linking of the gel. In the presence of water alone, the final steady state is such that there are no solutes in the water either in the pores of the gel or in the external phase. Water has the same activity (which is its concentration times an activity coefficient) in the two phases. The

condition for equilibrium distribution of water is that its chemical potential is the same throughout. The chemical potential of water is a function of its activity, and of pressure, temperature, and molar volume. For a bead in water, the activity of water is the same in both compartments, there is no pressure difference between compartments, so that water can come to equilibrium with activity, pressure, temperature, and molar volume all constant throughout. When a soluble polymer, larger than the pores of the gel, is added to the suspension, the gel visibly contracts, because the equilibrium distribution of water has been disturbed. Pressure and temperature are still constant throughout, but the concentration of water is now lower in the external phase, which contains solutes, than in the internal phase, which does not. A new steady state is established with some solute-free water remaining in the pores of the gel. Nothing has happened to change the temperature or the pressure, but water must equilibrate between the two compartments. With pressure, temperature, and water activities fixed, water has only one degree of freedom left: It can change its molar volume, which is the volume occupied by 1 mol of water. Thus, to equilibrate water must expand out of the pore, leaving behind a liquid which has a lower density than normal. Wiggins (Wiggins and van Ryn, 1986; Wiggins and MacClement, 1987; Wiggins, 1988) called this a form of stretched water, similar to water under tension (Henderson and Speedy, 1980).

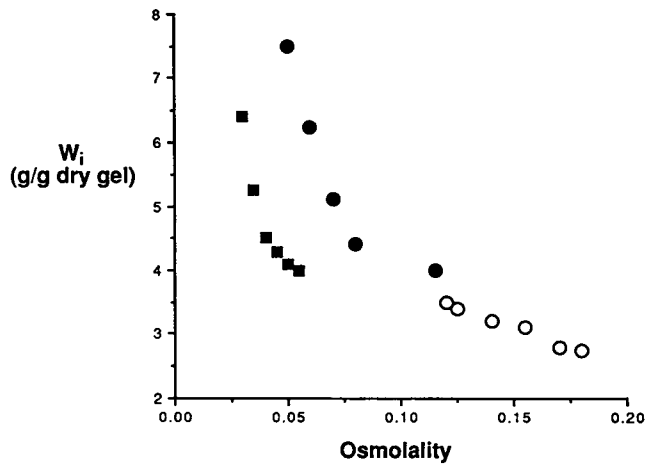

FIGURE 1 Water content of gels equilibrated with solutions of impermeant solutes of increasing osmolality. Increasing weights of each gel were equilibrated with the same weight of a polymer solution. (■) Sephadex G-100 in 6% wt/wt PEG 20 M (0.1 – 0.35 g gel + 2.2 g solution). (○) Biogel P-30 in 12% PEG 20 M (0.1 – 0.35 g gel + 3 g solution). (●) Biogel P-30 in 6% PEG 20 M (0.1 – 0.35 g gel + 3 g solution).

Reproducibility of results

Because many of the initial experimental results were unexpected and puzzling, all the experiments shown were repeated at least twice. The gels are synthetic; there was no built-in biological scatter, and reproducibility was excellent with standard deviations usually smaller than the symbol.

Fig. 1 shows the results of experiments in which various weights of Sephadex G-100 were equilibrated with solution containing 6% wt/wt PEG 20 M, and weights of Biogel P-30 were equilibrated with either 6% wt/wt or 12% wt/wt PEG 20 M. As shown by Edmond et al. (1968), as the osmolality of the external solution increased the water content of the gel decreased in a nonlinear fashion. Even at the highest concentration of polymer, however, water remained in the pores. These water contents were calculated from a calibration curve of concentration of PEG vs. its osmolality measured in a freezing point osmometer. The osmolality of the polymer solution before and after equilibration with the gel was measured, and converted to a concentration. Then, assuming that no PEG had entered the pores, the amount of water inside the pores was calculated. The effect was independent of the particular chemistry of the gel (dextran or polyamide). The effect was also independent of the particular polymer used, provided that it was too big to penetrate the pores of the gel. For example, PEG 6000 partially closed the pores of Biogel P-4, and dextran of average molecular weight 264,000, partially closed the pores of Sephadex G-100 and of Biogel P-100 (results not shown).

After equilibration with the polymer solutions the gels of Fig. 1 settled, so that a sample of the supernatant could be removed for measurement of its osmolality. The gels could also be remixed into a rather uniform slurry of water-filled beads suspended in polymer solution. Therefore, the osmolality of the slurry was measured in the freezing point osmometer before removal of the sample of supernatant for measurement of its osmolality. These two measurements of osmolality differed significantly: The value obtained for the gel/polymer solution slurry was less than the value obtained for the isolated supernatant, the difference increasing as the quality of gel relative to polymer solution increased. The difference in osmolality was found both by freezing point depression and by vapor pressure measurements. Fig. 2 summarizes these results by plotting the ratio of the weight of water in the pores of the gel to the weight of water in the external solution against the ratio of the osmolality of the isolated supernatant solution to the osmolality of the gel/polymer solution slurry. Again this effect was independent of the nature of the gel, and independent of the nature of the polymer (dextran gave a similar result). When Sephadex G-100

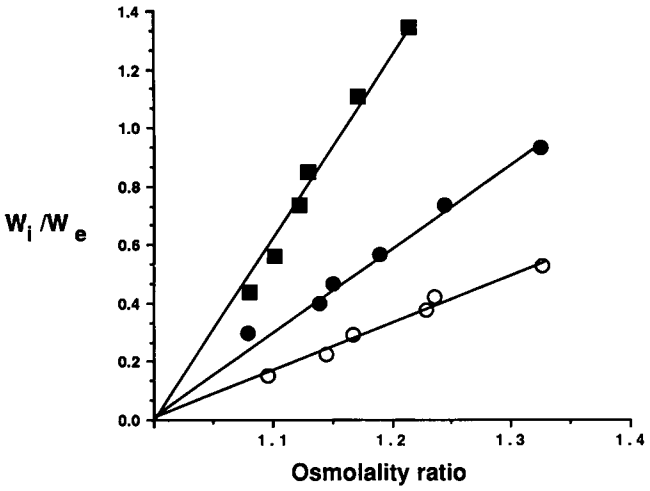

FIGURE 2 The ratio of the water content of the gel to the water content of the polymer solution at equilibrium as a function of the ratio of the osmolality of the gel/polymer solution slurry to the osmolality of the polymer solution removed from the gel. The symbols are the same as in Fig. 1.

was equilibrated with NaCl only there was no difference between the two values of osmolality at any ratio of gel to external solution, showing that the observed differences were a property of the gel/polymer solution system, and no artifact of measurement. One explanation of this result is that the water in the pores of the gel was not in equilibrium with the water in the polymer solution, so that the apparent osmolality measured in the gel/polymer solution slurry was an average of that in the pore water and that in the polymer solution. An alternative explanation is that the water was in equilibrium, with unequal activities but equal chemical potentials. Commercial osmometers, whether they are of the vapor-pressure or freezing-point depression type are misleading in that they record their findings in terms of osmolalities. This is valid for simple solutions, in which the chemical potential of water is determined primarily by its activity, and van't Hoff's equation is a good approximation, but it seems to fall down in these more complex gel/polymer solution systems in which activities of water only partially determine its chemical potential. Equality of chemical potential, which is the absolute criterion of equilibrium, must then be achieved by changes in the partial molar volume of water in one or both phases. Pore water, which has higher activity, may expand out and increase in specific volume, or water of lower activity in the polymer solution may contract and decrease in specific volume. When most of the water of the system is in the polymer solution and little in the gel, the structure of water in the polymer solution is little affected, but as the proportion of water in the gel increases the polymer solution is increasingly stressed and responds by decreasing its specific volume. This perturbation of the polymer solution is relieved when the solution is removed from the gel, and water resumes its normal density, with a decrease in chemical potential.

To distinguish between these two possibilities, it is necessary to measure properties specifically of water inside the pores. If that water is not in equilibrium with water in the polymer solution, but is still in the process of diffusing slowly out, it should have normal density, structure, and solvent properties, and none of these properties should change when it is removed from the polymer solution. If, however, pore water expands and stretches in the presence of the polymer solution, then its solvent properties should be those of stretched water in small hydrophobic pores (Wiggins and van Ryn, 1986), its density should be lower than that of normal water, and its degree of hydrogen bonding increased. All these properties should revert to normal when the gel is removed from the polymer solution.

Discrimination between Na^+ and K^+

One of the most interesting properties of stretched water in small hydrophobic pores is that it discriminates between Na^+ and K^+ ions in solution. K^+ tends to accumulate into stretched water, whereas Na^+ tends to accumulate into normal water. If pore water equilibrated with a polymer solution is also stretched, it should discriminate between the two cations also. Experiments similar to those of Fig. 1 were therefore repeated in the presence of solutions of PEG or dextran buffered with either Na^+ phosphate or K^+ phosphate (5 mM, pH 7).

Fig. 3 shows a typical result in which Biogel P-4 was

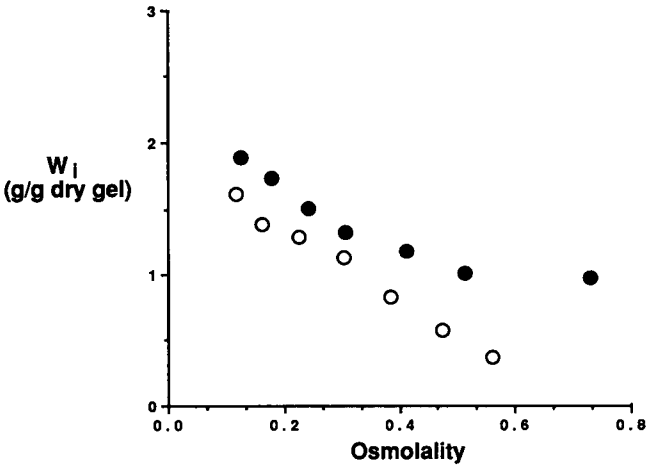

FIGURE 3 The water content of Biogel P-4 (0.4 g), equilibrated with 3 ml of increasing concentrations of PEG 20 M in 5 mM NaP_i (○) or KP_i (●).

equilibrated with phosphate-buffered solutions of PEG 6000, which is excluded by size from the pores of this gel. As the osmolality of the polymer solution (measured in the freezing point osmometer) increased, the amount of water in the gel decreased, as in Fig. 1, but for a given osmolality the amount of water inside the gel was always greater in the presence of K^+ phosphate, and this difference became more marked with increasing osmolality of the polymer solution. As in the experiment of Fig. 1 these water contents were estimated using the osmolality of the polymer solution before and after equilibration. This introduced little error, because the concentrations of Na^+ and K^+ phosphates which contributed to the osmolality readings did not change much with equilibration. This result suggests that K^+ was relatively accumulated into the pore water, so that the difference in water activity between the two phases was decreased or abolished, and less water left the pores for the polymer solution. Na^+, on the other hand, was excluded from the pore water, increased the difference in water activity between the two phases, and more water left the pores. There was little difference in concentration of either Na^+ or K^+ in the supernatant after equilibration, because both cation and water either went into the proes or stayed out of the pores. Because the concentration of PEG was used to calculate internal water content of the gel, the effect of removal of the polymer solution on the water contents in the presence of Na^+ and K^+ phosphate alone could not be estimated.

In many experiments the different volumes in the presence of Na^+ and K^+ phosphates were obvious from mere inspection of the gel. It was this very consistent finding that suggested the series of experiments represented by that of Fig. 3. Fig. 4 shows an example in which the height of the column of gel (Sephadex G-100, 0.1 g in 2.5 ml of solution) was measured relative to the height of the volume of solution at different concentrations of PEG 20 M in either 5 mM Na^+ phosphate or 5 mM K^+ phosphate. In the absence of PEG the heights were the same, but with increasing osmotic stress the height in the presence of K^+ at first increased and then decreased relative to that in the presence of Na^+. There are two opposing effects which result in this nonlinear behavior, which we have investigated in depth using other solutes (experiments to be described elsewhere). Stretched water selectively accumulates K^+ rather than Na^+. As the pore water took up more solute, its chemical potential decreased and it no longer needed to leave the pore or to stretch as much to equilibrate with the external solution. Therefore the bead swelled, relative to a bead in NaP_i, which was excluded from the pore water. Swelling of the bead tended to increase the height of the gel column. As the external osmolality increased, however, a bead accumulated relatively more KP_i than water, so that the internal solution (not the internal water) increased in

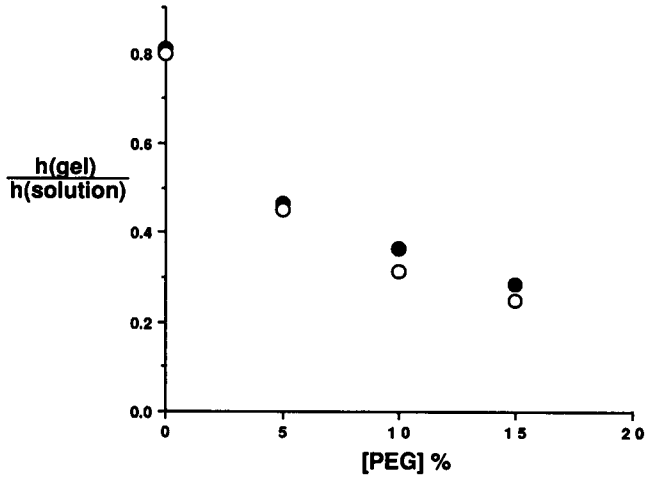

FIGURE 4 The ratio of the height of the gel column to the height of the solution when 0.1 g Sephadex G-100 was equilibrated with 2.5 ml of increasing concentrations of PEG 6000 in either 5 mM KP_i (●) or 5 mM NaP_i (○). The absolute heights of the gel plus solution were constant at 44 mm, whereas the height of the gel varied from 35 to 11 mm. Points are the means of duplicate determinations with standard deviations smaller than the size of the symbol.

density, while the external solution, depleted of KP_i, decreased in density. Therefore beads which had been rather floating in the external solution tended to sink. When the gel heights of the experiment of Fig. 3 were plotted in the same way the ratios which were equal in the absence of PEG, and diverging up to an osmolality of 0.4, were the same for the next pair of points, and had crossed over for the final pair.

The ratios in the presence of KP_i remained almost constant, but the gel height in NaP_i rose considerably; i.e., under great osmotic stress, the solution in the pores of a bead equilibrated in NaP_i was much less dense than the external solution, both because its water was less dense than external water, and because it had a much lower concentration of NaP_i than external water. Therefore, in spite of the smaller size of its beads, a gel column in NaP_i began to float, while the same column in KP_i began to sink. Similar trends were observed with Sephadex G-100 and Biogel P-100 in solutions of dextran 26400, and with Biogel P-30 and Biogel P-100 in solutions of PEG 20 M. This relatively crude measurement shows that, in the absence of polymer in the external solution the solvent properties of water were normal, but that they deviated from normality as the osmotic stress was applied.

Density of osmotically stretched water

Various weights of Biogel P-100 were equilibrated for 6 d at room temperature (24°C) with solutions of 12% (wt/

wt) PEG 20 M in density bottles. The effective density of the dry gel was estimated to be 1.566 ± 0.015 (mean and standard deviation of 25 determinations using weights of gel from 0.02 to 0.06 g). The density of the PEG solution used, measured on the same day was 1.056 g ml^{-1}. During equilibration the PEG solution concentrated as water entered the pores of the gel, the degree of concentration increasing as the weight of gel increased. Fig. 5 shows that as the weight of gel in the density bottle increased, the average density of the solution added to the gel decreased. This calculation involves no assumption concerning the distribution of PEG between inside and outside compartments. It uses only a constant value for the density of the dry gel, and the weights and volumes of the solution and gel. A possible explanation that can be considered is that as the gel contracted with increasing concentration of PEG, microcavities in the gel which had previously been accessible to water molecules closed, so that the effective density of the dry gel was not constant, but decreased in Fig. 5 with increasing gel weight. This possibility was tested by recalculating densities of the gel, assuming that the average densities of the solution before and after equilibration were the same. The result of this calculation was that the dry gel density had to increase, not decrease, with increasing gel weight from improbably low values of 0.243 to 0.963 g ml^{-1}. It seems probable, therefore, that the density of water in the pores of the gel was lower than that of normal water.

To calculate the density of water inside the pores of the gel, the concentration of PEG at the end of equilibration was determined by measuring the osmolality of the supernatant solution, and deriving the concentration from a standard curve. Densities of PEG solutions measured at the same temperature were a linear function of concentration. The density of water inside the pores of the gel could be calculated from the concentration of PEG put on the gel initially, the final concentration of PEG, the densities of those two solutions and the density of the dry gel. Two assumptions were necessary for this calculation: first, that no PEG entered the pores, and secondly, that the density of the external PEG solution was the same as the measured density of the same solution off the gel. The first assumption was not absolutely valid because the PEG 20M used had the usual range of molecular weights and it is likely that some of the smaller fragments penetrated the larger pores. The effective diameter of PEG 20 M was found by quasielastic scattering to be 7.6 nm (Georgalis, 1983) and the original maximal pore diameter of Biogel P-100 is ~6.1 nm. Leakage of PEG into pores of this size was demonstrated in the experiment illustrated in Fig. 4. Although the heights of the gel were clearly greater in the presence of K^+ than in the presence of Na^+, calculation of pore volumes using PEG 20 M as a marker of external volume showed no difference between Na^+ and K^+ except at the highest concentration of PEG. This illustrates a limitation in the use of a molecular marker to measure volumes of pores. If some marker leaks into the pores, the calculation then underestimates the volumes of those pores. This error will be greatest in the presence of K^+ when the pores are bigger, and can obscure a real difference when it exists. The impact of this error on the calculated values of the density of water in the pores must, however, decrease as the pores become smaller. Fig. 1 shows the calculated values of the weight of internal water per gram dry weight of gel as the osmolality of the external solution in a similar experiment increased. With such severely contracted pores inward leakage of PEG in a similar solution was unlikely.

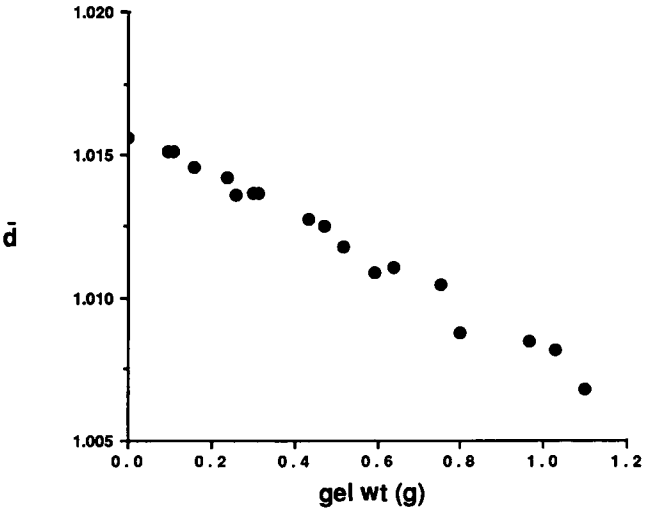

FIGURE 5 The average density in grams-milliliters^{-1} of the solution of 12% PEG 20 M equilibrated for 6 d with increasing weights of Biogel P-100 in 10-ml density bottles at 24°C.

The second assumption required for calculation of the density of pore water (d_i) is that the density of the PEG solution at equilibrium is the same as the density of a solution of the same concentration out of contact with the gel. Fig. 2 suggested that the chemical potential of water in the polymer solution was affected by the presence of the gel, presumably by means of a change in its density. In the absence of any information about the magnitude of that density change, d_i has been calculated using the density of a PEG solution off the gel. Calculated values are thus too high.

Fig. 6 shows the calculated d_i as a function of external osmolality. When the concentration of PEG was zero (i.e., in the absence of osmotic stress) the density of water in the pores of Biogel P-100 was 0.991 ± 0.006 g ml^{-1} ($n = 16$), which was significantly lower than that of liquid water (0.9973 at 24°C), and probably reflects the rather hydrophobic nature of the gel. As the external

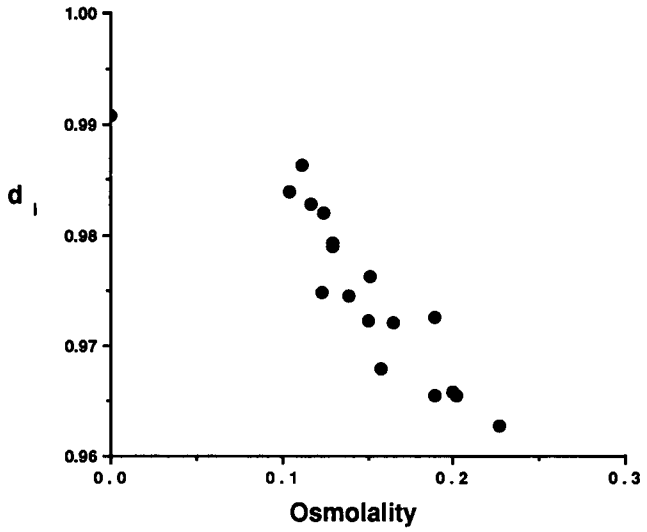

FIGURE 6 The density in grams-milliliters^{-1} of water inside the pores of Biogel P-100 under conditions of increasing osmotic stress.

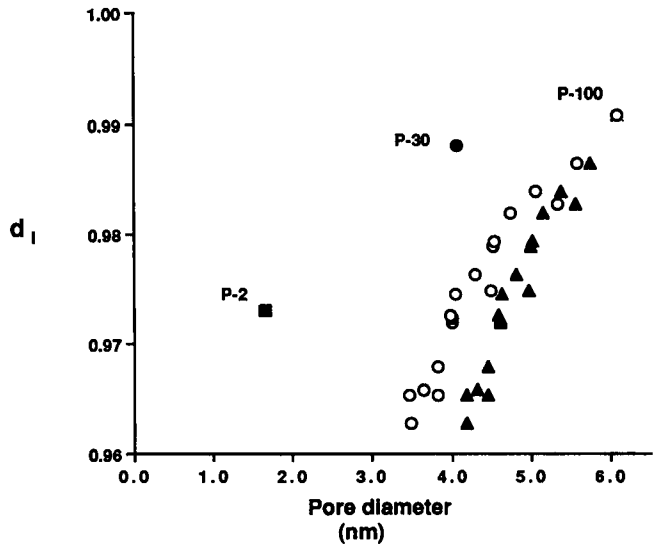

FIGURE 7 The density in grams-milliliters^{-1} of water in the pores of Biogel P-100, equilibrated with increasing concentrations of PEG 20 M, as a function of the diameters of the pores calculated from their water contents, assuming that the pores shrink only in diameter (O), or that they shrink both in diameter and length with loss of water (▲). Points labeled P-2, P-30, and P-100 show the densities of water inside Biogels P-2, P-30, and P-100 measured using the same density bottle technique with pure water. The pore diameters ascribed to them are those calculated from the exclusion limits (2,000, 30,000, and 100,000, respectively).

osmolality increased, however, the density of internal water decreased down to ~0.96; i.e., while the density of water in the gel was only slightly less than that of normal water in the absence of polymer, it decreased progressively under increasing osmotic stress. Biogel P-100 is a rather hydrophobic gel; it is possible therefore that the decrease in water density was due not to osmotic stress but to an increase in the fraction of water molecules in the pore under the influence of the hydrophobic surface. In Fig. 7, therefore, the diameter of the pores has been calculated from their water contents, assuming either that the pore shrinks only in diameter, or that it shrinks both in diameter and in length. These two diameters are plotted against the calculated d_i and compared with the measured densities of water in pores of Biogel P-2 and Biogel P-30. The densities of water in the osmotically contracted pores of P-100 are lower than those of rather hydrophobic pores of the same size; i.e., osmotic stress induced further stretching of water.

Equilibration of water and ions in charged gels

A charged gel tends to accumulate diffusible ions to a higher total concentration than that of the external equilibrating solution (Robinson, 1975). This increase in internal osmolality offsets, to some degree, the increase in external osmolality due to the presence of an impermeant solute: gel water, then expands less than in the absence of ions. Table 1 illustrates the interplay of these two effects. In the absence of impermeant solute an agarose gel in a low ionic strength medium (5 mM KP_i, pH 7) swelled to contain 5.2 g water per g dry weight. The sum of the concentrations of internal ions, Na^+, K^+, and P_i was greater than the external osmolality and the water was Na^+-selective ($K^K_{Na} < 1$). Addition of dextran sulphate of average molecular weight 500,000 increased both osmolality and ionic strength (dextran sulphate contained 4.4 mmol Na^+/g). Because much of the Na^+ associated with the dextran sulphate was essentially impermeant, because it had to stay outside to neutralize the negatively-charged dextran sulphate, the water content of the gel decreased as external osmolality increased, but with increasing ionic strength osmolality increased inside more than outside. ($\Sigma \cdot C_i >$ external osmolality). Water in the gel therefore remained Na^+-selective ($K^K_{Na} < 1$). When, however, the impermeant solute was PEG 20 M and the ionic strength held at the same low value ($Na^+ + K^+$) P_i = 5 mM, while Na^+/K^+ varied internal osmolality at equilibrium was much lower than external osmolality, gel water presumably expanded and stretched, and demonstrably became K^+-selective. As found previously (Wiggins and van Ryn, 1986) the K^+-selectivity increased as its concentration decreased. The switch in selectivity rules out the possibility that the Na^+-selectivity of the simple gel was due entirely to preferential interactions of Na^+ with the sulphonic acid and carboxyl groups.

TABLE 1 **Changes in the selective solvent properties of water in an agarose gel**

Impermeant solute	$[Na]_e/[K]_e$	$mOsM_e$	Σc_i	W_i/D.W.	K^K_{Na}
—	0.058	13	19.0	5.198	0.345
Dextran sulphate					
5%	39.9	58	68.7	4.074	0.813
10%	55.94	87	92.3	4.132	0.717
15%	119.05	180	224.7	2.778	0.773
20%	163.5	240	282.7	2.472	0.853
PEG 20 M					
20%	0.333	480	33.3	2.498	1.47
20%	0.855	492	31.2	2.497	1.508
20%	1.975	506	30.6	2.547	1.675
20%	13.29	531	34.1	2.363	2.184
20%	29.8	529	34.1	2.5063	2.312
20%	65.7	529	33.1	2.4192	2.623

Agarose gel encased in a dialysis membrane was equilibrated at 38°C for 5 d with constant mixing in solutions of either KP_i (5 mM, pH 7) to which dextran sulphate was added, or to 5 mM $(Na^+ + K^+)P_i$ (pH 7; $[Na^+]/[K^+]$ varying from 0.1 to 19 initially) to which 20% wt/vol PEG 20M was added. The extra Na^+ that appeared in the external solution at equilibrium came from the agarose. $mOsM_e$ is the osmolality of the external solution; c_i is the sum of the concentrations of Na^+, K^+, and P_i in the gel water; W_i/D.W. is the water content of the gel in grams per gram dry weight; K^K_{Na} is the selectivity coefficient given by $K^K_{Na} = [K^+]_i [Na^+]_e/[K^+]_e [Na^+]_i$.

OH stretch band of water in gels

Fig. 8 shows the OH stretch band of normal liquid water, displayed together with the same band for water in Biogel P-6 equilibrated with 12.5% PEG 20M. Rather than give average properties of the two populations of water molecules, this spectrum shows clearly that, relative to normal water, water associated with the gel contains more unbonded and weakly-bonded molecules (3,400–3,650 cm^{-1}) and more strongly bonded molecules (2,900–3,200 cm^{-1}). The spectrum does not indicate where these populations were, but partition and density measurements suggest that the strongly-bonded molecules were in the gel and the weakly-bonded molecules in the external solution.

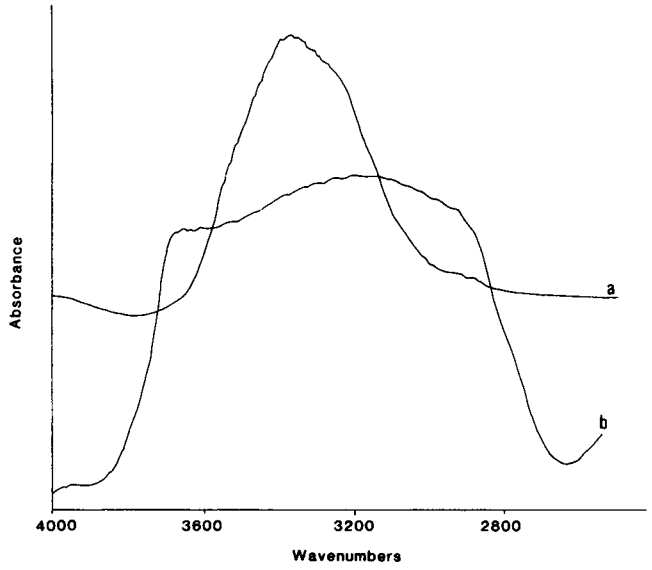

FIGURE 8 The OH stretch band of water. (*a*) Pure water, with a width at half height of 409 cm^{-1}; (*b*) water in a slurry of Biogel P-6 equilibrated with 12.5% PEG 20 M, with a width at half height of 997 cm^{-1}. This spectrum is representative of many. Each spectrum had an absorbance between −0.007 and 0.1. No substraction was necessary because the weight of water was five times the weight of gel.

DISCUSSION

A gel imbibes pure liquid until its polymeric chains assume their most favorable configuration under the prevailing entropic and enthalpic constraints. The activity of the liquid is unity both inside and outside the gel, and therefore, because the chemical potentials must be equal, the pressures on the liquid are the same. When an impermeant solute is added to the external solution the activity of the liquid in the pores of the gel is still unity, but that in the external solution is less than unity. The pressure in the two compartments is still the same. The only work that can be done to bring the water to equilibrium is expansion against the constant pressure. Therefore, the criterion for equilibrium is

$$dA = 0, \quad (1)$$

where A is the Helmholtz free energy, or the maximum total work that can be done by the system, including work of expansion.

$$dA = dG - PdV - VdP = -SdT - PdV + \Sigma \mu_i dn_i. \quad (2)$$

Because water is the only component of the system which can come to equilibrium, $dA = 0$ at constant temperature and pressure, when the chemical potentials of water in the two compartments are equal. In the gel compartment

$$\mu_g = \mu^\circ + RT \ln a_g + \int_{\bar{v}_o}^{\bar{v}_g} P d\upsilon = \mu^\circ + \int_{\bar{v}_o}^{\bar{v}_g} P d\upsilon. \quad (3)$$

In the polymer solution:

$$\mu_p = \mu^\circ + RT \ln a_p + \int_{\bar{v}_g}^{\bar{v}_p} P d\upsilon. \quad (4)$$

The condition of equilibrium is then

$$RT \ln a_p = \int_{\bar{v}_g}^{\bar{v}_p} P d\upsilon, \quad (5)$$

where a_p is the activity of water in the polymer solution, $\bar{v}_o$ the molar volume of water, and $\bar{v}_g$ and $\bar{v}_p$ the partial molar volumes of water in the gel and in the polymer solution at equilibrium. This reduces to

$$RT \varnothing m = - \int_{\bar{v}_g}^{\bar{v}_p} P d\upsilon, \quad (6)$$

where m is the molality of the polymer solution, and $\varnothing$ the osmotic coefficient. It has been assumed in this equation that the chemical potentials of water can be equalized partly by expansion of the gel water and partly by contraction of the water in the polymer solution. The freezing point and vapor pressure measurements of Fig. 2 suggested that when there was a large excess of polymer solution the third term in Eq. 4 became negligible, so that the chemical potential of water, measured by freezing point depression, was equal to $RT \ln a_p$ whether or not the solution was in contact with gel. Under these conditions all the difference in chemical potential must be made up by expansion of gel water. As the amount of gel relative to polymer solution increased, however, the third term in Eq. 4 apparently increased, and then the chemical potential of water in the polymer solution in contact with the gel was given by Eq. 4, whereas its chemical potential removed from the gel was equal to $RT \ln a_p$. In other words water, which contracts under the influence of the gel relaxes to its normal state on removal of gel. Similarly, water in the gel, which must always expand to some degree, relaxes to its normal density when the gel is removed from the polymer solution (Fig. 6).

This result is quite general, and should apply to any gel, solvent, impermeant solute system. A normal liquid would merely decrease its intermolecular interactions with expansion, and increase them with contraction, experiencing relatively trivial changes in physical and chemical properties. When water is the solvent, however, the effects of expansion or contraction on its chemical and physical properties are considerable. As molecules move further apart, the incidence of strong linear hydrogen bonds, which are rather rare in the crowded normal liquid water, increases. Thus, expanded water has stronger water–water hydrogen bonds, which increase its viscosity and change its properties as a solvent; it selects K^+ and univalent anions, and dissolves highly hydrated solutes poorly (Wiggins and van Ryn, 1986). Using the same arguments, one would expect that contracted water would be a better-than-normal solvent for highly hydrated solutes, and would exclude K^+ and small anions to some degree (see Table 1).

It is generally assumed that charged gels swell until further influx of water in response to the Donnan excess of diffusible ions is opposed by a pressure exerted on the solution by the gel matrix (Helfferich, 1962). The present experiments, however, suggest the possibility that, as in the absence of electrolytes, the final volume of solution imbibed by the gel is determined by the entropic and enthalpic constraints operating on the gel matrix: water and ions then equilibrate under that set of conditions, with no pressure difference between the inside and the outside of the gel. Table 1 shows that an agarose gel achieved a finite volume with internal osmolality greater than external osmolality. The Na^+-selectivity suggests that the gel water equilibrated by increasing its density.

Gels are extremely common in biology. In many tissues the interstitium is a proteoglycan gel; mucus which lines many epithelia such as the stomach, the gut and the urinary bladder, is a gel composed of proteins and polysaccharides; bacteria have cell walls which are polysaccharide gels. Frequently, there are soluble polymers of the same material associated with gels; for example, cells of the gastric epithelium secrete both soluble and insoluble mucus (Slomiany et al., 1987). The properties of these gels interacting with neighboring aqueous phases in vivo may be subtly different from their behavior in isolation, and have considerable functional significance. The most interesting gel, however, must be the cytoplasm; only the red blood cell perhaps, contains a solution; more complex cells with internal organelles and cytoskeletons must have the water-imbibing characteristics of gels. Regulation of the volume of mammalian cells is dependent upon the activity of the Na,K-ATPase, which continually uses energy to pump Na^+ ions out. Na^+ is thus an effectively impermeant solute, balancing the Donnan excess of diffusible ions drawn into the cell by the net charge on its macromolecules (Macknight, 1984). K^+ ions accumulate inside cells, but diffusible anions are usually held at low concentrations by the membrane potential, which is an indirect result of the activity of the pump. It is not possible to measure the intracellular osmolality, and the assumption is always made that it is equal to the extracellular osmolality, which can readily be measured. The justification for this assumption is in two parts: Water moves so rapidly across cell membranes that it could be held out of

equilibrium only by an energy expenditure so large that it could not escape detection; plasma membranes, even with the support of a cytoskeleton, could not withstand an appreciable pressure difference. The present experiments have shown, however, that with an external impermeant solute to oppose the swelling tendency of a gel, osmolalities can be different in the two compartments while the pressures are equal. Presumably the Na,K-ATPase can pump the intracellular osmolality down to any energetically accessible level, which could well be below that of the external solution. Equilibration of water is still possible, if the cytoplasmic water stretches.

Osmotically stretched cytoplasmic water would be less dense than normal water (Clegg, 1984) and less mobile (Trantham et al., 1984). Table 1 shows that the selectivity of stretched water for K^+ relative to Na^+ is so slight, even when the difference between external and internal osmolalities is ~500 mOsM, that it is unlikely to contribute significantly to distributions of Na^+ and K^+ across plasma membranes of cells under isotonic conditions. More highly hydrated ions, however, will be significantly affected. The difference between the free energies of hydration of Na^+ and K^+ is 73 kJ mol^{-1} (411–337), whereas the difference between those of H^+ and K^+ is 752 kJ mol^{-1}. Experiments in progress are showing that the activity of coefficient of the hydrogen ion in water similar to that in the agarose gel equilibrated with 20% PEG 20 M is greatly increased. The same would apply to Ca^{2+} (1,593 kJ mol^{-1}), Mg^{2+} (1,905 kJ mol^{-1}) and ATP (of the order of 798–1,750 kJ mol^{-1}, depending upon its charge (George et al, 1970). This may have profound implications for the kinetics and thermodynamics of enzyme reactions in cytoplasmic water, and in the energy available from proton, Ca^{2+} and Na^+ ion movements across membranes.

As a result of the continental operation of the Na^+ pump a whole cell has a limited amount of water to distribute among its various compartments. The nucleus, with its highly negatively-charged nucleic acids must attract a large Donnan excess of diffusible ions, decreasing the activity of nuclear water and reinforcing the activity of the Na^+ pump in stretching the water of the cytoplasmic compartment. Nuclear and cytoplasmic water then should have different densities, and different solvent properties. This may explain the finding that the activity of Na^+ inside skeletal muscle cytoplasm, as measured by ion-specific microelectrodes, is much lower than its concentration in total cellular water (Leader et al., 1984), and that ATP is more concentrated in nuclear than in cytoplasmic water (Miller and Horowitz, 1986). Each membrane-bound intracellular compartment might have its own characteristic activity of water: which would equilibrate among all the compartments by adjusting its density. This delicate balance would be upset by a change in the osmolality of any single compartment.

If cytoplasmic water is stretched, there is no longer any need to postulate "osmotically inactive water." This is invoked to account for the failure of cells to shrink in hyperosmotic solutions and swell in hypoosmotic solutions to the extent predicted for the case of equal intracellular and extracellular osmolalities (Dupre and Hempling, 1978). A cell containing stretched cytoplasmic water would respond to hyperosmotic conditions by losing some water and increasing the partial molar volume of what remained; it would respond to hypoosmotic conditions by taking up some water, and decreasing its partial molar volume. The changes in molar volume of water would have negligible effect on its total volume, but they would decrease its net movement. Thus, one physiological advantage of stretched cytoplasmic water would be as a buffer against wide swings in volume with changing osmolalities, before the secondary volume regulatory mechanisms have time to adjust.

Water in the relatively simple gel/polymer solution systems has been difficult to characterize, because in even slightly charged gels, Donnan accumulation of ions opposes stretching of water induced by impermeant solutes. It is hardly surprising, therefore, that there are many conflicting reports concerning water inside cells. Some measurements would yield average values of density and mobility, both of which might be higher than normal in the nucleus and lower than normal in the cytoplasm. Other measurements have been made in the absence of the external osmotically stressing solution, others under conditions of anoxia when autolysis, by increasing mitochondrial and cytoplasmic osmolality, allows water to relax toward its normal density, mobility and solvent properties. More detailed characterization of the complex interactions among ions, impermeant solutes, and water in gels might help to resolve these apparent contradictions.

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