THE POTENTIAL OF WATER IN MAMMALIAN TISSUES*, ‡

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ABSTRACT

Melting point depression was used as an index of the water potential of rat tissues and serum. Organs removed from anesthetized rats were immediately frozen in liquid nitrogen and ground with mortar and pestle. Aliquots of the resulting frozen powder were suspended in chilled liquid silicone. While the suspension was vigorously stirred and warmed at a constant rate, the temperature of the melting mixture was measured.

The melting curves of rat muscle, liver, heart, and brain were not significantly different from those of rat serum. The melting curve depression of whole kidney was greater than that of serum; this was demonstrated to be due to hypertonicity of the renal medullary area alone. It was demonstrated that autolysis will rapidly increase the depression of the melting curve of tissue. It is concluded that within the limits of the method used the melting point depression, and hence the water potential, of intracellular and extracellular fluids is the same.

Over the past half century there has been considerable interest in the possibility that there may be differences between the osmotic activity of water inside and outside the living mammalian cell. Using cryoscopic methods Sabbatani (1, 2), Collip (3), Leövey and Kerpel-Fronius (4), Gömöri and Molnár (5), Gömöri and Frenreisz (6), Pichotka *et al.* (7), and Opie (8, 9) obtained results indicating that tissues are hypertonic to blood or serum. However Conway, Geoghegan, and McCormack (10) and Brodsky and associates (11) have emphasized that tissue autolysis is capable of producing the high values for tissue freezing point depression reported. They demonstrated that tissue autolysis occurs even at 0°C. and by correcting for autolysis (12) or by boiling the

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tissue to avoid autolysis (13) they obtained results indicating that the freezing point of tissues was not significantly different from that of serum.

Because of the rapidity with which tissue breakdown will occur, it is apparent that freezing point measurements are unsuitable for directly obtaining the intracellular tonicity of tissues without recourse to uncertain correction factors for the expected autolysis, or without use of procedures to reduce tissue breakdown which themselves may introduce unknown variables into the measurement. Melting point measurements on quickly frozen tissue would seem preferable; in the frozen state molecular breakdown is minimal (14) and if the end point of the measurement were rapidly achieved, autolysis would not become significant.

There are definite technical difficulties, however, in accurately measuring the melting point of a chunk of frozen tissue. Valid measurements require absence of thermal gradients in the melting mixture. To achieve such thermal homogeneity two requirements must be fulfilled: (1) The ice particles must be of such small size relative to the rate of heat input that no significant temperature gradient will exist between their surfaces and their interiors. (2) With macroscopic amounts of tissue the frozen tissue must be maintained in a "fluid" state throughout the measurement so that complete mixing can be achieved to prevent thermal gradients between various portions of the melting tissue and the temperature probe.

By crushing the frozen piece of tissue while still at very low temperatures the ice particles can be rendered sufficiently small so that they present a large surface to facilitate heat exchange with their surroundings, and a small mass to minimize gradients from delayed heat conduction. A fine powder of frozen tissue, however, cannot readily be stirred to prevent thermal gradients from arising. Furthermore, when melting begins, the originally tiny particles of ice cling together to form larger agglutinates and temperature gradients rapidly arise. This difficulty can be avoided by suspending the finely crushed tissue in a precooled inert liquid. This suspension, being fluid, can be stirred vigorously to maintain thermal homogeneity throughout and to mechanically break up any groups of ice particles which tend to aggregate.

Temperature gradients in the melting tissue mixture could also be reduced by warming the tissue very slowly. Slow warming, however, may allow time for autolysis to occur near the melting point. Thus, though Opie (8, 9) used a melting point method rather than a freezing point determination, in various of his experiments 40 to 120 minutes elapsed during the final 1°C. rise in temperature prior to the melting point, sufficient time for considerable autolysis. With a finely divided suspension of frozen tissue in an inert liquid vehicle, heat can be introduced at a rapid rate so that the end point is quickly achieved and time for autolysis minimized. The optimal conditions of heat distribution described for such finely divided suspensions of frozen tissue will permit rapid warming with avoidance of temperature gradients

These principles have been incorporated into the technique described and utilized in the present study.

METHODS

The animals used in all experiments were mature white rats, both male and female. The animals were maintained on Purina chow with fluids *ad libitum*. To avoid tissue anoxia, light anesthesia was induced by the intraperitoneal injection of sodium pentobarbital. The desired organ was surgically exposed and portions or all of it were quickly removed and dropped immediately into liquid nitrogen.

In the case of the kidney, the organ was lifted from its bed and gently separated from any perirenal fat; the hilum was then clamped and immediately separated. With liver the major portion of each lobe was isolated by clamping with a hemostat and excised distally. Abdominal muscle was freed from the overlying rectus fascia, clamped superiorly and inferiorly, and excised. The heart was clamped at its junction with the great vessels and excised; one incision was then quickly made to open the chambers to remove any significant amounts of remaining blood. The brain was exposed by opening the superior aspect of the calvarium longitudinally with the aid of a small circular saw and by breaking off the remaining overlying bone with a hemostat; the brain was then scooped out with a sharp, curved spatula.

The maximum time that elapsed between compromising the blood supply to an organ and immersing the specimen in liquid nitrogen was estimated to be 10 seconds. Exceptions were brain, renal cortex, and renal medulla whose preparation required delays of 1 to 2 minutes. Following removal of the tissue, blood was promptly obtained by needle aspiration from the heart. If the heart had previously been removed, the clamp on the great vessels was released and escaping blood was sucked up from the pleural cavity. Usually, only one tissue was removed per animal.

The particular organ to be studied, obtained from 1 to 4 animals, was carried in the liquid nitrogen into a refrigerated room which was maintained at a temperature of $-10°C$. The pooled tissues were pulverized at the temperature of liquid nitrogen to a fine powder with a mortar and pestle. An amount of frozen tissue powder calculated to contain 488 mg. of water (an arbitrary amount) was quickly weighed out (precision balance, Roller-Smith Corporation, Bethlehem, Pennsylvania) and then dropped into a plastic melting tube which was at the temperature of dry ice. 10 ml. of liquid silicone (Dow Corning 200 fluid, 5.0 centistokes viscosity, Dow Corning Corporation, Midland, Michigan), also at the temperature of dry ice, was then added to the tube.

The tube containing the tissue-silicone suspension was then transported on dry ice from the refrigerated room to the laboratory and transferred to the apparatus shown in Fig. 1. The melting tube (*c*) was suspended in an evacuated, silvered jar (*h*) and thus largely isolated thermally from the surroundings. Rapid and reproducible mixing was accomplished by a rotary stirring device (*g*) of low heat conductivity (small blade of sheet steel; shaft, pulley, and screw cap of bakelite). The rate of stirring was controlled stroboscopically, with all determinations made at a speed of 1800 R.P.M. Baffle plates (*e*) present on the sides and bottom of the tube prevented stratification of the tissue suspension and helped maintain a homogeneous mixture. Heat was put into the system at a constant rate by the rapid, constant stirring. The tem-

perature of the warming mixture was measured with a thermistor (*d*) connected through a Wheatstone bridge (*i*) to a continuously balancing electronic potentiometer (*j*) (Type 153 electronik recorder, Minneapolis-Honeywell Regulator Company, Brown Instrument Division, Philadelphia, Pennsylvania), which recorded a permanent ink tracing. The electrical resistance of the thermistor varied inversely and essentially linearly with temperature over the range required for the experiments.

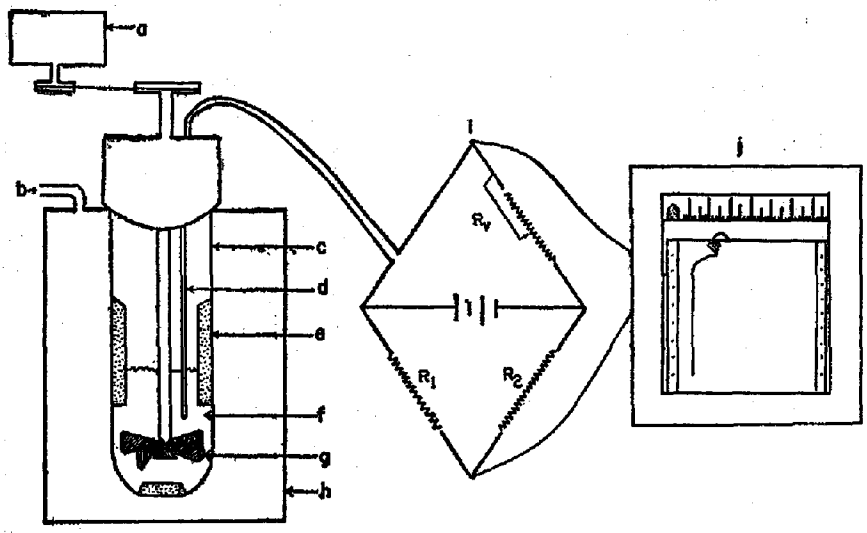

FIG. 1. Diagrammatic sketch of the apparatus utilized for melting curve determinations. The frozen tissue or serum particles were suspended in liquid silicone (*f*) contained in the plastic test tube (*c*). The stirring rod with attached blades (*g*) was rotated by an electric motor (*a*). Baffle plates (*e*) were attached to the sides and bottom of the tube which was suspended in a silvered, glass jar (*h*); suction was applied at (*b*). A thermistor (*d*) was wired to a Wheatstone bridge (*i*) variable resistance (R_v), fixed resistances (R_1) and (R_2); the unbalance of the bridge was recorded by a continuously balancing potentiometer (*j*).

Measurements of serum were carried out in an identical fashion. Five ml. of serum were dropped directly into liquid nitrogen, then pulverized and treated in the manner described for tissue. In addition the freezing point of the serum was determined with a cryoscope designed and constructed for us by Fiske Associates, Inc., Danvers, Massachusetts.

Usually four to six measurements were made with the same batch of frozen tissue or serum. Between determinations, the remaining frozen powder was kept in a covered tube immersed in liquid nitrogen.

The water content of the organs used was calculated from the difference in weight of tissue before and after drying for 24 to 48 hours in an oven at 90°C. Values determined and subsequently utilized for the per cent water content of rat tissues were:

abdominal muscle, 75 per cent; liver, 71 per cent; heart, 76 per cent; whole kidney, 76 per cent; kidney cortex, 75 per cent; kidney medulla, 76 per cent; serum 91 per cent. The brain, as obtained from the living animal, had a considerable and variable content of blood and therefore an estimated water content of 85 per cent was used.

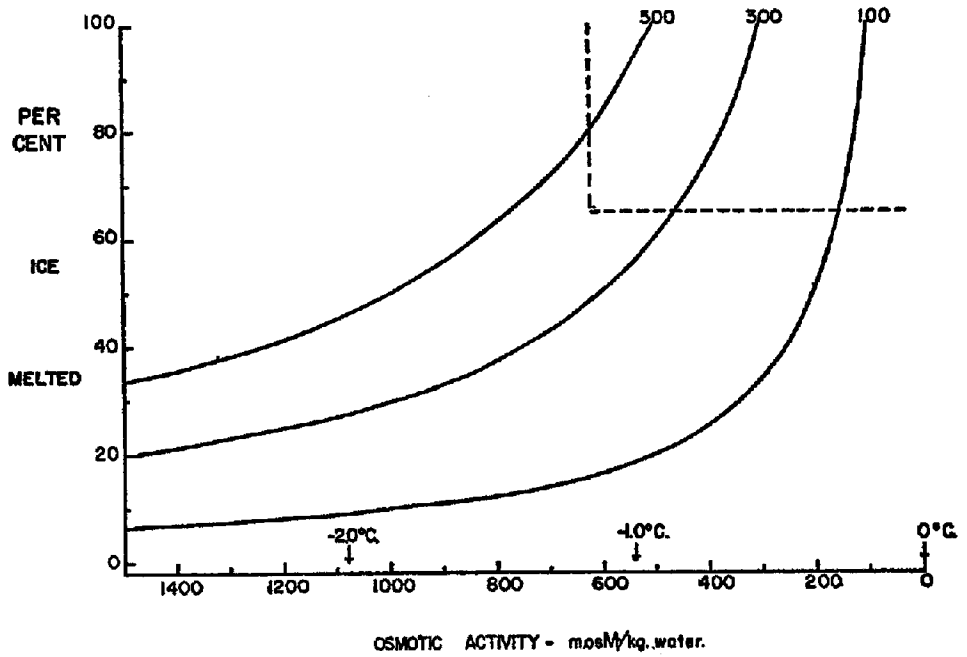

FIG. 2. Theoretical melting curves of three solutions of different osmotic activities. The calculated melting curves of solutions of 100, 300, and 500 m.osM/kg. water respectively are shown. The per cent of ice melted is the ordinate and the osmotic activity of the melting solution is the abscissa. Note that the abscissa is also proportional to temperature. The broken lines delineate the portion of the curves which has been used as the index of melting point depression in the subsequent measurements; it is this portion of the melting curves which is depicted in Figs. 5 through 10.

RESULTS

Evaluation of the results is facilitated by a consideration of what theoretically happens in a melting solution. As is well known, when an aqueous solution freezes pure water separates as ice crystals from the bulk of the solution. This results in a progressive increase in concentration of the remaining solution, as more and more water freezes, until the eutectic point is reached. Conversely, when the frozen mixture melts, the first ice that melts does so in a highly concentrated solution. Melting in turn results in progressive dilution of the solution. If the percentage of ice melted is plotted against increasing temperature of the

melting mixture—which is proportional to decreasing osmotic activity and to increasing water potential—a rectangular hyperbola is obtained. When solutions of different initial osmotic activities are compared, a family of rectangular hyperbolas results, as shown in Fig. 2. Changes in the value of osmotic coeffi-

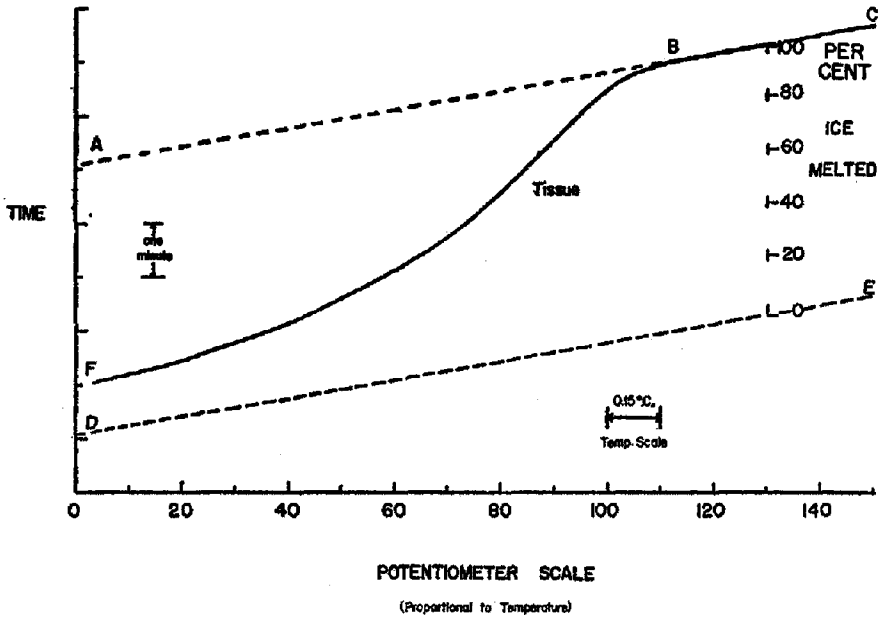

FIG. 3. A melting curve as recorded on the potentiometer graph. The curve FBC represents the tracing recorded during a measurement of tissue melting. All tracings were in fact begun at approximately −10°C., but only the final 2 to 3° are shown here. The ordinate is time, with a span of 1 minute indicated, and the abscissa is the potentiometer scale. The dashed line DE represents a continuation of the slope of the tracing prior to any tissue melting, and line ABC the slope after melting was completed. The per cent ice melted at any point on the curve is proportional to the vertical distance of that point above line DE, as indicated by the ordinate on the right side of the figure.

cients as the solution becomes more dilute will result in a slight—but, for our purposes, insignificant—deviation from a true rectangular hyperbola and this has been omitted in constructing the figure. The final particle of ice to melt does so in a solution of essentially the same concentration as the original solution and this point is therefore the "melting point."

Fig. 3 shows a tissue melting curve as recorded by the potentiometer. The ordinate on the recorder tracing is time (chart speed was 3 inches per minute for all measurements). The abscissa is the arbitrary potentiometer scale which

is proportional to temperature; movement of the recording pen from left to right indicates a temperature increase in the melting mixture.

The curve obtained represents the total calories acquired by the stirring blade and shaft plus the plastic tube and its contents as the ice melts and can be resolved into two components: (1) the calories absorbed by the ice as it melts, *i.e.* the latent heat of fusion, and (2) the calories absorbed by the com-

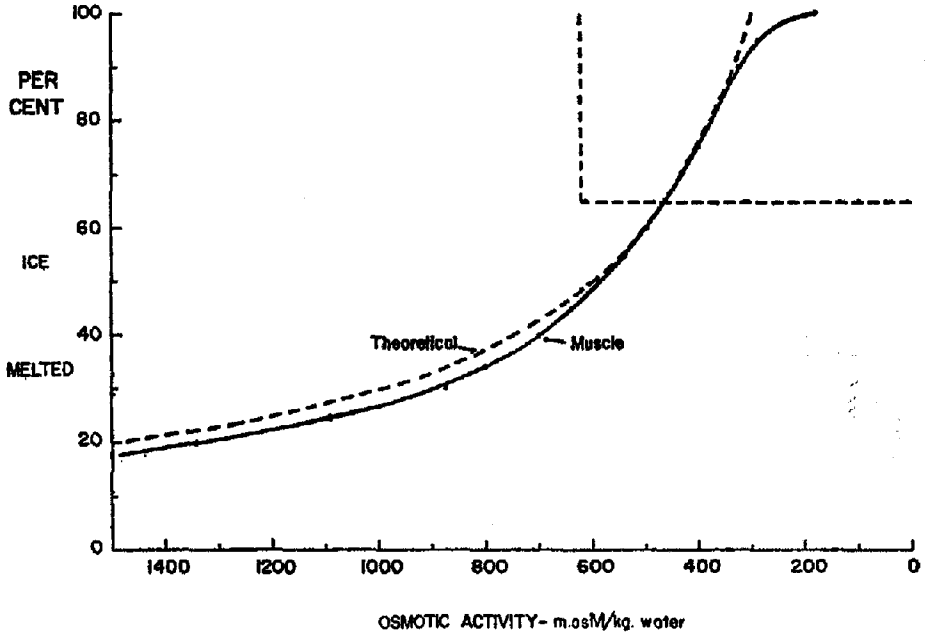

FIG. 4. The shape of an actual melting curve compared to a theoretical one. The solid line is a melting curve obtained with rat muscle and the dashed line the rectangular hyperbola of the theoretical curve. The dotted lines in the right upper corner delineate the portion of the curve represented in Figs. 5 through 10.

bined heat capacities of the various elements of the system as the temperature rises. The latent heat of fusion of water is 79.7 calories per gm. or 38.9 calories for the 488 mg. of tissue water or serum water used in each sample measured. The total heat capacity of the silicone, tissue, stirring blade, and stirring rod was estimated to be 8 calories per degree centigrade rise in temperature.

In order to obtain the melting curve of the frozen tissue, it was necessary to subtract out of the recorded curve the component due to the second or heat capacity factor of the system. When tracings were made of the warming curve of the chilled silicone and stirring device alone without frozen tissue, smooth curves were traced such as indicated by the broken line, DE, in Fig. 3. The

slope of this line remained fairly constant and was determined by the heat capacity of the system and the constant rate of heat input by the stirrer. When frozen tissue was added and the tracing remade, straight lines of constant slope were obtained both before any melting commenced, DE, and after all melting was completed, BC. The vertical distance between the two parallel

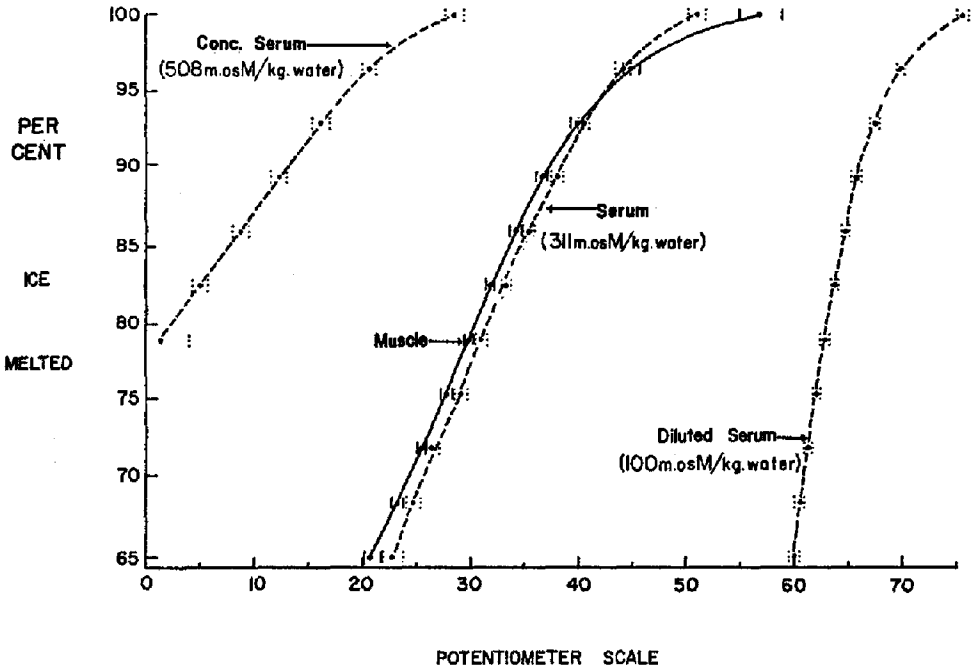

FIG. 5. The melting curves of rat muscle and serum. The solid line is the mean melting curve of rat muscle. The dashed lines represent the melting curves of concentrated, normal, and diluted rat serum. The ordinate is again per cent ice melted and the abscissa is the potentiometer scale which is proportional to temperature and osmotic activity. The standard error of the mean for each point is noted by the vertical bars and rows of dots.

lines shown in Fig. 3 thus represents the total heat of fusion on change in phase from ice to water.[1] As the same amount of water was contained in every sample measured, as the rate of heat input was constant, and as the heat of fusion of ice changes only negligibly over the temperature range studied (15), the vertical distance between the two parallel lines was the same for all measurements.

[1] All tracings were actually begun at $-10°$C. but only the final -2 to $-3°$C. are shown in Fig. 3. For this reason, at point F on the curve approximately 20 per cent of the water content of the tissue had already melted.

Thus the vertical distance upward from line DE to any point on the melting curve is proportional to the per cent of ice melted. Whereas the line DE meets the melting curve asymptotically making its point of origin difficult to determine, the line ABC is well defined. Hence all points used to reconstruct the melting curves were obtained from ABC as the reference line. By this technique the true melting curve was obtained with per cent ice melted as the

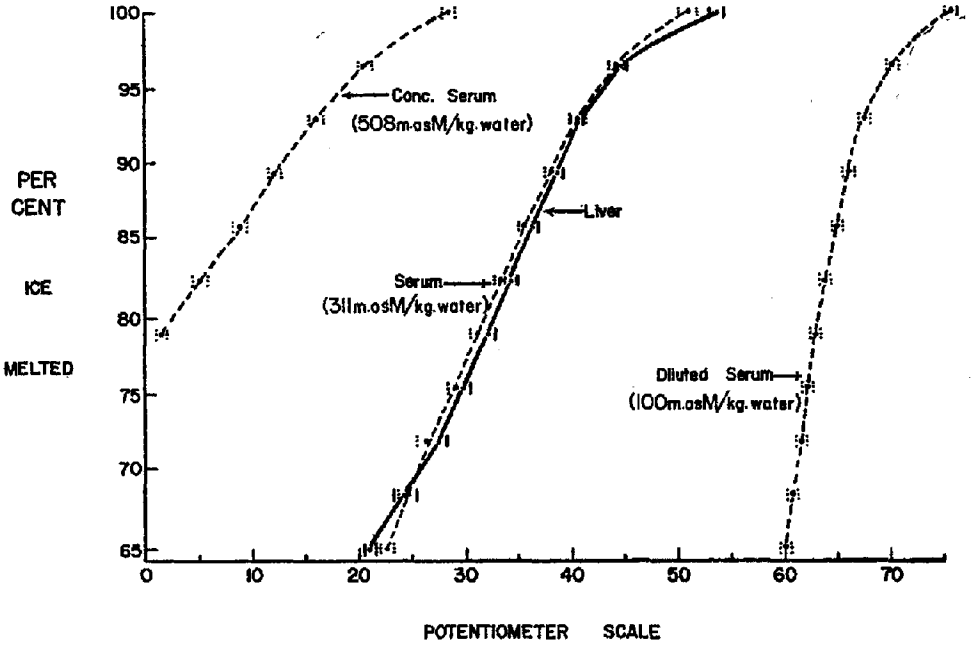

FIG. 6. The melting curves of rat liver and serum. The symbols have the same significance as in Fig. 5.

ordinate and with temperature, osmotic activity, or water potential as the abscissa.

Fig. 4 shows such a tissue melting curve superimposed on the rectangular hyperbola of a theoretical curve. It is apparent that the shape of the melting curve of rat muscle approximates quite closely that of the theoretical curve. It will be seen that the terminal portion of the tissue curve deviates to the right of the theoretical "melting point." Presumably this occurs because at this end of the curve the constant heat input exceeds the rate at which this heat can be absorbed by the very small amount of ice remaining unmelted; hence the temperature of the mixture rises before all the ice disappears. This phenomenon makes determination of the precise melting point difficult. Consequently, in

order to avoid the uncertainty of extrapolating to the point of complete melting, we have chosen the upper portion of the melting curve, enclosed by the broken lines in Fig. 4, as the index for comparing melting points of different solutions. Over this restricted range of the melting curve the effect of changes in osmotic coefficients will be minimized. Reference to Fig. 2 shows that, although the melting curves for solutions of different activities converge as the ice melts, the horizontal distance separating the melting curves at any given

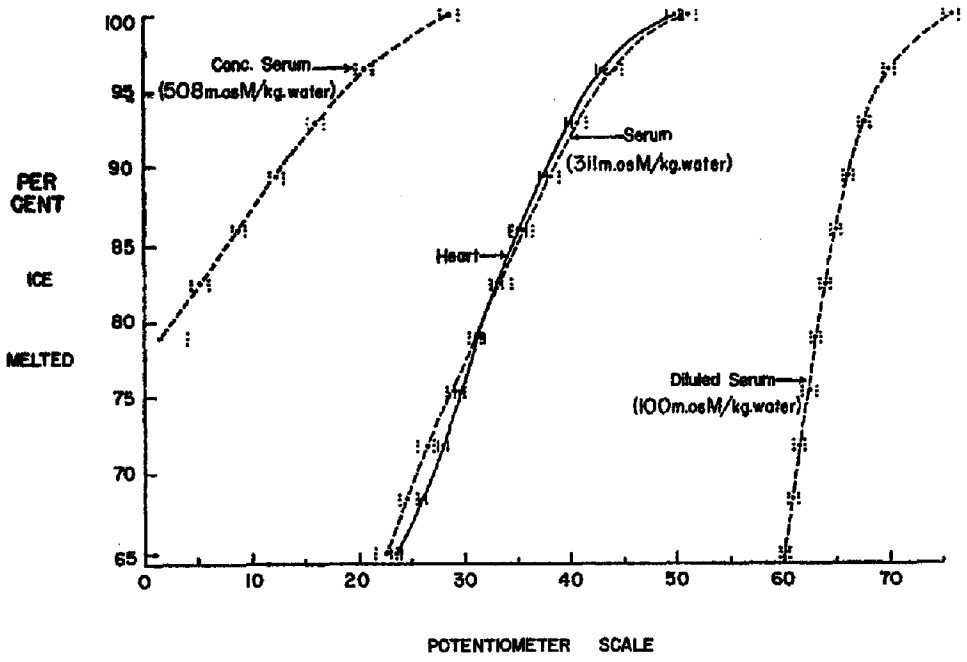

FIG. 7. The melting curves of rat heart and serum. The symbols have the same significance as in Fig. 5.

per cent of ice melted is proportional to the difference in activity between the solutions.

Fig. 5 shows the mean curve obtained from twelve separate measurements of the melting curve of rat muscle and of a similar number of measurements of rat serum. The mean osmotic activity of the rat serum was found to be 311 m.osM/kg. water by freezing point determination. To indicate the concentration scale, the melting curves of serum concentrated by drying to 508 m.osM/kg. water and diluted with distilled water to 100 m.osM/kg. water have been included. The small vertical bars or rows of dots indicate the standard error of the mean for each point. It is evident that the melting curve of rat muscle does not differ significantly from that of rat serum.

The results of twelve similar determinations made on rat liver are shown in Fig. 6. The three serum curves are shown for reference. Again, the melting curve of tissue is very close to that of serum. This finding is in marked contrast to the results of some previous workers who have reported values for osmotic pressure of liver up to and over twice that of serum (2, 6–8). Such values would have placed the liver curve to the left of the 508 m.osM/kg. water standard.

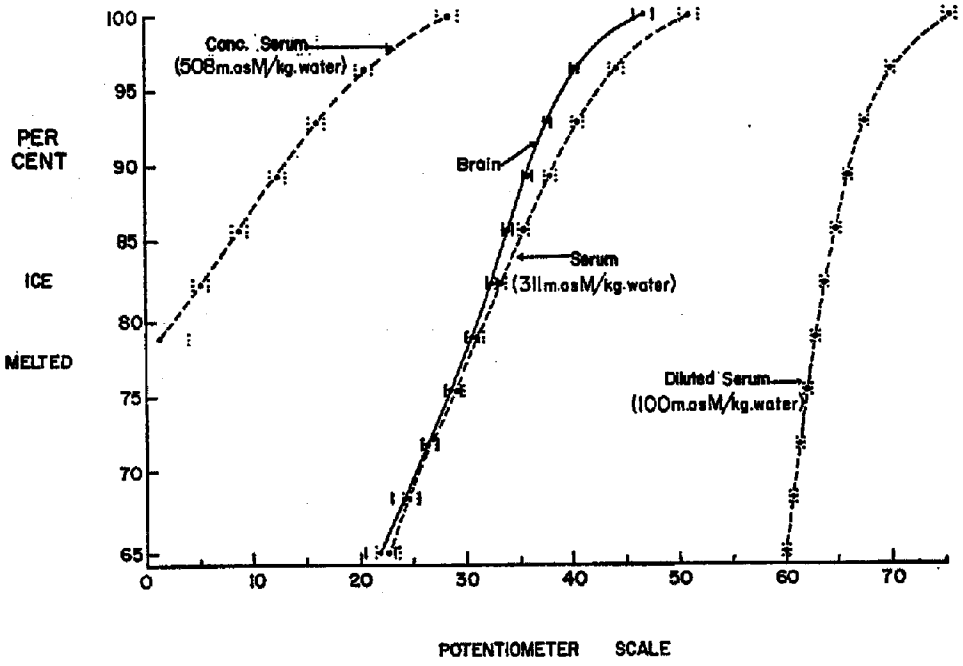

FIG. 8. The melting curves of rat brain and serum. The symbols have the same significance as in Fig. 5.

Figs. 7 and 8 depict the measurements made with rat heart and rat brain, respectively. The results again are very similar to those with serum. The explanation for the shift to the left of the melting curve of brain in its final portion is not entirely clear and will be discussed subsequently. In any case, the magnitude of the shift is not large, amounting to some 6 per cent of the depression of the melting curve.

Fig. 9 shows the melting curves of whole kidney, kidney cortex, and kidney medulla. The melting curve for whole kidney indicates that this tissue is significantly hypertonic to the rat serum. In order to evaluate separately the contributions of cortex and medulla to this result with the whole kidney, kidneys were removed as usual but cortex and medulla were separated by rapid dissec-

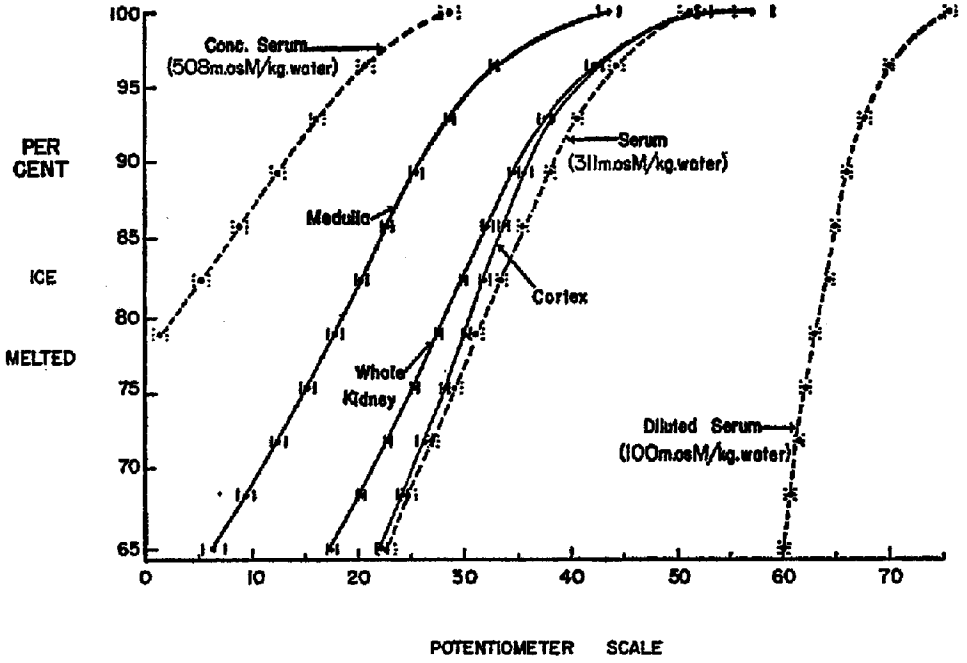

FIG. 9. The melting curves of whole rat kidney, renal cortex, renal medulla, and serum. The dashed curves are those obtained from diluted, normal, and concentrated serum of rats. The curve obtained for whole kidney is shown as well as the curves for kidney cortex and medulla.

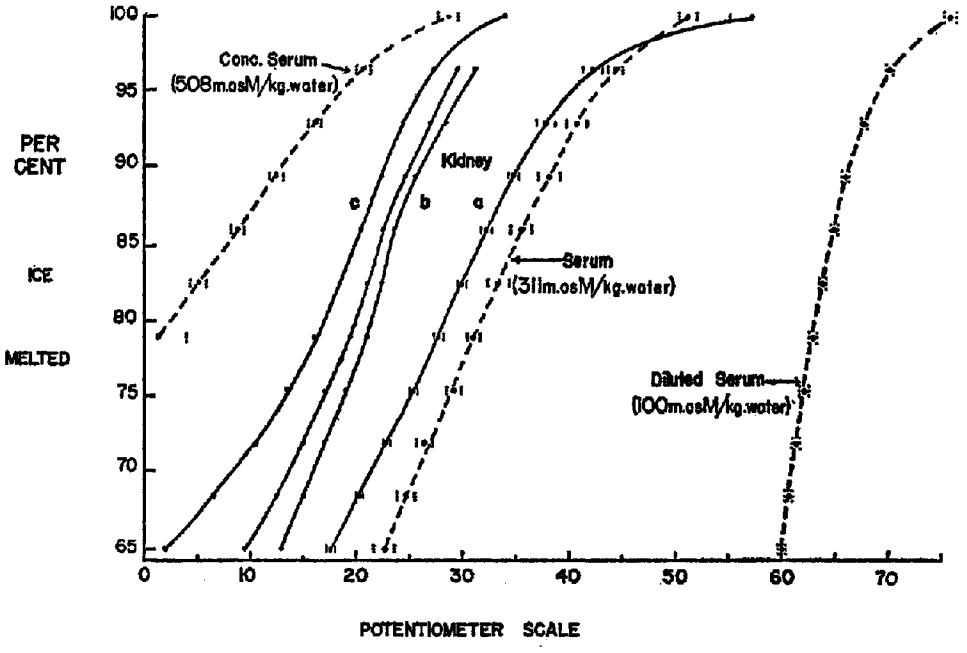

FIG. 10. The effect of autolysis on the melting curve of rat kidney. Curve *a* is the mean melting curve of whole rat kidney determined in the usual manner. The two curves *b* and the single curve *c* were obtained from the same frozen kidney after 10 and 20 minutes' exposure respectively to room temperature.

tion (requiring approximately 60 to 90 seconds). Both portions of tissue were dropped into liquid nitrogen simultaneously and their respective melting curves determined. It is evident that the high value obtained for the whole organ is the result of its content of hypertonic medullary tissue. This finding is not surprising in view of the role of this portion of the kidney in elaborating a concentrated urine and its tubular content of such urine (16).

Fig. 10 illustrates the effects of tissue autolysis on the melting curves of rat kidney. The curve for whole kidney, "*a*," and the three serum reference curves are taken from Fig. 9. The two curves labelled "*b*" were obtained from whole kidney tissue handled in the usual manner except that the frozen tissue powder was first exposed in a covered crucible to room temperature for 10 minutes, refrozen, and its melting curve measured. Curve "*c*" was obtained with tissue similarly exposed for 20 minutes. This treatment resulted in a marked increase in the depression of the melting curves despite the fact that after 10 minutes' exposure at room temperature the pulverized tissue had not yet completely thawed.

DISCUSSION

The purpose of the present study was to determine whether in fact gradients exist between intracellular and extracellular fluids which might produce net shifts of water alone across cell membranes. If a fluid is separated into two portions by a rigid diaphragm permeable to some components of the solution and not to others, the driving force which determines the flux of each species across the membrane is its gradient of total potential. Equilibrium exists for such a permeable species across the membrane, in a thermodynamic sense, when the potential of that species is equal on the two sides of the membrane and, from the kinetic standpoint, when the fluxes of the constituents are equal in both directions through the membrane. The thermodynamic definition of equilibrium has been generalized by Guggenheim (17) to indicate the separate contributions of concentration, pressure, and electrical forces to the total potential. As the concern of the present study relates to forces acting on the uncharged solvent, water, electrical forces are not involved. It can be assumed that any additional forces, such as gravitational ones, are acting equally on both sides of the cell membrane and thus contribute no net force to water movement. Hence pressure-volume and chemical activity forces remain as possible factors affecting net water movement across the cell membrane.

For simple dilute solutions Raoult's law defines the relationship between water activity (thus potential) and vapor pressure of the solution. In "solutions" as complex as tissues, however, there are many factors which will affect the potential of the solvent, water, and hence the vapor pressure of the "solution" other than those related simply to the concentration of a single solute; *i.e.*, the presence of macromolecules, water and solute "binding,"

mixtures of interacting solutes, surface effects, etc. Even when dealing with such a "solution" contained in a unit compartment it would be impossible at our present state of knowledge to evaluate the effect of these factors individually on the water potential within the compartment. However, their effects collectively will yield a single value for the water potential throughout this compartment. Thus, if a protein or surface within the compartment "binds" water so as to reduce the free movement of neighboring water molecules below that in the more distant ambient solution, a readjustment in the concentration of water molecules will result at this restrictive site until the potential of the water is the same there as in the ambient solution.

Measurement of the melting point depression of a solution yields the same information about the water potential of a solution as does measurement of the vapor pressure and consequently has been used in the present study to test for possible differences between the water potential of serum and of tissues. For convenience, it is customary to use as a scale of water potential a measure of a colligative property of simple standard solutions of known osmotic activity and to express the results as osmolality. It should be recognized, however, from the above discussion that equality of water potential between complex "solutions" such as tissue fluids and a standard of known solute concentration carries no implications regarding equality of total molecular concentrations, solute concentrations, or solute activity. Only the water potential of the two solutions may rigorously be said to be equal. Thus if two solutions of equal water potential are separated by a membrane permeable only to the solvent, no net movement of water will occur across the membrane regardless of how different or complex the two solutions may otherwise be.

Recently several workers (18–20) have emphasized that when a "leaky" membrane rather than a truly semipermeable membrane is used in osmotic pressure measurements, the rate of permeation of the membrane by solute becomes important in determining the rate and direction of solvent permeation and net flux as well as the potential of the solvent. It is known that cell membranes are permeable to many solutes of low molecular weight as well as to water and hence cell membranes fall into the category of leaky membranes. Therefore in treating a dynamic state one would have to know, in addition to the average potential of the solvent, the potential and permeability coefficient[2] for every solute on either side of the cell membrane in order to completely describe the movements of solvent through the membrane. Such information far exceeds current knowledge. The present study had the far less ambitious goal of determining whether there exist gradients of water potential which might contribute to net movements of water between cells and their

[2] The permeability coefficient for a solute need not be the same in the two directions across the membrane if some energetic process in the membrane directly or indirectly facilitates movement of the solute in one direction across the membrane (21).

environment of extracellular fluid. It is to this question that the results here reported give, within the limitations of the measurements, a negative answer.

The validity of the method used in this study as a measure of water potential requires first that it yield a true measurement of the melting point of aqueous solutions in general and second that it be applicable specifically to the measurement of the colligative properties of tissues.

1. Applicability of the Method to Aqueous Solutions.—The requirements for a valid melting point method set forth in the Introduction would seem to be largely fulfilled by the technique utilized in this study. Any thermal gradients existing between melting particles and solution during the measurement would be minimized by the vigorous stirring of the finely divided tissue suspension. Furthermore, as the interpretation of the results depends upon a comparison of tissue with serum, both handled identically, any such residual disturbance should affect both measurements similarly.

Very rapid freezing of the solutions might result in solute entrapment within the ice crystals rather than separation of ice from solute. This would result in a falsely small depression of the melting curve and overestimation of the water potential of the sample (22). To test this possibility equal portions of serum of known osmotic activity were frozen rapidly by the same technique as used for tissues or much more slowly over dry ice or in a refrigerator. In all instances, however, the melting curves subsequently obtained were identical. Furthermore it is known that very few solutes sufficiently mimic the size and configuration of the water molecule to be entrapped within the ice structure at the rates of freezing used in this study and such molecules, *e.g.* fluorides (23) are, at most, trace constituents of living tissues.

Liquid silicones seemed to offer an ideal vehicle in which to suspend the frozen particles of solution or tissue. They are non-polar liquids, highly immiscible with water. Consequently for the present purposes, the fragmented tissues may be considered to have been suspended in liquid glass. An appropriate silicone was selected for the following features: (1) a low freezing point (−65°C.) which permitted precooling on dry ice prior to mixing with the frozen tissue powder, (2) a low viscosity (5.0 centistokes) to reduce friction and to facilitate mixing, (3) a low specific heat (0.33 to 0.35 calories per gm.), and (4) a density (0.920 gm./cm.^{3}) close to that of frozen tissue to facilitate dispersion of the frozen particles. In spite of these favorable characteristics simple salt solutions produced erratic melting curves because of a tendency of the frozen powder to agglutinate during the melting process. However with tissues and serum this difficulty, though still present to a slight extent, did not interfere with satisfactory measurements.

Conway and McCormack (12) noted that care must be taken in handling tissue frozen in liquid nitrogen in order to avoid condensation of water on the cold tissue. In order to test this possible objection, the freezing point of serum and saline was determined before and after freezing in liquid nitrogen and a

small *in*crease in freezing point depression of from 1 to 3 per cent was regularly noted. No correction for this small effect has been attempted.

2. Applicability of the Method to Tissues.—It is possible that the process of preparing and melting the tissue would tend to alter factors in the tissue which affect the water potential, *e.g.* osmotic effects of macromolecules, "binding" of water, "binding" of solute. However, evidence for a significant effect of these factors on the water potential in tissues is lacking (24, 25) and, furthermore, the effect of freezing on these factors is unknown (14). If such changes were produced to any quantitatively significant degree by the freezing process, it seems unlikely that they would have just sufficed to bring the water potential in the tissue into equality with that of the serum.

The method used seemed to cope adequately with the problem of tissue autolysis. For most tissues a period of some 10 seconds only was required between interruption of blood supply to that tissue and immersion in liquid nitrogen. While in liquid nitrogen no measurable autolysis occurred as demonstrated by constancy of the subsequent melting curve of tissue kept in liquid nitrogen for as long as 8 hours. In spite of the rapidity of the melting process the shape of the melting curve is such that some thawed tissue, in which autolysis might occur, was present during the measurement. Thus at the beginning of the last 2 minutes of a measurement some 20 per cent of tissue water had already melted and at the start of the final minute this figure had increased to some 60 per cent. If autolysis had commenced in this melted portion of tissue the upper part of the melting curve would have been displaced to the left. Such an effect was seen only with brain and kidney cortex. A definite difference in the preparation of these two tissues from all others was that a delay of 1 to 2 minutes was required between compromising their blood supply and freezing them. Perhaps this time interval added to that during the melting measurement sufficed to allow significant autolysis in the final portion of the melting curve of these tissues. When kidney tissue was purposely exposed at room temperature to promote autolysis (Fig. 10) the subsequent melting curves showed a similar shift to the left in their terminal portions, supporting this suggestion.

The accuracy of the method can be evaluated from the standard error reported for the mean values. The standard error represented approximately ± 4 m.osm/kg. water, yielding a coefficient of variation for the mean of 1.3 per cent. Thus the method was precise enough to test the large discrepancies previously reported between tissue and serum tonicity but could not be used to exclude the possible existence of very small gradients of water potential.

As mentioned earlier the melting point determination would be affected by differences in pressure, as well as in concentration, between intracellular and extracellular fluids. If equal water potential between these two compartments were sustained, in spite of a lower intracellular water concentration, as a result of pressure exerted by the cell membrane, results similar to those obtained

would be expected so long as the pressure continued to be exerted on the intracellular fluid throughout the freezing and melting procedure. If such pressure differences did exist *in vivo* but disappeared during the measurement as a result of damage to the cell membrane, the results would have shown a lower water potential in tissues than in serum. However as 19 mm. of Hg pressure is equivalent to only 1 m.osM/kg. water concentration difference, a 5 per cent difference between the serum and tissue curves would have required a pressure of over 300 mm. Hg on the intracellular phase *in vivo*. Though plant cells with their cellulose sheaths may withstand pressures of this magnitude or even much greater, current evidence indicates that the membrane tension of animal cells is very much less than this (26).

The average water content of each tissue studied was determined as described and for each measurement a quantity of frozen tissue powder was used which was calculated to contain a constant weight of water. Melting point measurements made on portions of tissue and serum containing equal amounts of water yielded curves indicating equal total heats of fusion. This demonstrates that all the water in the tissue participated in the melting curve. It is well known that for most tissues some two-thirds of the tissue water is contained within the cells, while the remaining third comprises the extracellular fluid. Because of the possibility that the fluids in these two compartments had frozen separately and were at different water potentials, it was essential to determine the effect of a mixture of frozen solutions of different osmolalities on the melting curve. If the two frozen solutions are in equilibrium with the same liquid phase the expectation is that such a mixture will yield a curve representing the mean concentration of the two solutions.[3] For this purpose a volume of serum (288 m.osM/kg. water) and a volume of concentrated serum (508 m.osM/kg. water) were frozen separately in liquid nitrogen, pulverized separately, and then equal amounts by water content of frozen powder combined and the melting curve obtained by the usual technique. The resulting melting curve (389 m.osM/kg. water) was within 2 per cent of the expected (398 m.osM/kg. water). Therefore the melting point method yields a result representing the mean water potential in the sample measured. Because of the large fraction of intracellular fluid in tissues and the good agreement between the tissue and serum melting curves, it can be concluded that within the limits of accuracy of the method, the mean water potential within mammalian cells is equal to that of the extracellular fluids. Only in the case of the renal medulla were the results inconsistent with this conclusion, and this is to be expected because of the content of concentrated urine and the presence of the renal concentrating mechanism in this zone of the kidney.

[3] If intracellular and extracellular fluids remained separate during melting and if differences in water potential existed between them, a biphasic melting curve should have resulted. A single phase curve, as was observed, should result if the two fluids, though separate, were at the same water potential.

In addition to measurements of the colligative properties of tissues, indirect arguments of two types have previously been applied to the problem of the potential of water within cells. The volume of distribution of a large water load given to an animal was found just to equal the total body water content (27). The apparent volume of body water effective in diluting hypertonic solutions after their injection into the whole animal also yielded fair approximations of total body water content (12, 28–30). Though such results are most easily explained by the existence of a uniform water potential throughout the intracellular and extracellular compartments, they do not afford conclusive evidence for such a hypothesis.

A second approach has been the study of the swelling of tissues *in vitro* when placed in extracellular medium under conditions which inhibit metabolism (31–35). Such swelling is reversible if metabolism is resumed, and it can be prevented by increasing the tonicity of the incubating medium to approximately twice that of extracellular fluid (31, 36, 37). These observations, that tissue swelling is dependent upon tissue metabolism and that it can be prevented in hypertonic media, have been cited as evidence that the intracellular fluids have an osmotic pressure higher than that of the extracellular fluid and that this osmotic gradient is preserved *in vivo* and in metabolizing tissue slices *in vitro* by an active extrusion of water (31, 38). The fallacy of applying this argument to the question of the possible existence of gradients of water potential in tissues was first pointed out by Mudge (33) who demonstrated that solute as well as water from the medium enters the tissues during the swelling process. More recently, these results have been confirmed and it has been demonstrated that the swelling process is the result of movement of essentially isotonic medium into cells (39). Hence, as cell membranes are "leaky" (see above) it is clear that the study of tissue swelling is a separate problem which cannot give evidence for or against the possible existence of gradients of water potential between the extracellular and intracellular compartments *in vivo*. At present it is only by direct measurements such as those used in this study that this problem can be settled. Within the limits of the method the results confirm the view that gradients of water potential do not exist between intracellular and extracellular fluids. With the exception, perhaps, of the renal medulla and of glands with anisotonic secretions, transfers of solute must be accompanied by movements of solvent to maintain equal potentials of water on both sides of cell membranes.

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