THE FREEZING POINT DEPRESSION OF MAMMALIAN TISSUES IN RELATION TO THE QUESTION OF OSMOTIC ACTIVITY OF CELL FLUID*

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INTRODUCTION

The classic concept that intracellular fluid is isoosmotic with the extracellular fluid has never been rigorously established.

Evidence in favor of isoosmotic cell fluid has been adduced from the following: (*a*) The freezing point depression and vapor tension lowering of erythrocytes have been shown to be equal to those of plasma (1–6). Moreover, erythrocytes behave as nearly perfect osmometers when suspended in solutions of varying osmolarity (7); (*b*) the estimated sum of the molal concentrations of solutes in erythrocytes and muscle (8–11); (*c*) changes in serum osmolarity after injection of hypertonic solutions into intact animals (12–14).

On the other hand, evidence casting doubt on the concept of isoosmotic cell fluid has been accumulating for a long time. Sabbatani found that the freezing point depression of many dog tissues (removed 2 to 60 minutes after death) was greater than that of blood (15). His results were confirmed by Collip (16) who explained the high freezing point depression of excised tissue by assuming the activation of osmotically inert particles in the tissue after removal. Along other lines, the observation of tissue swelling in isotonic solutions has been made repeatedly by many early workers (17–20). Swelling was usually accounted for by the implicit assumption of cellular isotonicity initially with the subsequent production of osmotically active particles derived from the catabolic reactions of glycogen or proteins.

Recent data on swelling of tissue slices in isotonic solutions (21–31) and on melting point measurements of frozen tissues (32, 33) have posed a new challenge

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to the validity of the classic concept. Thus, freshly excised slices of mammalian tissue swell in isotonic, but not in hypertonic solutions, and the swelling in isotonic media could be reversed by oxygenation of the ambient fluid. These results led to the suggestion that the cell fluid *in vivo* is hypertonic to extracellular fluid, and that the osmotic gradient is normally maintained by continuously pumping water out of the cell (21, 22). However, Deyrup (29) found that tissues do not swell in isotonic sucrose, but do swell in isotonic sodium chloride, glucose, and Krebs-Ringer, and after oxygenation, return to normal volume in Krebs-Ringer or in isotonic sodium chloride solutions. She suggested that water pumps alone could not explain these phenomena, and that shrinking was apparently dependent upon the presence of ions in the ambient solution.

Ionic shifts occurring concomitantly with swelling or with oxygenation of tissue slices have been reported (34–39). Schwartz (30) placed tissue slices in a series of sodium chloride solutions and found that the sodium space of the slices after 10 minutes of immersion amounted to values close enough to those of extracellular fluid to suggest that the effect of immersion within the first 10 to 15 minutes was to equilibrate the interstitial fluid with the solutions. Subsequently, in studying temperature effects on the swelling of tissue slices immersed in solutions of sodium chloride, monosaccharides, and disaccharides, Itoh and Schwartz (31) were able to account for the observed volume changes of the tissues in terms of solute shifts between the slices and the ambient media.

Such data on swelling and composition changes of immersed tissues stimulated some to make direct measurements of the colligative properties of tissue juices obtained under more elegant conditions than those employed by earlier workers. Although all the recent cryoscopic data showed that the freezing point depression of freshly excised, frozen tissues was greater than that of plasma (32–33, 40–44), diametrically opposing conclusions were drawn by different investigators. Thus, Conway and McCormack (42) inferred that cell fluid is isotonic because the calculated tissue osmolarity of saline-dilute homogenates, extrapolated to zero time, approximated the plasma osmolarity. Similarly, Howard (40) claimed that cell fluid is isotonic because the freezing point of normal muscle and testicular tissue in rats was the same as that of plasma. However, she, like others, noted that the freezing point depression of liver was higher than that of plasma. On the other hand, Opie inferred that cell fluids must be hypertonic to plasma, since the melting point of frozen tissue powder was lower than that of plasma (32). Wirz *et al.* drew similar inferences on the tonicity of renal medullary cells on the basis of microscopic visualization of melting in the frozen tissue at a temperature less than that of plasma melting point (33).

None of the aforementioned experiments contained data pertinent to the effect of the technique of preparation on the structural or physical properties of the tissues. While tissue swelling experiments were performed on slices kept at room temperature or at 38°C., cryoscopic experiments were usually per-

formed on pulverized powder of tissues in liquid N_2 or O_2 kept at about 0°C. during the actual measurements. In short, the experimental conditions that differed among all reported data included the degree of disruption of tissue architecture in the sample used, the individual ion flux of slices, the time interval between tissue excision and determination, the effect of temperature during the actual measurement, and the possible effects of various diluent solutions.

The primary purpose of the present report was to study the cryoscopic behavior of tissue homogenates prepared in different ways. To obtain samples containing intracellular fluid, methods were devised to insure cell destruction in the homogenates. What follows will concern: (*a*) an attempt to establish the validity of the cryoscopic method as a measure of the osmotic activity of tissue homogenates; (*b*) an attempt to determine whether or not cells are destroyed in tissue homogenates prepared in different ways; and (*c*) the effect of various diluents on the calculated freezing point depression of different tissue homogenates.

Methods

Tissues, removed without hemostasis from dogs under the influence of nembutal or amytal, were immersed immediately (within 5 to 10 seconds or less) in liquid nitrogen or in isopentane cooled with liquid N_2. The frozen blocks of tissues were treated in one of two ways: (1) Some blocks were crushed in the Carver hydraulic press, and the tissue juice so obtained was placed immediately in test tubes for determination of freezing point depression. At first, the crushing was done with the press kept at room temperature, but the temperature of homogenates oozing out of the press reached +13° within 2 to 3 minutes. Therefore, crushing was performed with the press cooled to +2–3°C., so that the temperature of the homogenates obtained rarely exceeded +1–2°C. before they were placed in the osmometer. (2) Other blocks of frozen tissue removed from liquid nitrogen were placed in a mortar, covered with liquid N_2, and pulverized to a fine powder. Approximately 1.5 to 2.0 gm. of the frozen powder were quickly scraped into the appropriate tube for determination of freezing point depression. When aliquots of the same tissue of one dog were processed at the same time through "warm" and cool presses and by means of the mortar and pestle, the freezing point data from all three procedures showed no significant differences.

At first, all measurements of osmotic activity were made on homogenates without any added diluent solution. Later, when testing the cryoscopic method, and when testing the time course of osmotic activity in homogenates, various diluent solutions were added. Determinations of freezing point depression of tissue samples were performed in a Fiske osmometer using a thermistor as the temperature-sensing element.

RESULTS

Preliminary Tests

In earlier experiments, freezing point determinations were made on serum, plasma, red cells, whole blood, liver, kidney, pancreas, stomach, lung, muscle,

brain, and spleen. The data indicated that erythrocytes were isotonic to plasma or serum, while homogenates of all other tissue (except about one-half of the spleen samples) were hypertonic to plasma. Some of the high values (827 for liver, 538 for kidney) are from determinations on tissues which had remained at room temperature or at 0°C. for periods as long as several hours.

A superficial examination of the data in Table I might lead one to suspect the existence of a hypertonic tissue fluid. However, practical objections to the experimental conditions became apparent. (1) There was a wide scatter of data

TABLE I

Values of Osmotic Activity Determined Cryoscopically on Serum, Plasma, Erythrocytes, Whole Blood, and Various Tissues

Tissue		Osmolarity	
		Average	Range
Serum	(7)	286	266–300
Plasma	(32)	300	280–325
Red blood cells—clotted	(8)	310	285–392
Red blood cells—heparinized	(13)	325	266–342
Whole blood	(6)	289	277–300
Liver	(24)	413	314–827
Kidney	(23)	434	337–538
Spleen	(11)	312	286–399
Muscle	(9)	359	297–390
Pancreas	(5)	355	335–390
Gastric mucosa	(3)	328	315–340
Brain	(2)	313	306–319
Intestine	(1)	422	

Numbers in parentheses designate the number of experiments performed on each tissue listed. The value for each experiment was the average of several freezing point determinations on each tissue sample.

indicating poor reproducibility of freezing point values in tissues of different dogs. Occasionally, even the juice of a single organ would yield divergent data. On the other hand, standard salt solutions, or blood from a single dog would yield reproducible freezing point values. (2) The time required to reach the freezing point was longer for tissue juice than for plasma, red blood cells, or salt solutions. Perhaps the heat transfers between solid and liquid phases and environment at the freezing point were changed in a gel or protein system like tissue juice. Thus, the viscosity, high protein content, or gel state form of solutions in homogenates might have caused the observed freezing point depression to be spuriously high. Such objections led to considerable skepticism as to the validity of cryoscopic measurements of undiluted tissue homogenates. There-

fore, experiments were designed to check the validity of the freezing point technique under present experimental conditions. If tissue fluids were spuriously hypertonic because of the high protein content or viscosity of cell fluid, then dilution of the homogenate with water would be an obvious way to reduce these factors.

Testing the Validity of the Cryoscopic Technique Applied to Tissue Homogenates

Dilution tests were done as follows: Tissue juice from 100 gm. of kidney or liver processed in the Carver press was homogenized in a Waring blendor for 3 to 5 minutes. Weighed aliquots of the homogenate plus weighed aliquots of distilled water were placed in flasks immersed in an ice bath. Portions of the

TABLE II

Schematic Table Showing the Experimental Design Used in Dilution Tests

Tube No.	Tissue	Tissue water	Added water	Dilution factor
	gm.	*gm.*	*gm.*	
1	10	7.5	0	1
2	10	7.5	1.1	1.06
3	10	7.5	2.5	1.33
4	10	7.5	4.5	1.60
5	10	7.5	7.5	2
6	10	7.5	15.0	3
7	10	7.5	22.5	4
8	10	7.5	52.5	8

cooled liver or kidney homogenates were transferred to test tubes for cryoscopic measurements. Table II presents an hypothetical protocol for the scheme of serial dilution used. The chronologic order of determinations at different dilution levels was randomized. The interval between pressing the frozen block of tissue and the freezing point determination was approximately 30 minutes in most cases. When a single aliquot of tissue was subjected to the aforementioned treatment, it was found that osmolarity was 450 to 500 m.osM/liter and would show but small change after immersion in an ice bath for the next 30 to 60 minutes.

If the apparent freezing point depression of the undiluted homogenate were erroneously high due to factors interfering with heat exchanges in the freezing mixture, the progressive dilution of the homogenates should reduce the calculated values of osmotic activity so that they would approach those of plasma. Fig. 1 illustrates calculated values of tissue osmolarity *versus* dilution in a representative experiment. A total of 9 experiments was performed, and it was found that the calculated osmolarity did not change significantly over an eightfold dilution range. Such data conform with those expected on the assumption that

freezing point measurements of undiluted homogenates are accurate. The calculations did not correct for changing activity coefficients of the tissue electrolytes at varying degrees of dilution. But even had this correction been applied, the osmolarity calculated from the most dilute sample would have been but a few per cent lower than the value shown, and consequently, still over 1½ times the value of plasma osmolarity.

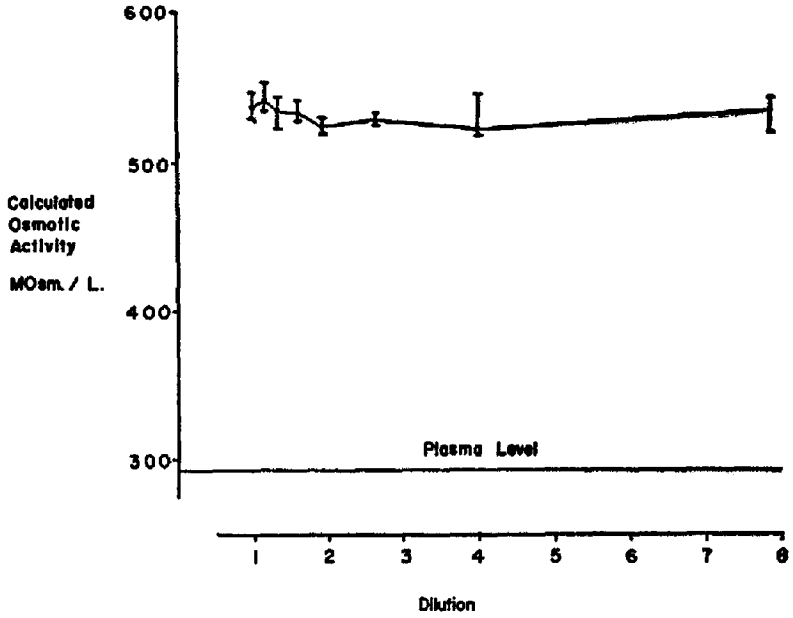

FIG. 1. Calculated osmotic activity of tissue *vs.* dilution. Each point plotted represents the average of 5 determinations of freezing point depression. The range of each set of values is indicated by the length of the vertical line.

The foregoing experiments indicate that the osmolarity of undiluted homogenates is apparently higher than that of plasma. This does not necessarily mean that the fluid within a living cell *in situ* is hypertonic to the surrounding extracellular fluid.

The Effect of Time and Temperature

Since time of standing and environmental temperature have been found to influence the osmotic activity of undiluted homogenates (41–44), a systematic investigation of the effect of such factors was undertaken.

Samples of liver homogenate were prepared in the prescribed manner as quickly as possible, and the first determination of freezing point depression was obtained within 5 minutes after removing the homogenate from the cooled press or from the mortar and pestle. The undiluted homogenates were kept in

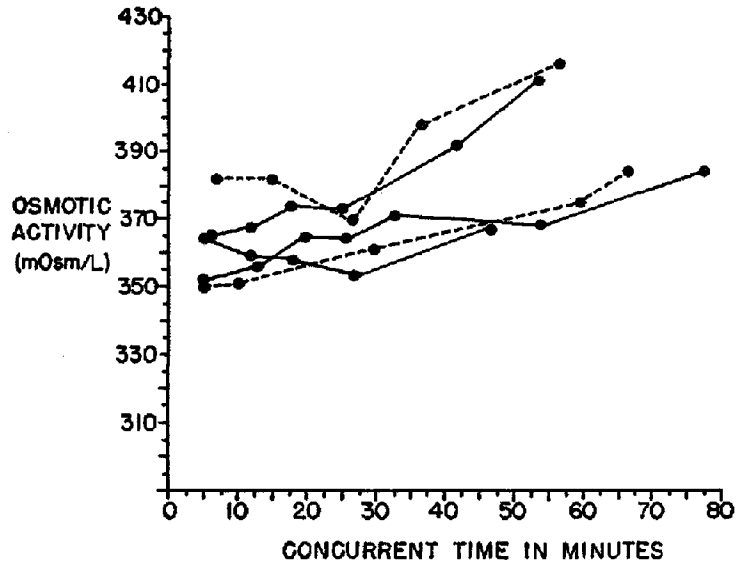

FIG. 2 *a*. Osmotic activity *vs.* time. Undiluted liver homogenate prepared in Carver press. Zero time refers to the instant pressure was applied to the blocks of frozen tissue in the press. Plasma osmotic activity = 287 to 314 m.osM/liter.

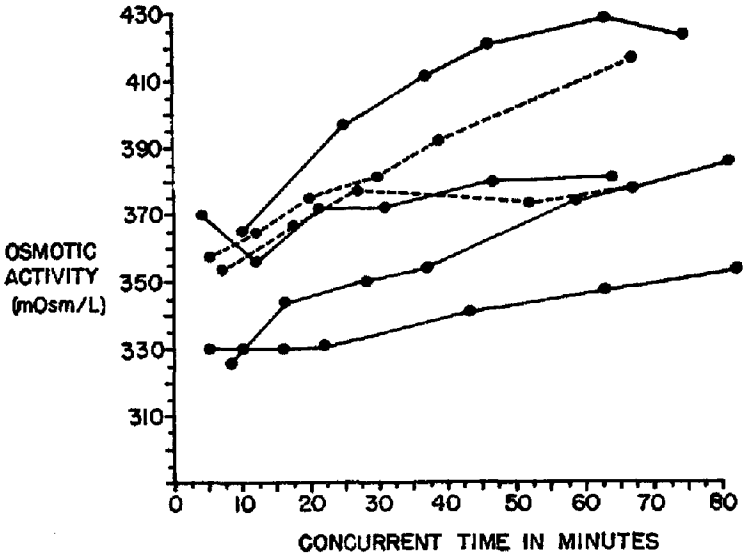

FIG. 2 *b*. Osmotic activity *vs.* time. Undiluted, pulverized liver powder prepared in the mortar under liquid N_2. Zero time refers to the time the powder, previously covered by liquid N_2, reached a temperature of 0°C. The time interval required for such thawing was no more than 1 to 2 minutes. Plasma osmotic activity = 287 to 314 m.osM/liter. Data of both Fig. 2 *a* and Fig. 2 *b* are from the same dogs.

an ice bath or at room temperature, and osmolarity measurements were made at 10 to 15 minute intervals for 1 to 2 hours or more.

Figs. 2 *a* and 2 *b* show patterns of concurrent increases of osmotic activity in undiluted liver homogenates prepared from the Carver press and with the the mortar and pestle and kept at 0°. The pattern about which data fell was similar in both instances. Inspection of both figures shows that the values after 3 to 10 minutes at 0° were 325 to 383 m.osM/liter. After 60 to 90 minutes at 0°, osmolarity had increased to a range of 340 to 430 m.osM/liter. Linear extrapolation of these curves to zero time, yielded osmolarities of 305 to 375 m.osM/liter, with 9 out of 10 extrapolated values in excess of 330 m.osM/liter. Such extrapolated values, higher than those of plasma osmolarity did not conform with the data or with the predictions of Conway based on extrapolations in saline-diluted homogenates.

The same experiments have been performed at room temperature (25°C.) and at 38°C. The rate of increase of osmotic activity of the warmed homogenates was definitely greater than that of the cooled homogenates. For example, at room temperature juice from the Carver pressed showed an osmolarity of 370 m.osM/liter in 5 minutes and over 500 m.osM/liter in 30 minutes. Results on frozen tissue powder from the mortar were closely similar to those of the Carver press data, both at 25°C. and at 38°C.

Effect of Isotonic Diluent

Because of the difference between our results with undiluted homogenates and those of Conway and McCormack with saline-diluted tissue homogenates (42), experiments were designed to test systematically the effects of saline diluents. Frozen blocks of tissue, pulverized in a mortar with a pestle under liquid N_2 (and in some cases under liquid O_2), were mixed with tared aliquots of isotonic saline at 0°C. The weight of saline added was about the same as the weight of whole tissue. Then, successive determinations of freezing point depression were made on the saline-tissue mixture (see Figs. 4 and 5). After obtaining the dry weight on an aliquot of the original batch of tissue powder, calculations of the tissue osmotic activity were made, and the results therefrom plotted graphically against time. Linear extrapolation of these calculated values to zero time yielded values of 310 to 350 mosM per liter of tissue water. The fairly wide scatter of data was similar to that reported by Conway. However, values extrapolated to zero time were about 10 per cent greater than that reported by Conway from the zero intercept of a linear regression equation (42).

Consideration of both the similarities and differences between results of the present report and those of Conway led to the following possibilities: Conway's calculations assume complete destruction of cells as well as instantaneous diffusion and mixing of intracellular with ambient fluids. However, if the cells of a tissue powder diluted in isotonic saline were intact or in clumps, and if diffusion of cell solute and water were slow, then even if the cell fluid were

hypertonic to the ambient, the initial freezing point depression of the cell-ambient mixture would approach that of the isotonic saline since it would freeze first. Even with a hypertonic cell-free homogenate diluted with isotonic saline, and in which there is incomplete mixing, the freezing point depression of the mixture would also approach that of isotonic saline.

Microscopic Examination of Tissues

Since some of the aforementioned considerations are based on the presence of intact cells, it was pertinent to examine the homogenates histologically. Smears of freshly prepared liver and kidney homogenates prepared with the mortar and pestle and from the Carver press, were fixed immediately in alcohol-ether and stained with hematoxylin and eosin. Figs. 3 *a* and 3 *b* are representative photomicrographs of liver homogenate. In the Carver press homogenate (Fig. 3 *a*) there appear many free floating nuclei and particulate bodies, but only an occasional intact cell. Several fields in each of 35 slides were examined. On the other hand, the mortar and pestle preparation (Fig. 3 *b*) had many clumps of intact cells. This tissue had been pulverized as vigorously as possible by hand for 15 to 20 minutes under liquid N_2. Only a fine powder remained in the mortar. The field shown in the figure is fairly representative of several fields examined in each of 35 such slides.

Thus, microscopic inspection of the tissues showed that the bulk of the cells had been disrupted by processing through a Carver press, while large numbers of cells and clumps of cells remained intact after pulverizing in a mortar with pestle as Conway and McCormack (42) and Opie (32) did. In view of the presence of clumps of intact cells in the mortar and pestle preparations, we questioned the validity of calculations like those of Conway which assume complete mixing of cell and ambient fluids as well as an instantaneous state of diffusion equilibrium between the two aqueous phases. Even if the cells were uniformly distributed through a perfectly homogeneous diluent solution, the rate of achievement of a diffusion equilibrium between the two phases could be delayed.

Effect of Varying Osmolarity of the Diluent

In Conway's experiments, a diluent fluid, isotonic saline, was added to frozen tissue which had been ground to a fine powder in a mortar, and the freezing point depression of the final mixture was determined repeatedly against time. The "tissue" osmotic activity was calculated from the assumption that the total number of osmotically active particles in the final mixture was equal to the total number of osmoles in the tissue and diluent. Mathematically,

$$(O_{\text{obs.}})(D_{H_2O} + T_{H_2O}) = (O_{\text{tissue}})(T_{H_2O}) + (O_{\text{dil.}})(D_{H_2O}) \quad (1)$$

or

$$O_{\text{tissue}} = (O_{\text{obs.}})\left(\frac{D_{H_2O} + T_{H_2O}}{T_{H_2O}}\right) - (O_{\text{dil.}})\left(\frac{D_{H_2O}}{T_{H_2O}}\right) \quad (2)$$

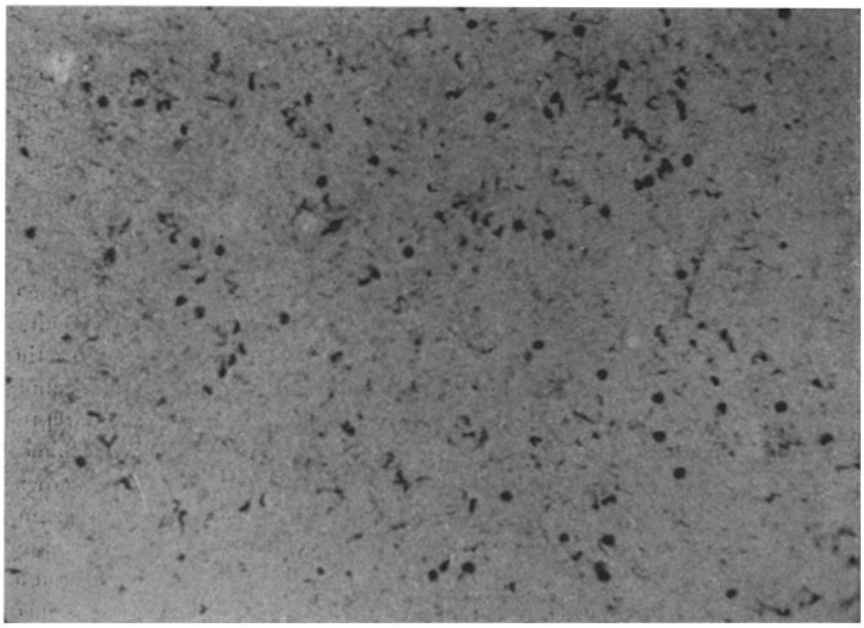

FIG. 3 *a*. Hematoxylin and eosin stain of liver juice after processing a frozen block of tissue through the Carver press.

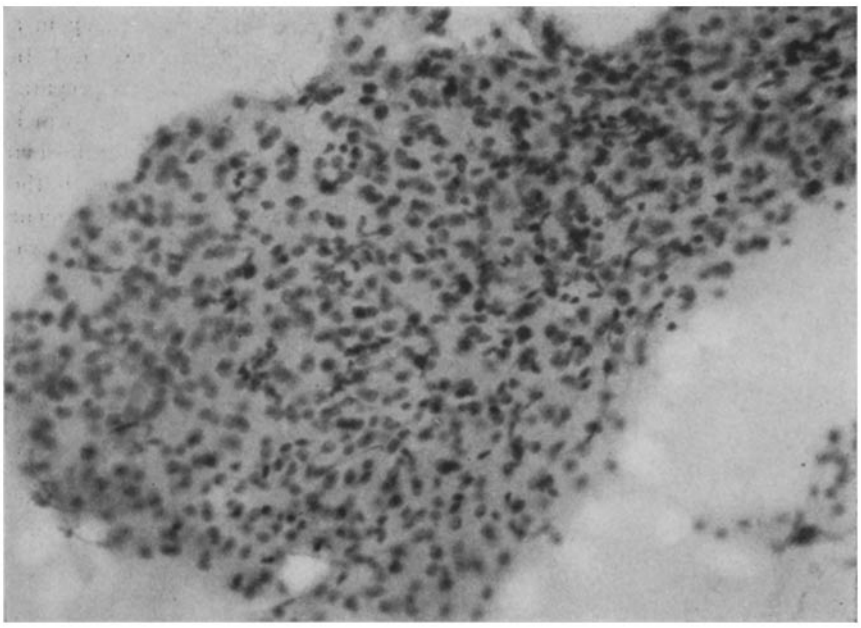

FIG. 3 *b*. Hematoxylin and eosin stain of liver powder after pulverizing frozen blocks of the tissue in a mortar.

in which O_{tissue} is the calculated tissue osmotic activity, $O_{obs.}$, the observed osmotic activity of tissue plus diluent, $O_{dil.}$, the osmotic activity of the diluent solution, D_{H_2O}, the weight of diluent solution, and T_{H_2O}, the weight of tissue water determined by dry weight measurement. The assumption implicit in this equation is that there is complete and instantaneous mixing and diffusion of all the water and osmotically active particles. If complete mixing did not occur, there would be two or more aqueous phases present. Even with com-

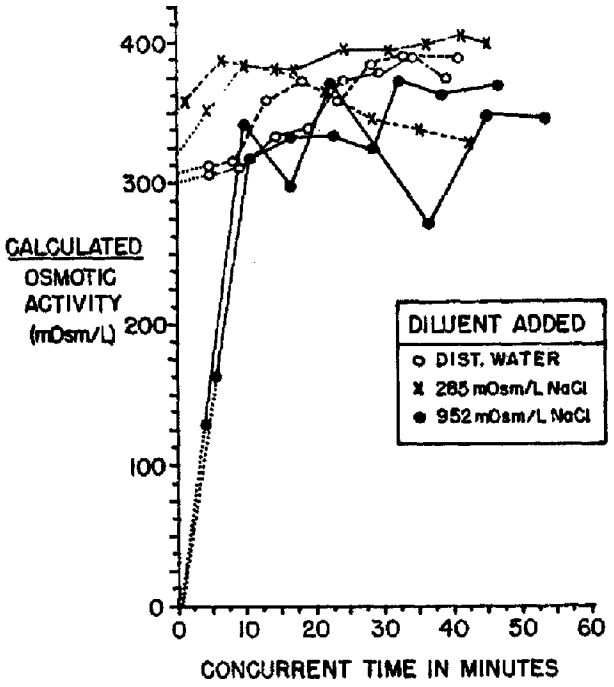

FIG. 4. Calculated osmotic activity *vs.* time. Liver powder prepared in the mortar was mixed with various diluents represented by symbols in the panel. Extrapolation shown by dotted lines.

plete mixing, an instantaneous diffusion equilibrium between the two phases would be required to satisfy the above equation. In either case, the freezing point of the final mixture would approach that of the phase of lowest osmotic activity.

If the added diluent were isotonic, and if no mixing or diffusion occurred between hypertonic cells and diluent, both the observed osmolarity of the mixture and the calculated osmolarity of the tissue would appear isotonic. In general, addition of an isotonic diluent to a hypertonic tissue phase would result in apparent isotonicity of both the mixture and the tissue, providing no equilibration occurred between the two phases.

With little or no equilibration, addition of a diluent of higher osmolarity than that of tissue could lead to a negative value for the calculated tissue osmolarity. In this case, the observed osmolarity would approach that of the aqueous tissue phase since it would freeze before a hypertonic ambient solution. Then the application of equation (2) would yield spuriously low, or even negative, values for $O_{t_{issue}}$ depending on the magnitude of the diluent osmolarity ($O_{dil.}$).

The following experiments were planned to determine whether or not the calculated tissue osmolarity was a function of the osmolarity of diluent. Tared aliquots of frozen tissue powder from the mortar and pestle preparation were mixed with tared aliquots of the following diluents: distilled water, 0.285 osM NaCl, 0.574 osM NaCl, 0.675 osM NaCl, and 0.931 osM NaCl. In each case, repeated determinations of freezing point depression were made on the diluted mixtures every 5 to 10 minutes for 90 to 120 minutes. Under such conditions, a total of 18 experiments covering 128 determinations of freezing point depression was performed on 6 dogs. Distilled water was used in 7 experiments, isotonic NaCl in 2, and hypertonic NaCl in 9.

Fig. 4 shows a plot of the calculated values of tissue osmotic activity *versus* time. For comparative purposes, the data in the figure were taken from 6 experiments on the liver tissue of one dog.

The linear extrapolations of osmotic activity values from the homogenate-diluent mixtures yielded the following values: 300 to 305 m.osM/liter for liver and distilled H_2O; 317 to 340 m.osM/liter for liver and isotonic saline; O, for liver and hypertonic saline. After 20 minutes of repeated determinations, all the six liver homogenates became hypertonic to plasma osmolarity of 295 to 300 m.osM/liter.

Figs. 5 *a* and 5 *b* are plots of the raw data from which the values of Fig. 4 were calculated. The derived and raw data are presented on the same scale for comparative purposes. It can be seen that the observed osmolarities of the tissue-water mixture were approximately 120 to 125 m.osM/liter in the first 5 minutes, and 150 m.osM/liter after 50 minutes. In the tissue–hypertonic saline mixture, osmolarities were 610 to 670 in the first 5 minutes and nearly 700 m.osM/liter after 50 minutes. In both diluents, small fluctuations in observed values become exaggerated in the calculated results because of the dilution effect.

The results with hypertonic saline are compatible with the predictions of a "delayed diffusion" hypothesis when a calculation, like that in equation (1) is applied to a system of intact cells in an ambient solution. Superficially, the data from water-diluted homogenates did not conform to the results expected on the basis of incomplete or delayed diffusion processes between ambient and cell fluid. One would expect that the extrapolated value of water-diluted homogenates would be zero, in the limiting case of zero diffusion, (*i.e.* impermeable cells); or would approach zero in the case of delayed diffusion. However, it is probable that distilled water can disrupt cells more effectively

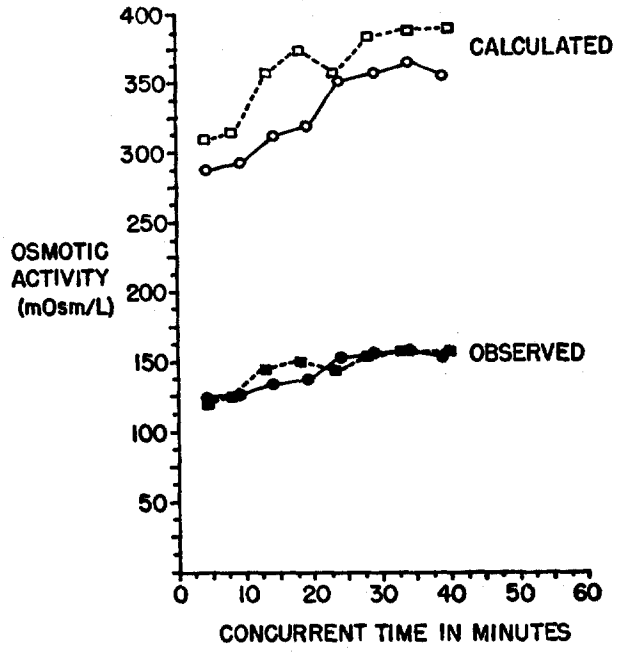

FIG. 5 *a*. Osmotic activity *vs*. concurrent time. Liver plus distilled water. Observed osmolarity of the mixture, and calculated osmolarity of the liver tissue.

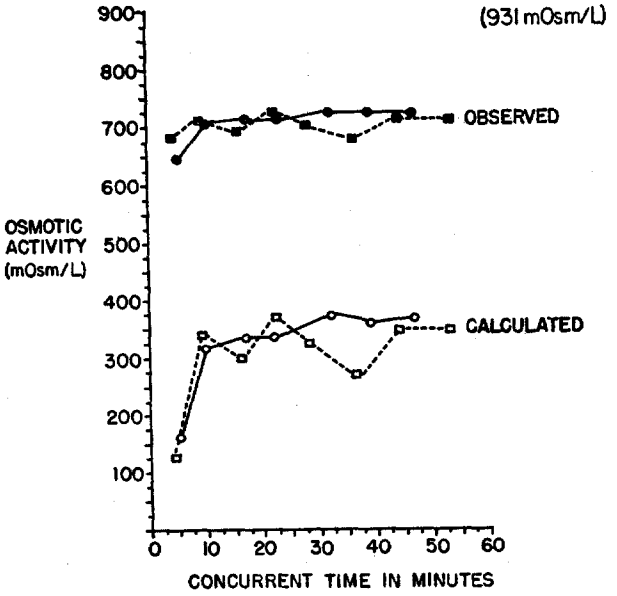

FIG. 5 *b*. Osmotic activity *vs*. concurrent time. Liver plus 0.95 osM NaCl. Observed osmolarity of the mixture, and calculated osmolarity of liver.

than can 950 milliosmolar NaCl. Disruption of significant numbers of cells by addition of distilled H_2O would reduce diffusion barriers and favor rapid diffusion of cell solute into the ambient water which would produce an osmolarity like that observed; *i.e.*, between that of distilled H_2O and cell fluid. On the other hand, hypertonicity of the ambient solution relative to cell fluid would tend to shrink cells, favor clumping, and consequently reduce diffusion processes between ambient and cell fluids.

A similar series of dilution experiments was performed on homogenates without intact cells. Such homogenates were prepared, as described previously in the Carver press. Sixteen experiments covering 121 determinations of freezing point depression were performed on 4 dogs. The results of calculated and extrapolated data were erratic from tissues diluted in both distilled water and 0.574 osM NaCl. However, the data after dilution of the cell-free juice in 0.675 osM NaCl were similar to those noted after dilution of the cell-rich powder from the mortar and pestle preparation. This suggests that clumping of homogenate, even without intact cells, delays mixing and diffusion between the two aqueous phases. Thus the calculated "tissue" osmotic activity extrapolated to zero time could be spurious in any experiment wherein a diluent is added to a tissue homogenate.

DISCUSSION

Although the freezing point depression of undiluted homogenates is greater than that of plasma, it does not necessarily mean that cell fluid of intact tissues in the animal is hypertonic to extracellular fluid. This consideration may imply that cryoscopic measurements on undiluted tissue homogenates yield spuriously high results, but our data suggest that the measurements are at least qualitatively valid. Our experiments do not exclude the possibility that factors such as low temperature or high pressure could alter physical properties of cell proteins and thereby increase the tissue osmotic activity. If this were the case, even an instantaneous determination of freezing point depression on an undiluted homogenate (processed through liquid N_2) would give a value higher than that of plasma. Thus, the experimental conditions present during preparation of, and during measurements on tissue must be examined in all experiments purporting to demonstrate either cellular isotonicity or hypertonicity.

Conway has stated that cell fluid is isotonic to plasma. He mixed equal weights of frozen tissue powder and isotonic saline, made successive determinations of the freezing point depression of the mixture kept at 0°C., and calculated, from such raw data, the freezing point depression of the tissue. His calculations assumed complete cell destruction and/or instantaneous mixing and diffusion of cell and ambient fluids. The estimated values were plotted against time, and extrapolated to zero time by calculated regression

line applied to the data. Since the extrapolated value at zero time approximated that of plasma osmolarity, Conway deduced that tissues must be isotonic (42). Obviously such calculations ignore the possibility of delayed mixing and diffusion of solutes between cell and ambient fluids in homogenates containing intact cells. The instantaneous freezing point depression (or the extrapolated freezing point) of isotonic or hypertonic cells in an isotonic ambient solution would have to be isotonic, since the isotonic phase freezes prior to the hypertonic phase. Consequently, Conway's experiment could not have distinguished between isotonic and hypertonic cell fluid.

Howard has also claimed that tissues are isotonic to plasma (40). Her data, obtained from inserting a thermocouple between pieces of intact tissue could have provided a measure of extracellular rather than of intracellular osmotic activity.

Other workers have claimed, on the basis of cryoscopic measurements, that cell fluids must be hypertonic to plasma. Opie measured the melting point of frozen pulverized blocks of tissue. However, it took about 60 minutes to reach the end point in Opie's experiments. A considerable proportion of such tissues must have been in the liquid phase by the time the end point was reached. Moreover, tissues thawed from the temperature of liquid N_2 to no more than 0°C. in 1 to 2 minutes will show increases in osmotic activity within 2 to 4 minutes, when kept (between freezing point determinations) at 0°C. Therefore, one would predict a significant increase of solute concentration in the liquid phase of Opie's tissues during the period required for determination of the melting point. Such experimental conditions will not allow a precise determination of osmotic activity of freshly prepared homogenates, so that Opie's results must be excluded as rigorous evidence of intracellular hypertonicity.

The objections to Sabbatani's (15) experimental data are: (1) no special device to homogenize tissues; (2) all tissues were removed after the death of the animal; (3) the time interval between removal of tissue and determination of freezing point depression was from 2 to 60 or more minutes, while the tissue was kept at room temperature. Data reported from this laboratory (45, 46) and elsewhere show that freezing point depression of fresh tissues kept at room temperature can increase over twofold in 20 minutes.

Recent data on swelling of tissue slices in isotonic media have led to the concept of intracellular hypertonicity. The fact that oxygenation of the ambient solution resulted in a decrease in size of swollen slices was considered convincing evidence in favor of hypertonic cell fluid (21, 22). Presumably an active water pumping mechanism maintains an osmotic gradient between cell fluid and surroundings in the living animal. After removal of oxygen, water moves passively in the direction of its chemical potential gradient, thus increasing the volume of cell fluid. However, Deyrup's findings (see above) are difficult to explain by water pumps alone. Many workers (31, 34–39)

have shown that certain ionic fluxes in tissue slices can be related to oxygenation or to swelling of the slice.

Therefore, the volume changes observed in slices immersed in isotonic media may be due to the movement of both solutes and water rather than to the movement of water alone between the slice and the ambient solution. This means that no osmotic gradient need be present to account for the phenomena of swelling or shrinking in the immersed slices.

On the basis of factual evidence to date, we know only that the freezing point depression of tissue homogenates is greater than that of plasma. The basic question of whether or not cell fluid is isotonic to its surroundings has been approached only indirectly (by extrapolation of cryoscopic values or by deductions from tissue slice experiments) and not directly. If cell fluid is isotonic, the problem would be to determine whether tissue homogenates become hypertonic because of physicochemical changes induced during their preparation, or because of metabolic processes active even at 0°C. If cell fluids are hypertonic, the problem would be to determine the precise nature of the water transport mechanism.

SUMMARY

The freezing point depression of freshly excised frozen tissues, pulverized in a hydraulic press or in a mortar, is greater than that of plasma. Even at 0°C. the freezing point depression of such homogenates increases significantly with time. Dilution data indicate that such freezing point data are valid. The presence of intact cells has been shown in smears of tissues pulverized in a mortar, but not in smears of those crushed in a hydraulic press. The osmolarity of various diluent solutions affects the calculated osmotic activity of tissue homogenates presumably because of delayed diffusion between the diluent and cell fluid. With a hypertonic NaCl diluent, spuriously low values of tissue osmotic activity are found from calculations assuming instantaneous mixing between homogenates and diluents. The limitations of data from cryoscopic experiments and from tissue-swelling experiments are discussed in relation to the basic question of whether or not cell fluid is isotonic to extracellular fluid.

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