THE FREEZING POINT DEPRESSION OF MAMMALIAN TISSUES AFTER SUDDEN HEATING IN BOILING DISTILLED WATER*

By JOHANNES W. TH. APPELBOOM, WILLIAM A. BRODSKY,‡ WILLIAM S. TUTTLE, AND ISRAEL DIAMOND

(From the Departments of Physiology and Pediatrics, University of Louisville School of Medicine, Department of Chemistry Graduate School, University of Louisville, and the Department of Pathology, Children's Hospital, Louisville)

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ABSTRACT

The calculated freezing point depression of freshly excised boiled mammalian tissue is approximately the same as that of plasma. The boiling procedure was chosen to eliminate the influence of metabolism on the level of the freezing point depression. Problems created by the boiling, such as equilibrium between tissue and diluent, change in activity coefficient by dilution, and loss of CO_2 content, are discussed.

A frozen crushed tissue homogenate is hypertonic to plasma. Boiling and dilution of such hypertonic homogenate exposed to room temperature for 5 to 15 minutes did not produce significant or unexplicable decreases in its osmotic activity. Moreover, freezing and crushing of a boiled diluted tissue did not produce any increase of the isoosmotic level of freezing point depression.

It is possible to explain these data either with the hypothesis of hypertonic cell fluid or with that of isotonic cell fluid. In the case of an assumed isotonic cell fluid, data can be explained with one assumption, experimentally backed. In the case of an assumed hypertonic theory data can be explained only with the help of at least three *ad hoc* postulates. The data support the validity of the classical concept which holds that cell fluid is isotonic to extracellular fluid.

INTRODUCTION

Data to be reported show that the freezing point depression of boiled mammalian tissue is approximately the same as that of plasma. This finding has to be reconciled with that of our previous report (1) showing that the freezing point depression of frozen tissues, pulverized in a hydraulic press or in a mortar, was greater than that of plasma.

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On the basis of the available data in the literature, the basic question of whether or not cell fluid is isotonic to its surroundings has been approached only indirectly even with cryoscopic measurements. Those who claim that cell fluids are hypertonic, suggested that their measurements of freezing point depression or of melting point were truly direct measurements of the tissue osmotic activity (2, 3). On the other hand, those who claim that cell fluids are isotonic, suggested that measurements of the freezing point depression were indirect and spuriously high because of experimental conditions. Therefore, such data were ambiguous with respect to answering the basic question of whether cell fluid is isotonic to interstitial fluid (1, 4, 5).

Data on tissue swelling (6–18) cannot be considered as direct measurements of cell osmolarity, particularly in view of data showing that swelling or shrinking phenomena are associated with movement of water and solute rather than with movement of water alone (19–28).

If cell fluid were 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 fluid were hypertonic, the problem would be to determine the precise nature of the water transport (1).

Purpose

Since autolytic or metabolic action could contribute to the hypertonicity of frozen-crushed homogenates, we searched for a technique that could stop metabolism immediately after excision of the tissue. Elimination of one of the possible interfering factors would permit a determination of the osmotic activity of non-metabolizing tissues. Sudden heating to the temperature of boiling water was chosen as the method of stopping tissue metabolism. Obviously, such treatment would produce physicochemical changes in tissue proteins different from those produced by the freezing-crushing method.

Boiling of tissue had been performed in 1901 by Sabbatani (29), who found that brain and liver, excised from a dead animal were hypertonic to serum, both before and after heating the tissue in a boiling water bath. However, objections to these data were: (1) removal of tissues after the death of the animal; and (2) the time interval between the removal of tissue and the determinations of the freezing point depression was from 2 to 60 or more minutes, while tissue was kept at room temperature. It appeared that the two reported values were hardly adequate as estimates of the freezing point depression of boiled tissues.

The approach of the present experiments was to measure the freezing point depression of freshly excised tissue subjected to sudden heat by immersion in boiling water. Assuming that diffusion equilibrium between tissue and ambient water was attained, one would be justified in calculating the freezing point depression of the undiluted fresh tissue.

Methods

Pieces of liver and kidney[1] were removed without hemostasis from nembutalized dogs. Two separate procedures, designated A and B, were used.

A. Five to ten bits of tissue, weighing 100 to 200 mg. each, were transferred quickly one by one into tared beakers (15 ml. capacity) filled with boiling water. The beakers with boiling water and tissue bits were kept in a surrounding boiling water bath for 30 to 60 minutes during which time the temperature in the beakers averaged 95–98°C. Care was taken to keep the tissue completely immersed in the water. After boiling, the beakers with tissue and water were cooled, weighed, and the freezing point depression determined on the supernatant. Then the aliquot of supernatant was returned quantitatively to the original beaker containing the tissue bits and placed in a drying oven at 105° for 24 hours. After the heat drying procedure, the beakers with dried tissue were weighed, thereby providing a measure of dry tissue in the original boiled sample. Whole tissue weight and tissue water were calculated from four to six determinations of dry weight on separate specimens of the same tissue removed from the same dog at the beginning of the experiment.

B. In later experiments, the small pieces of tissue were transferred quickly into tared dry beakers and weighed accurately on a Sartorius balance, a procedure taking less than a minute. Then boiling water was poured on the tissue, the beaker and its contents were transferred to the boiling water bath, and heated for 30 to 60 minutes. No significant difference was observed in data obtained with the two procedures, suggesting that no breakdown of organic material occurred in the intact tissue in the first 1 to 2 minutes after removal from the animal.

Knowing the weight of the whole tissue and the weight of the added water, as well as the dry weight of the tissue (determined from separate aliquots of the same tissue of the same dog), we were able to determine the dilution factor, from which the osmotic activity of the undiluted tissue could be estimated. The furmula employed was:

$$O_{\text{tissue}} = \left[\frac{T_{H_2O} + D_{H_2O}}{T_{H_2O}}\right][O_{\text{obs.}}], \tag{1}$$

in which O_{tissue} is the calculated tissue osmotic activity, $O_{\text{obs.}}$, the observed osmotic activity of tissue plus added water, D_{H_2O}, the weight of the added water, and T_{H_2O}, the weight of the tissue water determined by dry weight measurement. Obviously, this equation ignores the changes of activity coefficient with dilution. This factor will be considered in detail subsequently.

Each observation was the result of six replicate determinations made on a given tissue. Serum osmotic activity was determined on fresh serum of the same dogs by direct measurement of the freezing point depression. Values for freezing point depression were expressed in milliosmols per liter with the assumption that a solution of 1 osM/liter has a freezing point depression of −1.86°C. The terms, freezing point depression, osmotic activity, or osmolarity, will be used interchangeably throughout this report.

[1] The *renal cortex* only and not the medulla was the source of *all* kidney tissue of the present report.

Analytical techniques used included determinations of freezing point depression of the tissue, serum and saline standards in the Fiske osmometer using a thermistor as the temperature-sensing element; weights of tissue, containers, and diluents on a Sartorius balance; CO_2 content of serum and of the supernatant fluid of boiled tissue by the method of Van Slyke and Neill (30); CO_2 content of tissue and whole blood by the method of Danielson and Hastings (31). In a few instances, barium-soluble CO_2 content of whole blood was determined by a modification of the method of Ferguson and Roughton (32).

RESULTS

Tissues investigated were blood, liver, and kidney. Data to be reported herein will indicate that the calculated osmotic activity of the boiled liver and kidney was isotonic or even slightly hypotonic to that of the concurrent

TABLE I

Values for Calculated Osmotic Activity of Tissues and Blood, CO_2 Content of the Tissues and Blood before and after Boiling with pH after Boiling

Numbers in parentheses designate the number of determinations on each tissue.

Tissue	Calculated osmotic activity of tissue		Osmotic activity of serum	CO_2 content before boiling	Calculated CO_2 content after boiling	Loss of CO_2 with boiling	pH after boiling
	Average	Range					
Liver	290 (36)	273–306	293 (6)	17.6	10.5	7.1	7.900
Kidney cortex	289 (18)	285–294	303 (3)	18.9	4.7	14.2	7.700
Whole blood	268 (54)	246–285	296 (9)	18.1	9.9	8.2	8.100

serum, and that boiled whole blood was hypotonic to serum. This iso- or hypotonicity of boiled *intact* tissues contrasted sharply with the previously reported data (1, 33), which had shown a consistent hypertonic value for the osmotic activity of frozen *non-intact* tissue homogenates.

The observed hypotonicity could have been due to a change in the CO_2 content of the tissue during boiling, in which case osmotically active particles (CO_2, HCO_3^-, $CO_3^=$) could have disappeared.

Level of Freezing Point Depression of Liver, Kidney, Whole Blood, and Plasma

Table I shows average values for calculated osmotic activity of the tissue indicated, osmotic activity of serum, change in CO_2 content with boiling, and pH of the diluted tissue after boiling. In six dogs, the average value for calculated osmotic activity of six livers was 290 m.osM/kg. of tissue water, or but 3 m.osM/kg. less than that of the directly determined osmotic activity of six samples of serum from the same dogs. In three other dogs, the average value of calculated osmotic activity of three kidneys was 289 m.osM/kg., or 14 m.osM/kg. less than that of the corresponding samples of serum.

The table shows that an average of 7.1 mM/kg. of CO_2 was lost by boiling the six livers, while 14.2 mM/kg. were lost by boiling three kidneys. This means that the observed differences between calculated osmotic activity of liver or kidney *versus* that of serum (3 and 14 m.osM respectively) could be explained adequately by the observed amount of CO_2 content lost from the tissues during boiling. The average loss of CO_2 content of whole blood was 8.2 mM/kg., a value too small to account for the observation that osmotic activity of boiled blood was 28 mM/kg. less than that of serum. To explain the discrepancy in osmotic activity of boiled *versus* unboiled blood, one may invoke the following possible factors: (1) ion-binding with the hemoglobin or precipitation of phosphates, associated with the high level of tissue pH found after boiling; (2) the presence in boiled blood of osmotically inactive carbamino compounds, suggesting that the loss of osmotically active CO_2 was greater than that observed. Preliminary experiments have been performed to test the possibility of the presence of carbamino- or barium-soluble CO_2 in blood. However, results of such determinations in blood have been erratic, and therefore, we have not been able to draw any conclusions with respect to the precise chemical form of CO_2 remaining in the boiled extracts.

The boiling of the tissues and the dilution with distilled water created special conditions and problems which had to be tested. Two questions raised were: (1) whether or not a diffusion equilibrium between the tissue and the supernatant was reached within the time allotted for boiling; and (2) whether or not activity coefficients of the tissue electrolytes were changed significantly at various levels of dilution required for the boiling process.

Diffusion Equilibrium between Tissue and Diluent

Small pieces of liver were quickly transferred from the dog to tared beakers, weighed, covered with boiling water, and heated in a boiling water bath. Beakers were allowed to remain in the 100° bath for periods varying from 0.5 to 60 minutes. After removal of each beaker from the hot bath at a specified time, it was cooled quickly in an ice bath, and determination of the freezing point depression made on the supernatant fluid. Calculations of the apparent osmotic activity of the undiluted tissues were made in the usual manner described herein. As diffusion progresses, one would expect an increase in the observed freezing point depression of the supernatant. When the diffusion process approaches equilibrium, the fluid of the supernatant would have a freezing point depression approaching that of the fluid within the bits of tissue. Such a condition corresponds to the assumptions (see Equation 1) inherent in the calculations of osmotic activity of undiluted tissues. Obviously, this technique cannot exclude the presence of osmotically active particles "sealed" in unknown regions of the intact tissue; *i.e.*, within impermeable barriers created in some unspecified manner by the boiling process.

Fig. 1 shows results of a typical experiment, wherein the apparent osmotic activity calculated from the freezing point depression of the supernatant fluid was plotted against duration of time of boiling. Two other experiments, not shown, yielded almost identical patterns of osmotic activity *versus* time.

The calculated osmotic activity of tissue asymptotically approached the serum value. At 20 minutes, calculated osmolarity was 94 per cent of the empirically assumed equilibrium value (serum) and at 30 minutes, calculated osmolarity was 95 per cent of equilibrium value. Since most of the tissues of the present report had been subjected to 30 minutes of boiling, they could

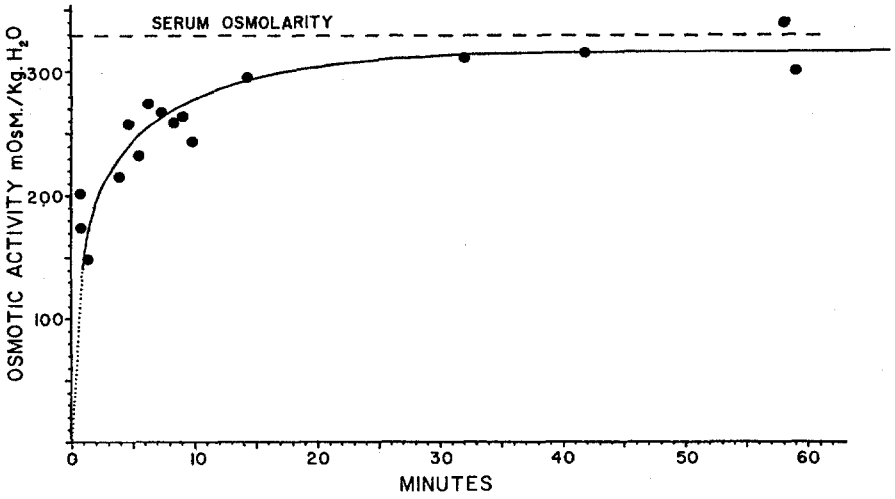

FIG. 1. Osmotic activity of supernatant diluent *versus* time of boiling. Extrapolation of osmotic activity of supernatant to zero time shown by dotted line. It is assumed that the osmotic activity of the supernatant at zero time is that of distilled water.

have equilibrated with the ambient fluid to the extent of 95 per cent. If so, an additional correction of 5 per cent of the total osmolarity should be applied to the values of calculated osmotic activity of boiled diluted tissues.

Stability of Heat-Treated Tissues

The asymptotic nature of the curve of Fig. 1 suggests that the boiled extracts were stable with time for periods of ½ to 1 hour. Other observations, not presented herein, have shown that the osmotic activity of such boiled extracts remained constant for at least 2 hours. In experiments reported elsewhere (34), frozen, crushed homogenates, dried in a 105°C. oven for 24 hours, were reconstituted to their original volume with distilled water. Such reconstituted homogenates were hypertonic to plasma, but were stable for 24 hours with respect to osmotic activity. Similar experiments were performed on

boiled diluted intact bits of tissue (treated in the manner described under Methods). The heat-dried bits of tissue, reconstituted with distilled water, were isotonic with serum and stable for 24 hours with respect to osmotic activity.

Change of Activity Coefficient of Tissue Electrolytes with Dilution

The next question to be tested was whether or not the activity coefficient of the tissue electrolytes could change by virtue of the dilution required by the present experimental technique. For this purpose, pieces of liver were boiled with distilled water for 30 minutes and the supernatant filtered. The filtrates so obtained were concentrated by evaporation so that the level of their freezing point depression reached a value corresponding to *ca.* 300 m.osM/liter. Serial dilutions were made of the concentrated filtrate. Deter-

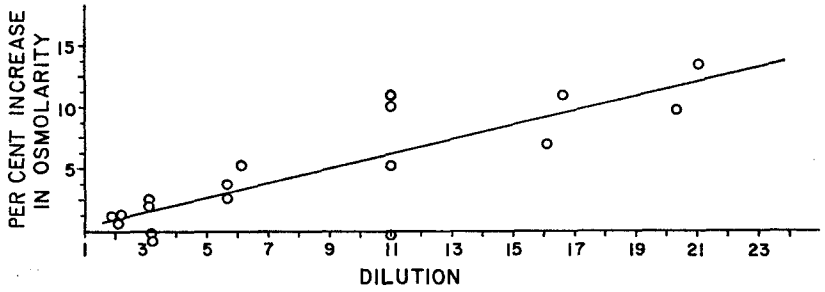

FIG. 2. Influence of dilution on the activity coefficient of tissue electrolytes. Ordinate, per cent increase in calculated osmolarity over the original osmolarity of tissue fluid. Abscissa, degree of dilution.

minations of the freezing point depression were performed on the filtrates at several levels of dilution.

The change of activity coefficient of the tissue electrolytes determined empirically, was expressed as the percentage increase in osmotic activity of the original undiluted filtrate. In mathematical terms,

$$\text{Per cent } \Delta\text{osm.} = \frac{(O_{\text{dil.}})\,(f.) - (O_{\text{conc.}})}{(O_{\text{conc.}})} \times 100 \qquad (2)$$

in which per cent Δ osm. is the per cent increase of osmotic activity of filtrate caused by dilution; $O_{\text{dil.}}$ is the observed osmotic activity of diluted filtrate; $f.$ is the dilution factor; and $O_{\text{conc.}}$ is the observed osmotic activity of the original concentrated filtrate. This expression (per cent Δ osm.) is an empiric definition of the change in activity coefficient of tissue solutes over a wide range of concentrations. If there were no change of activity coefficient with dilution, then $(O_{\text{dil.}})\,(f.) = O_{\text{conc.}}$, whence per cent Δ osm. $= O$.

Fig. 2 shows the percentage increase of osmotic activity (or of activity co-

efficient) *versus* degree of dilution over a one- to twentyfold range of dilution. It can be seen that empirically determined activity coefficient of the tissue electrolytes increased by 3 per cent at the fivefold level of dilution and by 11 per cent at the twentyfold level of dilution. Identical experiments with a NaCl solution at 300 m.osM/liter were performed. The observed change of osmotic activity of the NaCl solution *versus* dilution yielded results practically identical with those obtained from the concentrated tissue filtrate. The per cent increase of activity coefficient of our standard saline solutions agreed with that reported in the International Critical Tables (35). Such agreement was within 1 per cent up to a tenfold dilution; and to within 5 per cent at a twentyfold dilution. In most of the experiments in the present report, the increase of calculated osmotic activity coefficient could not have exceeded 3 per cent, since the usual level of dilution for the boiled tissues was between four- and sixfold. Since the error of determination of freezing point depression could have been as much as 2 m.osM/liter, a sixfold dilution could magnify this error to 12 m.osM/liter, or to 4 per cent of the total osmolarity—a value of the same order of magnitude as the observed change in activity coefficient of tissue electrolytes. At first, calculated values of tissue osmotic activity did not include a correction factor for the change in activity coefficient. Subsequently, certain findings to be presented herein, led to the application of such corrections.

Effects of Boiling on Non-Intact Tissue Homogenates of Known Osmolarity

Experiments were designed to test the possibility that boiling, in some unspecified manner, reduced the osmotic activity of the tissue. This would imply that tissues are really hypertonic to serum, but that the heat of boiling either binds osmotically active particles or produces impermeable regions or barriers within the tissue. The following procedure was an attempt to determine the effect of boiling on the osmotic activity of a tissue preparation on which the osmotic activity had been determined before boiling. This was accomplished by measuring the freezing point depression of a tissue homogenate both before and after immersing it in boiling water at specified times.

Tissues were frozen in liquid N_2 and homogenized by crushing in a Carver press. The non-intact tissue homogenates so prepared, were kept at room temperature. Keeping homogenates at such a temperature favors a high rate of increase of their osmotic activities (1, 4). At specified times, direct measurements of the freezing point depression were made on aliquots of the homogenates. At the same time, corresponding weighed aliquots of the same homogenate were diluted with boiling water, immersed in the boiling water bath, and carried through the procedure of the present report. The freezing point depression of the boiled homogenates was obtained in the manner described under Methods. In other words, both types of aliquots were identical with

respect to having been frozen, crushed, and allowed to remain at room temperature for specified time intervals, and differed only with respect to boiling and dilution prior to the determination of the freezing point depression.

Fig. 3, a plot of the values of osmotic activity *versus* time, shows the results of such experiments on non-intact homogenates of liver. The osmotic activity of the homogenate, determined directly, increased from a value of 355 m.osM/K after standing at room temperature for 6 minutes, to one of 470 m.osM/K after 30 minutes. The osmotic activity of the same homogenate,

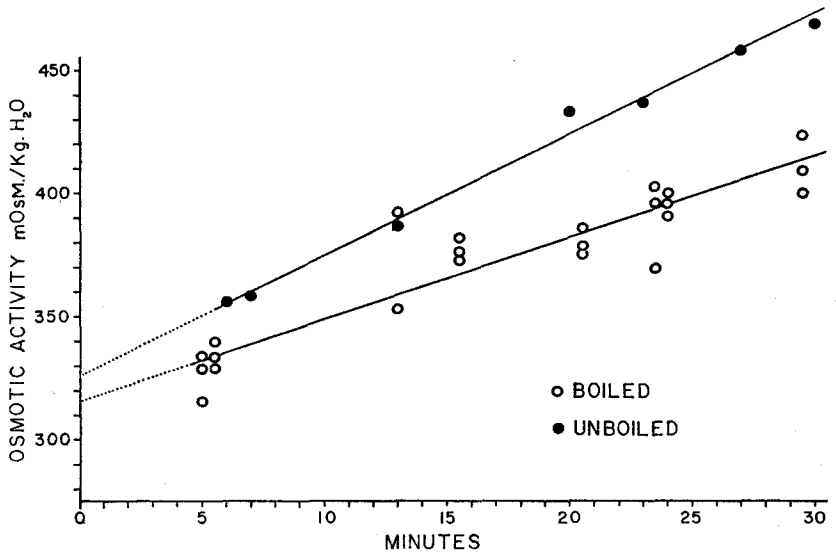

FIG. 3. Effect of boiling on the osmotic activity (ordinate) of liver homogenate kept at room temperature for varying periods of time (abscissa). Extrapolation to zero time shown by dotted lines. The homogenate had been prepared by crushing frozen tissue in the Carver press. Zero time was defined as that of liquefaction of solid tissue in the press.

left standing at room temperature for identical times, and determined after dilution and boiling, also increased with time, but reached lower levels than those of the unboiled homogenate. For example, after boiling, values of osmotic activity were: 330 m.osM/kg when boiling was performed after 5 minutes; and 415 m.osM/kg when boiling was performed after 30 minutes. Straight lines were fitted to each group of points by the method of least squares. Extrapolation of both lines to zero time showed a difference between the osmotic activities of 10 m.osM/liter, while at 10 and 20 minutes the difference was respectively 26 and 43 m.osM/liter. Two explanations for this difference and its increase in time can be proposed: (1) The diffusion equilibrium after 30

minutes of boiling was 95 per cent complete, whence the observed value of osmolarity could be corrected upward by 5 per cent over the entire time range. (2) The CO_2 loss induced in tissues by boiling (8 mM/liter for liver) indicated a loss of osmotically active particles, whence the observed value of osmolarity could be corrected upward by 3 per cent during the first minute of standing.

CO_2 determinations on fresh tissue were performed by quickly immersing the freshly excised bits into the Danielson-Hastings' solution (31) prior to the analysis. CO_2 determinations on boiled tissue were performed by direct analysis (30) of the supernatant fluid. Such tissues had been immersed in boiling water 0.5 to 1.0 minute after excision. No determinations on CO_2 content were made on homogenates allowed to stand at room temperature. Hence, the correction for loss of CO_2 could be applied only to freshly excised bits of tissue, and to tissues subjected to boiling after being at room temperature for less than 1 minute.

The application of the two corrections (8 per cent) shows that there had been no unexplained reduction of osmotic activity after boiling homogenates which had been exposed to room temperature for as long as 15 minutes. However, such a correction would be slightly excessive owing to the change of activity coefficient, 3 per cent occasioned by the four- to sixfold dilution (see Fig. 2). The application of all three corrections (*i.e.*, 95 per cent of diffusion equilibrium, loss of CO_2 content of tissue during boiling, and change of activity coefficient of tissue electrolytes produced by dilution) shows that boiling did not produce unpredictable changes of osmotic activity of hypertonic homogenates which had been allowed to stand for 10 minutes.

Osmotic Activity of Boiled Diluted Tissue in Light of Data on Diffusion Equilibrium, Loss of CO_2 Content, and Change of Activity Coefficient

Table II presents data on predicted *versus* actually determined osmotic activity of tissues subjected to boiling and dilution. Predicted values were obtained as follows: (1) Osmotic activity of the fresh tissue was assumed equal to that of plasma. (2) All corrections were applied to the actually determined value of osmotic activity of plasma. (3) Corrections used were based on actual data concerning degree of diffusion equilibrium attained, loss of CO_2 content, and change of activity coefficient.

It can be seen that predicted values of osmotic activity of boiled liver and kidney were 97 to 98 per cent of the actually determined values. The close approximation between predicted and actual values supports the validity of the one assumption used; *i.e.*, that tissue fluid is isotonic with plasma.

The predicted value of osmotic activity of boiled blood was 105 per cent of the determined value. Although this is a fair approximation, certain problems with respect to osmotic activity of boiled blood remain to be solved. These problems have been mentioned under the first subsection of Results.

Influence of Freezing, Crushing, and Homogenizing on the Osmotic Activity of Boiled Diluted Tissues

It appeared that boiling of a non-intact tissue homogenate of known osmotic activity did not decrease its osmotic activity significantly. It remained to be seen, why non-intact tissue homogenates were consistently hypertonic to plasma, while boiled intact tissues were isotonic.

Assuming that tissue is hypertonic, one can explain the hypertonicity of a tissue homogenate. Boiling the intact tissue could produce diffusion barriers, which rendered the boiled tissue apparently isotonic. This is because such impermeable barriers might enclose regions containing osmotically active solutes. On the other hand, tissue homogenates, consisting of destroyed tissues with no histologic barriers, do not present conditions favorable for the production of barriers by boiling. Hence, boiling would have no influence on the osmotic activity of tissue homogenates.

TABLE II

Values for Plasma Osmolarity, Predicted Tissue Osmolarity, and Determined Tissue Osmolarity

The predicted tissue osmolarity is calculated from the plasma osmolarity with the assumption that tissue is isotonic to plasma.

Tissue	Plasma osmolarity	Predicted osmolarity	Determined osmolarity
Liver	293	280	290
Kidney cortex	303	282	289
Whole blood	296	282	268

These considerations led to certain predictions: (*a*) If boiling creates a diffusion barrier in intact tissues, and if one can destroy such barriers with freezing, crushing, or glass-homogenizing, one might increase the osmotic activity of the boiled tissues by freeing "trapped" solutes; (*b*) on the other hand, if tissues are really isotonic, and no barriers are created by boiling, then freezing, crushing, or glass-homogenizing of the boiled tissues would not increase the osmotic activity of boiled tissues.

The following experiments were designed: Small pieces of fresh dog liver were heated in a beaker of distilled water immersed in a 100°C. water bath for 30 minutes and the osmotic activity of the supernatant fluid was measured. The mixture was then: (1) frozen in liquid nitrogen, crushed in a Carver press, and the osmotic activity redetermined on the resulting homogenate; or (2) the mixture was homogenized in a glass homogenizer, and the osmotic activity redetermined on the resulting homogenate.

Microscopy in both cases showed complete tissue and cell destruction after crushing or homogenizing the boiled tissue. Figure 4 *a* shows that the histologic structure of the liver remained intact after boiling for 30 minutes in distilled water. Figure 4 *b* shows histologic evidence of the architectural de-

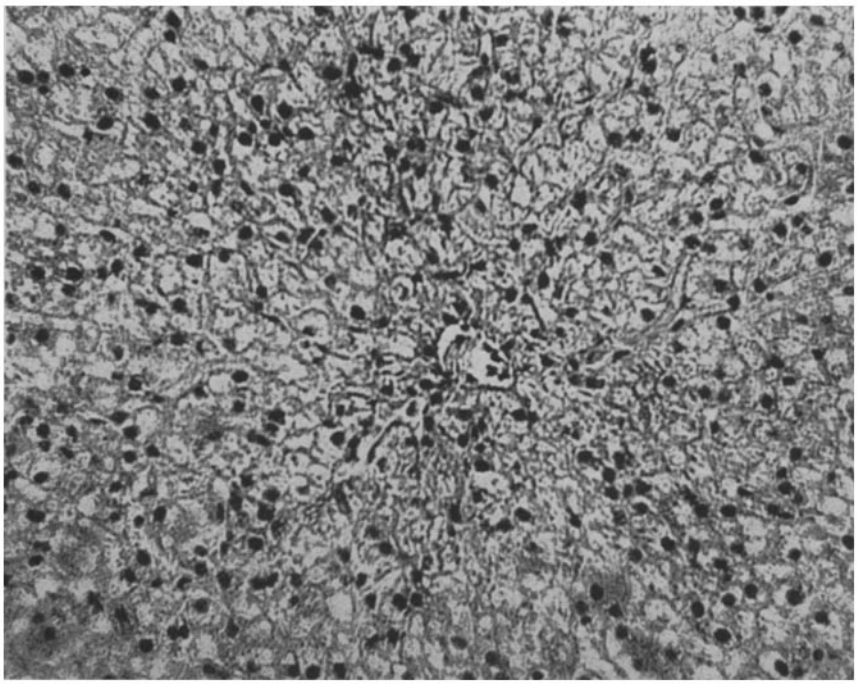

FIG. 4 *a*. Hematoxylin and eosin stain of boiled intact liver tissue.

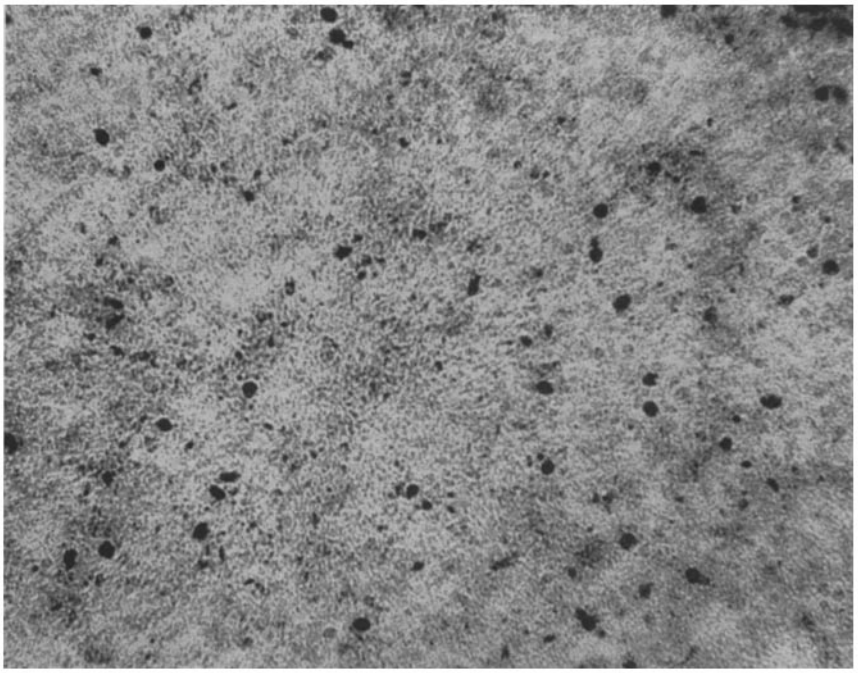

FIG. 4 *b*. Hematoxylin and eosin stain of liver tissue after boiling and crushing in a Carver press.

struction produced in the same batch of tissue after crushing and homogenizing.

Neither of the two procedures resulted in an increase in the osmotic activity of boiled liver tissue, suggesting that no isolated impermeable regions are present in such boiled tissues. Tables III and IV present raw data on measurements of osmotic activity of boiled liver before and after freezing-crushing or after glass-homogenizing. The change in osmotic activity induced by freezing-crushing or by glass-homogenizing was usually 0 to 2 m.osM/liter in both directions—a change within the limits of error of the analytical technique.

TABLE III

Raw Data Showing No Effect of Freezing and Pressing on the Freezing Point Depression of Diluted Boiled Liver

Experiment	Osmotic activity of boiled mixture	Osmotic activity after freezing and pressing
Liver 1	189	190
Liver 2	157	159
Liver 3	174	172
Liver 4	187	187

TABLE IV

Raw Data Showing No Effect of Glass-Homogenizing on the Freezing Point Depression of Diluted Boiled Liver

Experiment	Osmotic activity of boiled mixture	Osmotic activity after homogenization
Liver 1	206	206
Liver 2	197	193
Liver 3	176	177
Liver 4	190	190
Liver 5	168	174
Liver 6	167	170

Moreover, the average change induced by mechanical destruction of boiled tissue in ten sets of aliquots (forty determinations) was zero.

It appeared that mechanical destruction of an intact but dead (boiled) tissue failed to produce a change in its osmotic activity. However, previous data from this laboratory (1) and from others (2, 4) indicated that mechanical destruction of an intact living (frozen) tissue produced an hypertonic, metabolizing homogenate. Such data do not appear to be compatible with an hypothesis requiring that boiling of tissues creates isolated impermeable regions containing osmotically active solutes.

Although the freezing-crushing process applied to boiled tissue may induce changes in its proteins, such changes do not affect its osmotic activity (Table III). On the other hand, the freezing-crushing process applied to freshly ex-

cised (living) tissue may change its proteins in such a way that the resulting homogenate becomes hypertonic. However, the data in this report are not sufficient to detect physicochemical changes in tissue proteins.

DISCUSSION

Any valid hypothesis concerning the osmotic activity of intracellular fluid must explain the results of the following experiments: (1) Boiling of a fresh tissue yields an extract isotonic with plasma. (2) Destruction of tissue by freezing and crushing yields a homogenate hypertonic to plasma. (3) Boiling of a hypertonic homogenate does not reduce its osmotic activity significantly. (4) Destruction of an isotonic boiled tissue by freezing and crushing, or by glass-homogenizing without freezing does not change its osmotic activity.

TABLE V

Summary of the Experiments, Results, and Assumptions Required to Explain the Data on the Basis of the Two Hypotheses: Tissue Isotonicity versus Tissue Hypertonicity

1	2	3	4
Process	Result	Assumptions	
		Isotonic tissue	Hypertonic tissue
A. Boiling	Isotonicity	None	1. Barriers produced 2. Ion binding
B. Freeze-crush	Hypertonicity	1. Autolysis + metabolism 2. Physicochemical	None
C. Freeze-crush + boiling	Hypertonicity (no change)	None	1. No barriers produced 2. No ion binding
D. Boiling + freeze-crush	Isotonicity (no change)	None	1. No barriers destroyed 2. Ion binding irreversible

Table V presents a summary of results of the experiments, as well as of assumptions required to explain the data on the basis of two opposing hypotheses—isotonic cell fluid *versus* hypertonic cell fluid. The implications of the two hypotheses, indicated in columns 3 and 4 of the table, will be spelled out in the following discussion.

Isotonic Hypothesis.—If we assume that intracellular fluid is isotonic to plasma, we should expect the extracts of boiled tissue to be isotonic, whether or not they are subjected to destructive measures such as freezing, crushing, or homogenizing. This implies that boiling would have no influence on tissue osmolarity, an implication verified experimentally since boiling did not produce unpredictable reductions in the freezing point depression of hypertonic, non-intact homogenates (Fig. 3). To explain the hypertonicity of fresh tissue homogenates, we need either of two assumptions: (1) metabolic and/or autolytic activity in the tissue homogenates; or (2) changes induced in the proteins

of fresh tissues by freezing and pressing, with a resulting increase in the number of osmotically active particles. Assumption 1 has experimental foundation, since it is well known that homogenates metabolize and autolyze even at 0°C. (36, 37). If such autolysis were the origin of increased osmotic activity in homogenates derived from isotonic tissues, one could account for all the data on freezing point depression without any *ad hoc* assumptions.

Hypertonic Hypothesis.—On the other hand, if we assume that intracellular fluid is hypertonic to plasma, we should be forced to introduce several assumptions, not experimentally supported and of a complicated nature. The hypertonic homogenate produced by freezing and crushing of an hypertonic tissue would not need further explanation. However, to explain the observed isotonicity of boiled intact tissue, one must assume that boiling induces, in some way, disappearance of osmotically active particles[2]—either by the formation of impermeable barriers or by the binding of ions to tissue proteins.[3] Use of these arbitrary assumptions leads to an apparent discrepancy in the behavior of geometrically intact tissue *versus* that of non-intact tissue homogenates. Whereas boiling of an intact and allegedly hypertonic tissue reduces its osmolarity, boiling of a non-intact tissue of known hypertonicity does not reduce its osmolarity. This requires the *ad hoc* postulates that barriers or ion binding occur only in geometrically intact tissues, and not in destroyed tissue homogenates. Even if boiling does influence the osmotic activity of an hypertonic intact tissue, it is difficult to understand why the osmotic activity would fall consistently to isotonic or to nearly isotonic levels.

Once boiling has decreased the osmotic activity of an allegedly hypertonic tissue to an isotonic level (see Table V, process A-4) by the formation of barriers or by ion-binding, it is not possible to reverse the process and to produce an hypertonic osmotic activity by mechanical destruction of the boiled tissue; *i.e.*, by freezing, crushing, or homogenizing and freeing trapped solutes. The explanation of such apparent irreversibility would require the *ad hoc* postulates, that barriers surrounding osmotically active solute, once formed, cannot be destroyed, or that ion-binding, when and if it occurs, is irreversible after boiling.

[2] Ferry *et al.* (38) have cited data showing that heat treatment of solid proteins may produce changes such as breaking of bonds (hydrolysis) as well as formation of new cross-links in the protein molecule, depending on the degree of dilution of the system. In fibrin, dilution plus steam treatment favored predominance of hydrolytic reactions, whence an increase of osmotically active particles would result.

[3] Postulates on the binding of ions in living cells have been proposed to explain potassium accumulation in cells (Ling (39)), to account for the disappearance of active base in the body (Elkington *et al.* (40)), and to explain ion and water transport (Goldacre (41), Ling (39)). The schemes of Goldacre and of Ling, involving the folding and unfolding of protein molecules could be invoked to account for reduction of osmotic activity by boiling.

The formation of barriers to Hb diffusion has been found after steaming of fibrin film—a preparation that had been freely permeable to Hb before such treatment. However, the permeability of this film for small molecules (glucose, NaCl) was not affected by the steam heating (Ferry *et al.* (38)). If a similar barrier had formed in our boiled tissue, it would not produce significant changes in calculated osmotic activity. To account for production of osmotic gradients across films, a postulated diffusion barrier must block the movement of small solute molecules (ion, glucose) and water.

Current schemes on ion-binding[2] demand a reversible binding process, which does not fit our observations in boiled tissues. We are not aware of experimental or theoretical data on irreversible types of ion binding in tissues.

The possibility of the existence of bound water, reviewed extensively by Gortner (42) and by Blanchard (43) might be reconsidered in order to account for present cryoscopic observations. The application of the concept of bound water to either of the two hypotheses of Table V would require several second and third order assumptions for a valid explanation of the data. For example:

Bound water in cells assumed to be isotonic would demand that the bound water remained bound during boiling, freezing, and crushing, while the hypertonicity of the homogenate after freezing and crushing could be explained either by binding of more water or by metabolism.

Bound water in cells assumed to be hypertonic would explain the isotonicity of cell fluid after boiling only by assuming that the bound water was released and the hypertonic cell fluid diluted to an isotonic level. Freezing and crushing would not influence the bound water of the allegedly hypertonic tissue, since the resulting homogenates were hypertonic. Boiling of the hypertonic homogenate fails to release bound water and to dilute the tissue, as was presumably the case when intact tissue was boiled. It is not clear why the bound water released by boiling would dilute the tissue exactly to the osmolarity level of plasma.

The above considerations do not prove or disprove rigorously either of the concepts; *i.e.*, of isotonic or of hypertonic cell fluid. However, the data do support, with a minimum of *ad hoc* postulates, the validity of the classical concept that intracellular fluid is isoosmotic with the extracellular fluid.

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