Kinetics of Water Loss from Cells at Subzero Temperatures and the Likelihood of Intracellular Freezing

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ABSTRACT The survival of various cells subjected to low temperature exposure is higher when they are cooled slowly. This increase is consistent with the view that slow cooling decreases the probability of intracellular freezing by permitting water to leave the cell rapidly enough to keep the protoplasm at its freezing point. The present study derives a quantitative relation between the amount of water in a cell and temperature. The relation is a differential equation involving cooling rate, surface-volume ratio, membrane permeability to water, and the temperature coefficient of the permeability constant. Numerical solutions to this equation give calculated water contents which permit predictions as to the likelihood of intracellular ice formation. Both the calculated water contents and the predictions on internal freezing are consistent with the experimental observations of several investigators.

INTRODUCTION

One theory of low temperature injury ascribes cell death to the formation of intracellular ice crystals and predicts, therefore, that survival following low temperature exposure will be maximal when the occurrence of intracellular ice is minimal. An attractive feature of this hypothesis is its ability to provide a rational explanation for the fact that the survival of many cells is improved using low cooling velocities of about 1°/min. (Mazur, 1960; Smith, 1961).

The rationale for believing that cooling velocity could affect the probability of intracellular freezing is the following. Consider a hypothetical cell being cooled in an aqueous medium that has just begun to freeze and in which the temperature has dropped below the freezing point of the protoplasm. Assume that the intracellular water has supercooled and as a result has a higher vapor pressure than that in the external medium. If it remains supercooled, and if no water moves out of the cell and no solutes into it, the ratio of the internal to external vapor pressure would increase progressively with decreasing temperature. But since cell membranes are, in fact, permeable to water (and we

shall assume them to be impermeable to the solutes in the system), the vapor pressure differential causes water to leave the cell and so concentrate its contents. Qualitatively, it is clear that with sufficiently slow cooling, enough water would leave the cell to eliminate the vapor pressure differential and keep the protoplasm at its freezing point. Under these conditions, it would not freeze. The question is, how slow is "sufficiently slow"? Or to put it differently, are the cooling velocities that most affect survival (usually 1 to 1000°C/min.) the same as those determining the amount of water in a cell, which, in turn, affects the degree to which the cell water is supercooled and the likelihood of its freezing?

The amount of water in a cell and the degree of supercooling will also be influenced by the rapidity with which water can leave under a *given* vapor pressure differential. Two factors affecting this rate are the inherent permeability of the cell to water and its surface area–volume ratio.

This picture is supported qualitatively by observations that a number of cells become shrunken or plasmolyzed when cooled at 1°C/min., but remain more normal in appearance at cooling velocities of several hundred degrees/min. or higher. These cells include yeast (Nei, 1960; Mazur, 1961 *a*), liver (Meryman and Platt, 1955), and higher plant cells (Asahina, 1956). In yeast, shrinkage is due to the loss of water and not solutes (Mazur, 1963), so that with these cells at least, cell volume is a measure of water content. The volume of rapidly cooled cells is sufficiently large to indicate that they contain ice; the smaller volume of cells cooled slowly at 1°C/min. suggests that they contain no ice.

Such experimental data are available for only a few cells, and it was the purpose of the present study to derive a quantitative expression for water loss that hopefully would apply to cells in general. The derivation will be considered first. Then the equation will be solved for specific values of the various parameters that seem most applicable to several types of cells. Finally, the calculated behavior of some cells will be compared with their actual behavior. The calculated and observed behaviors agree sufficiently to indicate that the qualitative picture just outlined has quantitative physicochemical support.

THEORY

Intracellular and Extracellular Vapor Pressure as a Function of Temperature

VAPOR PRESSURE OF SUPERCOOLED PROTOPLASM   If protoplasm is assumed to be an ideal dilute solution, Raoult's law will apply:

$$p_i = p^o x_i \tag{1}$$

where p^o, p_i, and x_i are the vapor pressure of pure water, of water in the protoplasm, and the mole fraction of intracellular water, respectively. Taking

logarithms of both sides and differentiating with respect to temperature, we obtain

$$\frac{d \ln p_i}{dT} = \frac{d \ln p^o}{dT} + \frac{d \ln x_i}{dT} \tag{2}$$

but, according to the Clausius-Clapeyron equation,

$$\frac{d \ln p^o}{dT} = \frac{L_v}{RT^2} \tag{3}$$

where L_v is the molar heat of vaporization. Therefore,

$$\frac{d \ln p_i}{dT} = \frac{L_v}{RT^2} + \frac{d \ln x_i}{dT}. \tag{4}$$

CHANGE IN VAPOR PRESSURE OF THE EXTERNAL MEDIUM WITH TEMPERATURE   The change in vapor pressure of ice in the external medium (p_e) will be

$$\frac{d \ln p_e}{dT} = \frac{L_s}{RT^2} \tag{5}$$

where L_s is the molar heat of sublimation. If the external medium contains solutes, as would be true with even deionized water, some liquid solution will be present at temperatures above the eutectic point. We shall assume that any such solution remains in equilibrium with ice so that its vapor pressure will also be p_e and will obey Equation 5.

DIFFERENCE BETWEEN INTERNAL AND EXTERNAL VAPOR PRESSURES   By subtracting Equation 4 from 5 and remembering that $L_s - L_v = L_f$, the molar heat of fusion, one obtains

$$\frac{d \ln p_e/p_i}{dT} = \frac{L_f}{RT^2} - \frac{d \ln x_i}{dT} \tag{6}$$

but

$$x_i = \frac{n_1}{n_1 + n_2} = \frac{n_1 \bar{v}_1}{n_1 \bar{v}_1 + n_2 \bar{v}_1} = \frac{V}{V + n_2 \bar{v}_1} \tag{7}$$

where n_1 and n_2 are moles of water and solutes, $\bar{v}_1$ is the partial·molar volume of water, and V the volume of water in the cell. Substituting Equation 7 in 6, substituting v_1^0, the molar volume of pure water, for $\bar{v}_1$, and carrying out the indicated differentiation assuming v_1^0 and n_2 to be constant (see Appendix), yields

$$\frac{d \ln p_e/p_i}{dT} = \frac{L_f}{RT^2} - \frac{n_2 v_1^0}{(V + n_2 v_1^0)V} \frac{dV}{dT} \tag{8}$$

which gives the ratio of the vapor pressure of supercooled water inside the cell to that of water at thermodynamic equilibrium outside the cell, as a function of the volume of intracellular water and temperature.

THE RATE OF WATER LOSS   The rate at which the volume of intracellular water changes is

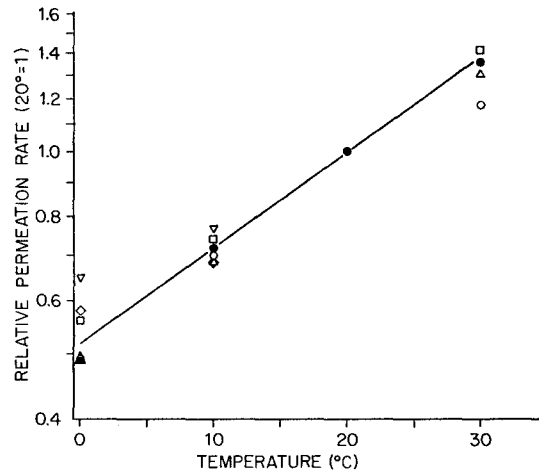$$\frac{dV}{dt} = kA(\Pi_i - \Pi_e) \tag{9}$$

FIGURE 1. Effect of temperature on the relative permeability of various mammalian red blood cells to water. The relative permeation rate was calculated from data of Jacobs *et al.* (1935) as 1/Time for hemolysis taking the time at 20°C as unity. The curve was fitted by eye. ●, rat; ○, man; ◇, rabbit; □, guinea pig; △, dog; ▽, ox.

where t is time (min.), k the permeability constant (μ^3 of water per μ^2 of cell membrane surface per min. per atmosphere difference in osmotic pressure between inside and outside the cell), A the cell membrane area, and Π_i and Π_e the internal and external osmotic pressures (Davson and Danielli, 1952, p. 41).

But Π is a function of vapor pressure:

$$\Pi \bar{v}_1 = RT \ln p^o/p \tag{10}$$

where p^o and p are the vapor pressures of pure water and water in a solution, respectively. Substituting this vapor pressure equivalent for Π_i and Π_e in Equation 9 and substituting the molar volume of water (v_1^o) for its partial molar volume, yields

$$\frac{dV}{dt} = \frac{kART}{v_1^0} \ln p_e/p_i \tag{11}$$

Change in Permeability Constant (k) with Temperature

Jacobs, Glassman, and Parpart (1935) have measured the effect of tempera-
ture on the permeability constant of water in mammalian erythrocytes, and
calculations from their data are expressed in Fig. 1 as the logarithm of the
relative rate against temperature. The relation is reasonably linear up to
30°C, and the data are adequately fitted by the equation

$$\ln k = a + bT \tag{12}$$

where k is the permability constant at temperature T and b the slope. If k_g is
the known permeability constant at a known temperature T_g, then

$$\ln k_g = a + bT_g \tag{13}$$

and

$$\ln \frac{k}{k_g} = b(T - T_g) \tag{14}$$

Expressed exponentially, we have

$$k = k_g e^{b(T-T_g)} \tag{15}$$

Rate of Cooling

The simplest assumption is that the cooling rate is constant or

$$\frac{dT}{dt} = B \tag{16}$$

Change in the Volume of Intracellular Water with Temperature

Equations 11 and 16 can be combined to eliminate t, in which case

$$\frac{dV}{dT} = \frac{kART}{Bv_1^0} \ln p_e/p_i . \tag{17}$$

If k is replaced by its equivalent given by Equation 15, and Equation 17
then solved for $\ln p_e/p_i$ and substituted for that quantity in Equation 8, the
result is

$$\frac{d}{dT} \left(\frac{v_1^0 B}{ARk_g} \cdot \frac{1}{T} \cdot e^{-b(T-T_g)} \frac{dV}{dT} \right) = \frac{L_f}{RT^2} - \frac{n_2 v_1^0}{V + n_2 v_1^0} \cdot \frac{1}{V} \frac{dV}{dT} \tag{18}$$

If one assumes that V and T are the only variables, the differentiation

yields

$$Te^{b(T_g-T)} \frac{d^2V}{dT^2} - \left[(bT + 1)e^{b(T_g-T)} - \frac{ARk_g n_2}{B(V + n_2 v_1^0)} \cdot \frac{T^2}{V} \right] \frac{dV}{dT} = \frac{L_f Ak_g}{Bv_1^0} \quad (19)$$

This, then, is the desired equation relating the volume of intracellular water to temperature, to cooling rate, and to four parameters characteristic of the cell; namely, A, k_g, b, and n_2.

RESULTS

Solution of Equation 19, Parameters and Units

Equation 19 is not solvable analytically. Its numerical solution requires stating intial values, which are (a) $dV/dT = 0$ when $T \geq T_f$, the freezing point of protoplasm; and (b) at temperatures $T \geq T_f$, $V = V_i$, a constant representing the initial volume of intracellular water. The equation was solved on an IBM 7090 digital computer by M. T. Harkrider of the Mathematics Division, Oak Ridge National Laboratory, using the Runge-Kutta method for second-order differential equations (Korn and Korn, 1961). I am most indebted to him for this essential part of the study. Because of the broad range of the parameters, the solutions required iteration in steps of 10^{-3} degrees K. Typical values for the various parameters, the values for the constants, and the units are summarized in Table I.

The numerical solutions are presented graphically as the percentage of the initial intracellular water remaining in the cell at temperatures from $-1°$ to -28 or $-30°C$. Most graphs also include a curve labeled "equilibrium." This represents the fraction of water that would remain in a cell at any given temperature if cooling were infinitesimally slow; *i.e.*, it represents the fraction of water remaining in cells that have equilibrated with the surrounding ice at a constant temperature by losing sufficient water to bring the protoplasm to its thermodynamic freezing point. The equation describing this equilibrium water content could be derived from Equation 18 by setting $B = 0$, but it is more direct to derive it from Equation 6: Since equilibrium means that the internal and external vapor pressures are equal ($p_i = p_e$), Equation 6 becomes

$$\frac{d \ln x_i}{dT} = \frac{L_f}{RT^2} \quad (20)$$

Integrating between limits

$$\int_{x_i}^{1} d \ln x_i = \frac{L_f}{R} \int_{T}^{273} \frac{dT}{T^2} \quad (21)$$

TABLE I

VARIABLES, PARAMETERS, AND CONSTANTS IN EQUATION 19*

Symbol	Meaning	Units	Typical values
V	Volume of water in cell	μ^3	Dependent variable
T	Temperature	Degrees K	272 to 242
m	Molality of protoplasm	Moles/1000 gm H_2O	0.5
T_f	Freezing point of cytoplasm	Degrees K	272
V_i	Initial volume of internal water at $T = T_f$	μ^3	0.42, 88, 6920
n_2	Osmoles of solute in cell	Moles	2.1×10^{-16}, 4.4×10^{-14}, 3.45×10^{-12}
b	Temperature coefficient of permeability constant	Degree^{-1}	0.0325
A	Area of cell protoplast	μ^2	3.04, 107, 1964
k_g	Permeability constant at temperature T_g	$\mu^3/(\mu^2$ min. atm)	0.15, 0.3, 3
T_g	—	Degrees K	293
B	Rate of temperature change	Degrees K/min.	-1, -10, -100, -1000, and $-10,000$
R	Gas constant	μ^3 atm/(mole degree)	82.057×10^{12}
v_1^0	Molar volume of pure water	μ^3/mole	18×10^{12}
L_f	Molar heat of fusion of ice	μ^3 atm/mole	5.95×10^{16}

* Some of the units and values need explanation. (1) The value of 0.5 used for the intra-cellular molality (m) is that of yeast (Conway and Armstrong, 1961; Eddy and Williamson, 1957; Mazur, 1963). The freezing point, T_f, of such a solution is ideally $= -0.93°C \approx 272°K$. (2) The initial volumes, V_i, of 0.42, 88, and 6920 μ^3 are those of the water inside hypothetical cells (or, more properly, protoplasts) 0.98, 5.84, and 25 μ in diameter containing 84.6 per cent (v/v) of water. This value of the water content and the value of 88 μ^3 are from calculations and measurements made previously on yeast cells (Mazur, 1961 a, 1963). (3) the values of n_2 were obtained as mV_i (V_i in liters) assuming the density of water to be one. (4) The value $b = 0.0325$ is that calculated from the data of Jacobs *et al.* (1935) (Fig. 1). (5) The areas, A, are those of spheres of 0.98, 5.84, and 25 μ in diameter. (6) The value of $k_g = 0.3$ was chosen as one typical of the permeability constant for many cells (Davson and Danielli, 1952; Dick, 1959). The value $k_g = 3$ is that given for human erythrocytes by Jacobs (1932). (A more recent estimate by Sidel and Solomon (1957) is $k_g =$ about 5 at 20°C.) The value of $k_g = 0.15$ is approximately that for yeast cells according to my calculations from the osmotic volume measurements by Ørskov (1945).

Other values of the parameters were used in specific cases.

one obtains

$$\ln x_i = \frac{L_f}{R}\left(\frac{1}{273} - \frac{1}{T}\right). \tag{22}$$

And if we substitute the volume equivalent for x_i from Equation 7, and substitute v_1^0 for $\bar{v}_1$, we get (assuming L_f and n_2 to be constant)

$$\ln\left(\frac{V}{V + n_2 v_1^0}\right) = \frac{L_f}{R}\left(\frac{1}{273} - \frac{1}{T}\right) \tag{23}$$

which was used to calculate the equilibrium curve.

Fig. 2 a (1) depicts the behavior of an "average" cell with a diameter of 6 μ and a permeability constant (k_g) of 0.3. We note that at a cooling rate of 1°C/min., the intracellular water content remains close to the equilibrium value, the protoplasm supercooling by at most 0.2° (horizontal distance from
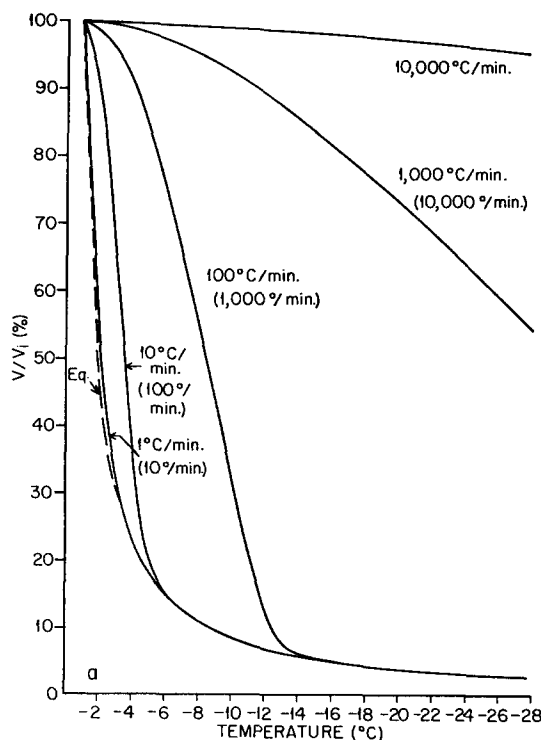

FIGURE 2. Calculated percentages of supercooled intracellular water remaining at various temperatures in 5.84 μ diameter spherical cells cooled at several rates. Initial volume of intracellular water = 88 μ^3.

a (1), k_g = 0.3 (unbracketed cooling velocities);

(2), k_g = 3 (cooling velocities in parentheses);

b, k_g = 0.15. Values of other parameters are given in Table I.

equilibrium curve). However, at higher cooling rates the water is unable to leave rapidly enough to maintain the protoplasm at its freezing point, and the amount of supercooling increases. Thus, with a cooling velocity of 10°C/min., the protoplasm is supercooled about 1.6° at −3.6°C; with 100°C/min., a maximum of 7° at −10°C; and with 1000°C/min., about 26° at −28°C.

The curves in this figure can also be described in terms of the percentage of water remaining in the cell at various temperatures. Thus, with cooling

rates of 1, 100, and 1000°C/min., the cells have lost half their water at −2, −8.4, and −29.6°C, respectively. At −8°C, cells cooled at 1, 100, and 10,000°C/min. contain, respectively, 11, 55, and 99.5 per cent of their initial intracellular water.

A cell with higher permeability to water loses water more rapidly. Equation 19 indicates that if k_g is increased tenfold, the cooling rate has to increase a
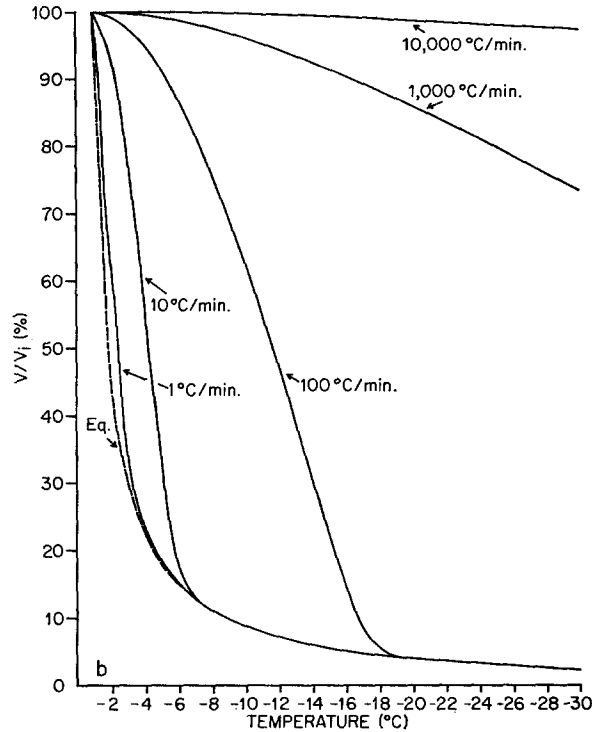

FIGURE 2 b

like amount to yield the same relation between water volume and temperature; e.g., the curve in Fig. 2 a labeled 1000°C/min. for k_g = 0.3 becomes 10,000°C/min. for k_g = 3. If the permeability constant is halved, the curves for a given rate move to the right, as shown in Fig. 2 b.

Large spherical cells have smaller surface-volume ratios than do smaller cells, and would, therefore, be expected to lose less water in a given interval of time. The calculations shown in Fig. 3 are consistent with this expectation. Shown in this figure is the fraction of original cell water remaining in spherical cells 1, 6, and 25 μ in diameter (with A/V ratios of 7.2, 1.2, and 0.28, respectively). At a given rate of cooling, the larger cells retain a higher percentage of their water than do the smaller cells, and are, therefore, more supercooled at a given temperature.

Assumptions in Equation 19

A number of assumptions underlie the derivation of Equation 19. Their validity is discussed in the Appendix. Fortunately, the numerical solutions are relatively insensitive to the potential errors introduced by most of the assumptions. However, they are sensitive to the temperature coefficient of the permea-
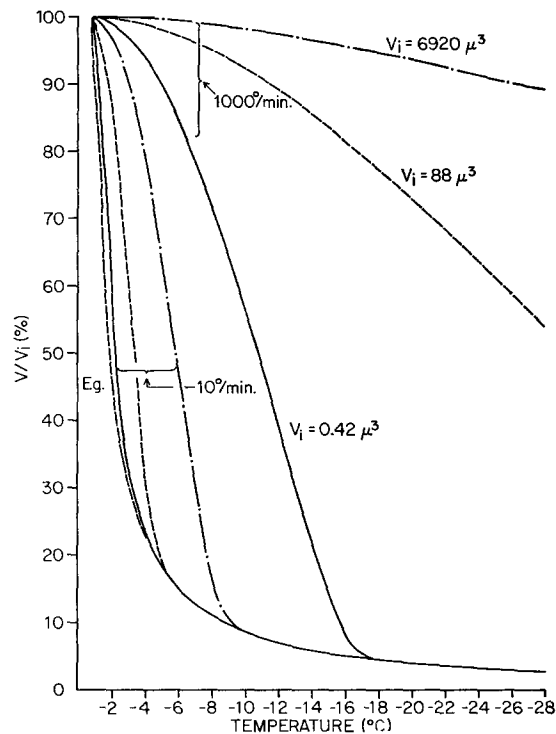

FIGURE 3. Calculated percentages of intracellular water remaining at indicated temperatures in cells of various sizes undergoing cooling at 10° or 1000°C/min. The value of k_g is 0.3; the values for the other parameters are given in Table I.

bility constant. This temperature coefficient (b in Equation 15) was derived empirically from data on red blood cells over the range 0 to 30°C and extrapolated to subzero temperatures. Since there appear to be no published data bearing directly on the validity of this extrapolation, this simpler equation was used instead of an Arrhenius equation of the form, log $k = J - \dfrac{c}{T}$, which fitted the available data at above zero temperatures no better.

The Q_{10} for the permeability constant for water in red blood cells (1.38) is appreciably lower than the values of 2 to 3 for sea urchin eggs (McCutcheon

and Lucké, 1932) or cells from onion and dandelion (Delf, 1916). (Note that
$\ln Q_{10} = 10b$.) The curves in Fig. 4 show how the calculated volumes of intra-
cellular water are affected by the choice of several Q_{10} values; namely, 1, 1.38,
and 3 (b = 0, 0.0325, and 0.1099, respectively). The volume-temperature
relation is seen to be decidedly sensitive to the value of b. Hence, obtaining a
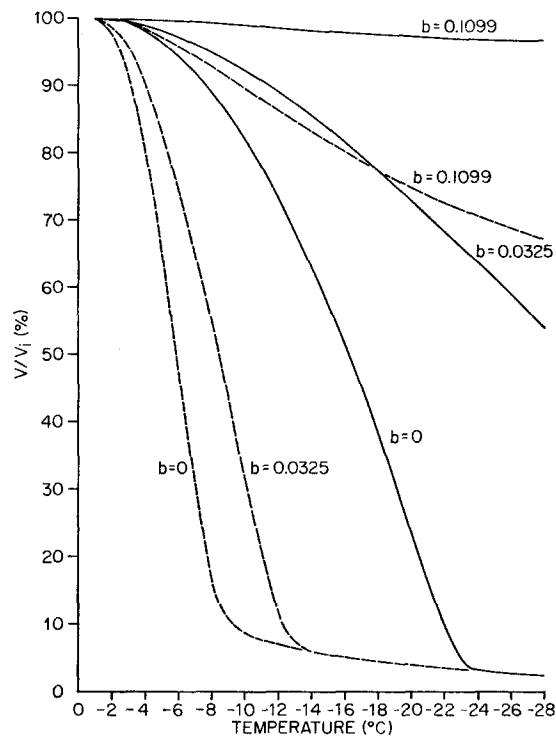reasonably accurate value of b from above zero temperature measurements will

Figure 4.   Effect of various values of b (= 0.1 ln Q_{10}) on the calculated percentages of
intracellular water remaining in cells at indicated temperatures. Solid line, cooling at
1000°/min.; dashed line, cooling at 100°/min.; V_i = 88; k_0 = 0.3. The values of the
remaining parameters are given in Table I.

be at least as important to the applicability of Equation 19 as will errors
introduced by assuming that Equation 15 is applicable at subzero tempera-
tures. In yeast and in unhemolyzed red cells, there is no abrupt *increase* in
permeability as the temperature drops below 0°C during freezing since the
intracellular solutes do not leak out of the cell. Nor does an abrupt *decrease*
in permeability appear likely, for even if the protoplasm were to gelate, the
diffusion rate of small molecules, including water, in an aqueous gel is little or
no different from that in a sol (Hermans, 1949).

DISCUSSION

Fate of Supercooled Water in Cells

With cooling rates above about 1°C/min., the calculations indicate that the protoplasm will become supercooled. Three phenomena can then occur.

1. Supercooled water can continue to leave the cell as the temperature drops further and eventually can reduce the cell water content to the equilibrium level. For example, equilibrium would be attained at $-14°C$ when cells with the properties listed in Fig. 2 a (1) are cooled at 100°C/min.

2. Before equilibrium is attained, the temperature might drop below that of glass transformation. At that point, the viscosity of water is so high (10^{13} poises; Jones, 1956) that no further diffusion occurs, and the liquid remains metastable or vitrified indefinitely. For pure water, that temperature appears to be -130 to $-160°C$ (Dowell and Rinfret, 1960), but it is considerably higher in concentrated gelatin solutions (Dowell, Moline, and Rinfret, 1962).

3. The supercooled water may freeze. Such freezing would eliminate the vapor pressure differential between the inside and outside of the cell, whereupon the outflow of water would cease.

Available data are insufficient to state precisely which would happen, but certain predictions can be made. Nucleation rate, and hence the probability of initiating crystallization, increases with the degree of supercooling. With pure water, the probability approaches 1 at about $-40°C$, even if no foreign nucleating sites are present (Fisher, Hollomon, and Turnbull, 1949). Apparently, there are no efficient nucleating agents in cells, for Salt (1950) has supercooled many insects in air to below $-20°C$, and occasionally to below $-30°C$. But we are considering cells surrounded by a medium containing ice, and ice, of course, would be the best nucleating agent if it were able to penetrate the cell to effect seeding. Experimental observations indicate it is unable to do so at temperatures above -5 to $-10°C$ (Chambers and Hale, 1932; Smith, 1961, p. 409). This inability of ice to penetrate may stem from the fact that crystals small enough to grow through narrow aqueous channels in the cell membrane would have too small a radius of curvature to exist at temperatures above $-10°C$ or thereabouts (Mazur, 1960). Below these temperatures, they can exist and can pass through the channels to initiate intracellular freezing. This picture is consistent with a number of observations showing that supercooled cells surrounded by ice usually do undergo internal freezing when the temperature drops rapidly below -5 or $-10°C$ (Asahina, 1961).

When these comments are considered in the light of the numerical solutions to Equation 19, they lead to the prediction that no intracellular ice can form in any cell in which the water content has been reduced to the equilibrium value,

or to below 10 per cent, by the time its temperature has dropped to around
$-10°C$. The "10 per cent" restriction is added because this proportion of the
water in a number of cells is incapable of freezing (Wood and Rosenberg,
1957). For the average cell in Fig. 2 a (1) these requirements would be met at
cooling rates of about 50°C/min. and less. For a similar cell with a tenfold
greater permeability to water (Fig. 2 a (2)), intracellular freezing would not
occur at rates below about 500°C/min. For the 25 μ cell, a cooling rate of
10°C/min. would be marginal (Fig. 3). And for a 1 μ cell (Fig. 3), intracellular
freezing should not occur at cooling rates of less than about 200°C/min.

If cooling velocities exceeded these values, there would be a distinct possi-
bility for the initiation of internal freezing. And if crystallization was once
initiated, the fate of the intracellular water would depend on the balance
between the rate of crystal growth, the rate at which water leaves the cell,
and the rate of cooling. Suppose freezing were initiated at $-12°C$, a tem-
perature at which cells containing 50 per cent of their initial water would be
supercooled some 10°C. Lusena (1955) has found that with this amount of
supercooling, ice crystals grow 4500 μ/sec. in a 16 per cent lysozyme solution;
therefore, provided cells behave like 16 per cent lysozyme, it would take about
0.0001 min. for the ice to grow across a 25 μ cell and, of course, proportion-
ately less time for smaller cells. Even with cooling at 10,000°C/min., the
temperature would drop only 1.0°C during the 0.0001 minute. The numerical
solutions to Equation 19 for k_g = 0.3 indicate that during a drop of 1.0°C,
the water content of a 25 μ cell will change less than 0.1 per cent (*i.e.*, it will
be 99.7 per cent before freezing begins at $-12°C$ and 99.7 per cent after
freezing is completed). Similar calculations were carried out for cooling rates
of 100 and 1000°C/min., for 1 μ and 6 μ diameter cells, and for a permeability
constant of 3.0—all showed that intracellular freezing will be completed
before the temperature has dropped as much as 1°C and before as much as 1
per cent of the water originally in the cell has diffused out. (The calculations
assumed that the latent heat of fusion is too small or is dissipated too quickly
to affect the cooling rate, assumptions that are discussed in the Appendix;
however, they did not correct for the fact that, as the cell freezes, a progres-
sively smaller fraction is supercooled and able to diffuse out of the cell. The
assumption, if wrong, will tend to give an underestimate of the amount of
water leaving the cell during freezing, while the lack of correction for progres-
sive freezing will tend to give an overestimate.)

The highest protein concentration Lusena studied was 16 per cent. Extrapo-
lation of his data to a 25 per cent lysozyme solution yields 3200 μ/sec. as the
growth rate of ice, a decrease that would not affect the conclusions just stated.
Hence, once intracellular freezing begins, the cell will not be further dehy-
drated nor can its internal water be vitrified, at least within the range of
conditions considered here.

Observed Fate of Intracellular Water

A number of microscopic observations have been made on living cells during cooling, both directly during freezing and indirectly by subjecting cooled cells to freeze-substitution or freeze-drying. Most of the observations on animal cells have been reviewed by Smith (1961, pp. 409–419). In this discussion we
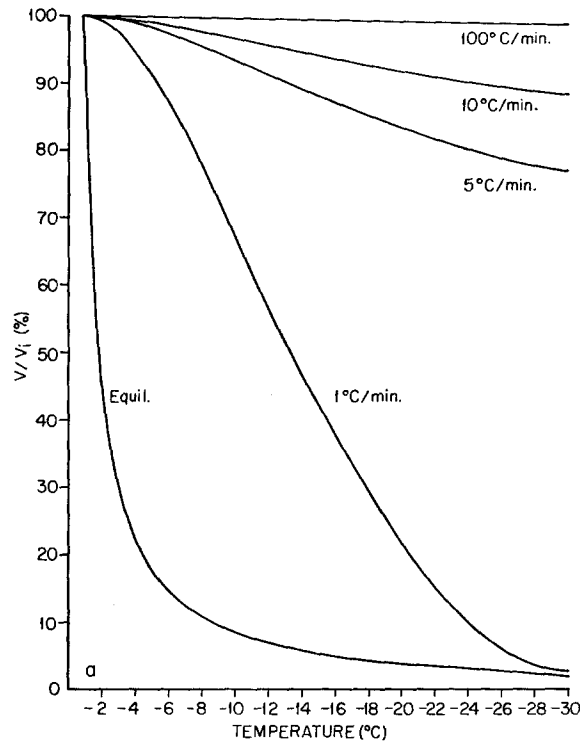

FIGURE 5. Calculated percentages of intracellular water remaining at indicated temperatures in (a) unfertilized *Arbacia* eggs, (b) human red blood cells cooled at indicated rates. The parameters for *Arbacia* were $V_i = 2.08 \times 10^5$, $A = 1.86 \times 10^4$, $b = 0.1034$, $k_g = 0.11$, $n_2 = 1.04 \times 10^{-10}$. They were calculated from data of McCutcheon and Lucké (1932) and Lucké, Hartline, and McCutcheon (1931). The parameters for red blood cells were $V_i = 61$, $A = 163$, $b = 0.0325$, $k_g = 5$, $n_2 = 1.83 \times 10^{-14}$, and $T_f = 272.4°K$. They were calculated from data of Ponder (1955), Jacobs *et al.* (1935), and Sidel and Solomon (1957). The values of the other parameters are those given in Table I.

will consider only a few representative studies, emphasizing observations on those cells for which data on permeability and volume are available.

VERY LARGE CELLS Smith, Polge, and Smiles (1951) cooled amebae to −10°C. They report that above −8° the cells appeared unfrozen even though the surrounding water was frozen, and that no change in volume occurred

during the short period of observation. At lower temperatures, internal freezing did occur. Asahina (1961, 1962) cooled eggs of the sea urchin *Strongylocentrotus*. He, too, observed that above about $-5°C$ the cell interior was unfrozen. When he continued cooling the eggs at 1 °C/min., they remained unfrozen and decreased in volume; when he cooled them at 10°C/min., they froze internally. Fig. 5 *a* shows the calculated fraction of water remaining in eggs of the related genus, *Arbacia*, during cooling (curves were computed for
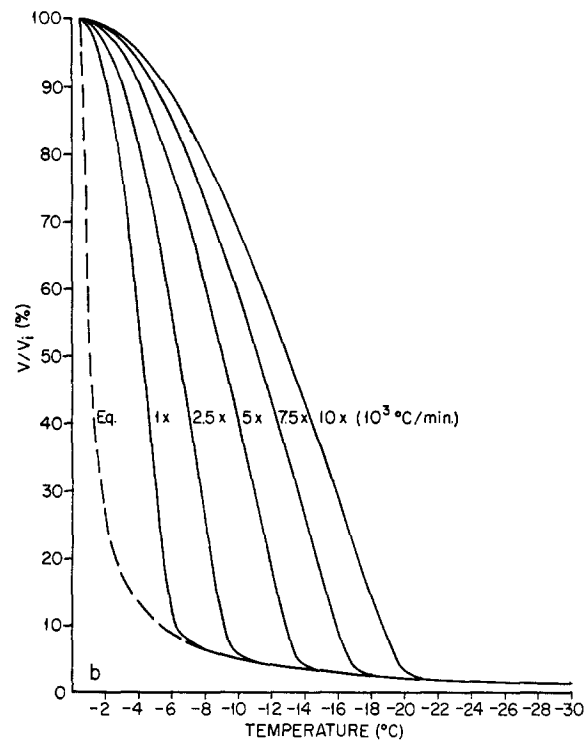

FIGURE 5 *b*

Arbacia because no data on the temperature coefficient of *k* could be found for *Strongylocentrotus*). The results are consistent with Asahina's observation in that the eggs lose considerable water when the temperature is decreasing at 1°C/min. but lose practically none when the temperature is dropping 10°C/ min. Therefore, intracellular freezing of most of the protoplasmic water would be much more likely at the higher cooling rate. However, in *Arbacia* eggs the surface-volume ratio and the permeability constant for water are so low (about 0.09 and 0.11, respectively), and the Q_{10} of the permeability constant is so high (about 2.8) that nearly 70 per cent of the intracellular water is calculated to be still in the egg at $-10°C$ and to be supercooled some 9°C even with cooling as slow as 1°C/min. This water would be expected to freeze. That

internal freezing did not occur in *Strongylocentrotus* eggs cooled at 1°C/min. could be due to the fact that its permeability constant is 0.38, nearly fourfold higher than that of *Arbacia* (Shinozaki, 1951).

Amoeba is still larger and has an appreciably lower A/V ratio and permeability to water (0.04 and 0.016, respectively, according to Mast and Fowler, 1935; and Prescott and Zeuthen, 1953). On the basis of Equation 19, therefore, it should lose water considerably more slowly than the sea urchin eggs, in agreement with the observations of Smith *et al.* (1951).

RED BLOOD CELLS   Rapatz and Luyet (1960, 1961) have examined the effect of cooling velocity on red blood cells of the frog using light and electron microscopy. A 450 μ layer of blood cooled in a bath at $-30°C$ yielded shrunken, distorted cells with no evidence of intracellular ice, whereas layers frozen in baths at $-80°C$ or below yielded cells that were similar in shape and size to untreated cells and showed evidence of internal ice in the thicker central portions. If 15 μ layers of blood were used, the ice crystals were dispersed throughout the cytoplasm. The authors do not state numerical cooling velocities; however, because 0.1 ml of water in a tube 5 mm (inside diameter) cools from -5 to $-25°C$ in a $-30°C$ ethanol bath at about 50°C/min. (Mazur, 1961 c), an estimate of 500°/min. for the cooling rate to $-30°C$ of the 450 μ layer in the less efficient coolant, isopentane, is probably reasonable. The 15 μ layers most probably cooled at rates in excess of 10,000°C/min. If the permeability of frog red cells is similar to that of mammalian red cells, the curves in Fig. 2 a (for $k_0 = 3$) would represent the calculated volumes of intracellular water. The figure shows that cells cooled at less than 1000°C/min. will have dehydrated to the equilibrium value before the temperature has reached $-14°C$, whereas those cooled at rates exceeding 10,000°C/min. will still contain over 80 per cent of their water at $-14°C$. This water will be supercooled some 13°, and will, therefore, be susceptible to crystallization.

YEAST   Nei (1960) has examined yeast under the microscope during freezing and finds that the cells become flattened in suspensions frozen slowly at -2 to $-10°C$, and that there is no evidence of intracellular ice. On the other hand, the cells are more nearly normal in size and shape in suspensions frozen rapidly to $-78°$ or $-190°C$ and appear to contain ice. I have freeze-substituted yeast with ethanol at -32, -65, and $-79°C$ after cooling them slowly (1°C/min.) or rapidly (50 to 270°C/min.). The volumes of the substituted cells are about 33 and 50 per cent of unfrozen controls with the two cooling rates, respectively (Mazur, 1961 a). Since the volume decrease appears to be due to water loss alone (see Introduction), the calculated percentages of the original water still remaining in the slowly and rapidly cooled cells are 10 and 33 per cent (Mazur, 1963). The curves in Fig. 2 b represent

the water contents of yeast, calculated according to Equation 19. At a cooling rate of 1°C/min., the water content remains close to the equilibrium value so that the cells dehydrate and cannot freeze. But at a cooling rate of 100°C/min., cells at −14°C still contain a calculated 30 per cent of their initial water which is supercooled some 11° and is, therefore, likely to freeze. With rapid cooling, most yeast cells apparently do freeze internally between −10 and −20°C, and survival decreases from 100 per cent to less than 10 per cent in that temperature interval (Mazur, 1961 *c*). Thus, the calculated water contents are consistent with the observed volumes and with the occurrence or non-occurrence of internal ice.

Survival and Intracellular Freezing

Although intracellular freezing is not the sole cause of low temperature death (Smith, 1961), the presence of large, internal ice crystals almost invariably appears to be lethal (Mazur, 1960, 1961 *a*). The avoidance of internal ice, therefore, seems to be one prerequisite for achieving high survivals after low temperature exposure. Qualitatively, slow cooling should decrease the likelihood of intracellular freezing or at least reduce its extent, and as was noted in the Introduction, slow cooling does, in fact, favor high survival. Now we see that Equation 19 provides quantitative physicochemical support for this assumed correlation between slow cooling, the avoidance or reduction of intracellular ice, and obtaining high survival. Furthermore, the numerical solutions indicate that the cooling rate of 1°C/min. so often used is one which should prevent internal ice formation in almost all cells except very large ones.

In some cells, ice formation is not the only cause of injury. For example, with *Escherichia coli* (Harrison, 1956) and human red blood cells (Lovelock, 1953), a low cooling rate, while preventing internal ice, increases lethality, possibly by overexposing the cells to gradually concentrating solutes. In such cases, an intermediate cooling velocity may be more successful; *i.e.*, one slow enough to minimize internal ice formation and rapid enough to minimize the toxic effects of concentrated solutes. Equation 19 permits an educated guess as to what such a rate should be. With human red blood cells, for example, one would predict an optimum rate of 2500 to 5000°C/min., since at higher rates the cells begin to contain appreciable amounts of supercooled water at temperatures below −10°C (Fig. 5 *b*). Interestingly, an intermediate optimum rate for minimum hemolysis has been found by Gehenio and Luyet (1958), Rinfret and Doebbler (1960), and Luyet (1960). The first named authors estimate it to be on the order of 6000°C/min.

CONCLUSIONS AND IMPLICATIONS

In view of the number of assumptions required to formulate Equation 19, it is gratifying that the calculations derived from that equation seem to be con-

sistent with the observed behavior of some cells to within an order of magnitude. The agreement is especially satisfying since all the parameters have definite physical meaning and none is an arbitrary constant. However, a critical test of the applicability of the equation will require considerably more precise values than now exist for some of the parameters, especially for V, B, and b.

Experimental observations that depart markedly from predicted behavior would be intriguing, for they would indicate either that one or more of the assumptions is not applicable to a given cell or that the supposed value of a parameter is wrong. On the latter point, we have noted the great sensitivity of Equation 19 to the value of the permeability constant and its temperature coefficient. While this sensitivity is probably the greatest source of potential error in using Equation 19 to predict the ability of cells to survive low temperatures, it also presents a potential approach to estimating the permeability to water of certain cells, such as bacteria, for which few such estimates exist.

APPENDIX

The derivation of Equation 19 required making a number of assumptions:

1. The plasma membrane was assumed to remain intact during cooling. It must remain intact in those cells that survive low temperature exposure. The absence of hemolysis supports this assertion for surviving red blood cells, and electrical conductivity (Mazur, 1963) and interferometric measurements (Mazur, 1961 b) support it for yeast. Moreover, even when yeast are killed by low temperature exposure, resistivity measurements suggest that the plasma membrane remains relatively impermeable to solutes during cooling and most of the subsequent warming (Mazur, 1963).

2. The plasma membrane was assumed to be permeable only to water. While not strictly true, the assumption should cause no appreciable error in most instances. The permeability of cells to water is usually much higher than to even the most rapidly penetrating solutes (Davson and Danielli, 1952), and is, of course, several orders of magnitude higher than to the non-permeating solutes normally in protoplasm. The leakage rate of potassium in yeast, for example, is only 1 per cent per hour (Rothstein, 1959).

3. Protoplasm was assumed to be an ideal dilute solution in Equation 1. Actually it is not, but the errors introduced by deviations from Raoult's law should not be serious. The van't Hoff equation, which is predicated on Raoult's law, describes the osmotic behavior of cells reasonably well (Dick, 1959; Mitchell and Moyle, 1956). Moreover, frozen suspensions of yeast have been found to absorb quantities of heat during warming that show a relation to temperature and to solute concentration similar to that shown by frozen solutions that obey Raoult's law (Mazur, 1963).

4. Any liquid solution in the external medium was assumed to be in thermodynamic equilibrium with ice, and the cell water was assumed to be supercooled and at the same temperature as the suspending medium. Since the problem here was to derive expressions for the behavior of supercooled water in cells surrounded by an aqueous

medium containing ice, these two assumptions need no justification. As for the assumptions on thermodynamic and thermal equilibrium, consider a spherical microregion around an individual cell. The time (τ) for a pulse of heat to travel from the edge to the center of a sphere of radius, r, is

$$\tau \approx r^2/\pi^2 D \qquad (24)$$

(Strong, 1938)[1] where D is the thermal diffusivity, which for water at 0°C and ice between 0 and -30°C equals 0.00136 and 0.011 cm²/sec., respectively. (D was calculated as $\kappa/\rho s$ from data in Dorsey, 1940, where κ, ρ, and s are the thermal conductivity, density, and specific heat.) Thus, τ is 7.8×10^{-6} and 9.6×10^{-7} min. for spheres of water and ice 25μ in radius. Clearly then, even with the highest cooling rates considered (10^4°/min.), all regions within a 50μ diameter sphere will have the same temperature to within a small fraction of a degree, the temperature difference between surface and center being $\tau \dfrac{dT}{dt}$ (Stephenson, 1960) or 0.08°C and 0.01°C for spheres of water and ice, respectively.

The establishment of thermodynamic (vapor pressure) equilibrium between ice and any residual unfrozen solution in the external medium has been assumed to be instantaneous with change in temperature. Maintaining this equilibrium would require conversion of water in the external solution to ice. Stephenson (1956) has calculated that this conversion is complete in pure water whenever cooling is slower than 300,000°C/min.; *i.e.*, none of the water remains metastable (supercooled). Therefore, no metastable water should be present in the external medium at the maximum cooling rate considered here (10,000°C/min.) as long as that medium does not contain solutes like gelatin which in sufficient concentration can diminish crystal growth rate extensively (Dowell *et al.*, 1962). Whatever external liquid is present will be in equilibrium with ice.

5. Substitution of the molar volume of water for the partial molar volume in Equations 8, 11, and 23 produces little error. For example, the partial molar volume of water ($\bar{v}_1$) at 20°C in 3.25 M solutions of NaCl, KCl, CaCl₂, sucrose, and glycerol, is 17.97, 17.86, 17.68, 17.73, and 18.02 cm³/mole, respectively. The molar volume (v_1^0) of pure water is 18.048 cm³/mole. (The values of $\bar{v}_1$ were calculated as $\bar{v}_1 = \dfrac{M}{\rho - cd\rho/dc}$ (Glasstone, 1946, p. 670), with ρ, the density at various values of molar concentration, c, obtained from the Handbook of Chemistry and Physics (1961), and $d\rho/dc$ estimated by drawing tangents to the graph of ρ *versus* c. M is the molecular weight of solvent (water).)

6. The assumption that the cooling rate is constant (Equation 16) introduces no conceptual error but could cause experimental difficulties. A constant rate of heat withdrawal by a coolant will not produce a constant rate of cooling in suspensions containing appreciable concentrations of solutes or cells. This non-linearity would be

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[1] Stephenson (1960) states that the thermal time lag in a sphere is $\dfrac{r^2}{6D}$ which yields values about 50 per cent larger than the relaxation times calculated according to Strong's equation.

due to the necessity for removing the latent heat of fusion. However, the error could be made vanishingly small by using very dilute suspensions of cells in dilute media and seeding and equilibrating them between 0°C and the freezing point of the protoplasm before cooling is begun.

7. In the differentiation of Equation 19, only V and T were considered variables. R is an exact constant. T_f, V_i, k_0, and T_0, are constant for specific cells, their numerical value, however, being subject to experimental error. The value of v_1^0 changes less than 0.05 per cent/°C and, therefore, is "constant." The constancy of the cooling rate, B, and of b has already been discussed.

For cells with non-leaky membranes, n_2 will remain constant as long as there is sufficient intracellular water to keep the protoplasmic solutes in solution. Electrical resistivity measurements of frozen suspensions of yeast indicate that liquid protoplasmic solution is present as low as $-30°C$, and measurements by differential thermal analysis of heat absorbed during warming indicate that most of the intracellular solute molecules are in solution at temperatures above $-10°C$ (Mazur, 1963). The equilibrium concentration of an ideal solution at $-10°C$ is about 5 molal ($10°/1.9°/$ mole). If the initial concentration of intracellular solutes is 0.5 molal, as assumed here in most cases, the water content has to be reduced to 10 per cent of its initial value before the protoplasm becomes 5 molal; therefore, the value of n_2 should remain approximately constant at water contents above 10 per cent. Furthermore, any reduction in the value of n_2 by precipitation of solutes similarly affects both the numerical solutions to Equation 19 and the equilibrium water volume calculated by Equation 23, and, therefore, has a smaller effect on the differences between equilibrium and non-equilibrium curves.

The area, A, of the cell may or may not remain constant. It is constant in mammalian red cells (Ponder, 1955), but appears to vary as the two-thirds power of cell volume in sea urchin eggs (Lucké, Hartline, and McCutcheon, 1931). In the latter case, in place of Equation 9 we have

$$\frac{dV}{dt} = k(36\pi)^{\frac{1}{3}}V_{\text{cell}}^{\frac{2}{3}}(\Pi_i - \Pi_e). \tag{25}$$

Obviously, the already formidable Equation 19 would become more so if Equation 25 were used in lieu of Equation 9. A rough estimate of the possible error introduced if it were wrong to assume a constant area would be as follows: A yeast cell protoplast has a volume (V_{cell}) of $104\mu^3$ and contains a volume (V) of $88\mu^3$ of water. Its area is $107\mu^2$, and A/V is, therefore, 1.22 (Mazur, 1963). If V_{cell} decreased to $38\mu^3$, corresponding to a loss of $66\mu^3$ of water, A/V would be $107/22$ or 5 assuming A to be constant. But if $A = (36\pi)^{\frac{1}{3}}V_{\text{cell}}^{\frac{2}{3}}$, it would become $54.7\mu^2$, and A/V would be $54.7/22$ or 2.5. The two assumptions thus lead to a 50 per cent difference in A/V when 25 per cent of the initial water remains in the cell. Actually, Lucké et al. tested Equation 25 by observations on cells expanded beyond their normal volume in hypotonic media. In the case treated here, in which cells are smaller than normal, the possibility of folding of the plasma membrane would seem to make the assumption of constant plasma membrane area as probable as the assumption that $A \propto V_{\text{cell}}^{2/3}$.

Finally, the molar heat of fusion (L_f) is actually not constant with temperature,

but varies according to Kirchhoff's law as

$$\left(\frac{\partial L_f}{\partial T}\right)_p = C_{p_{H_2O}} - C_{p_{ice}} \tag{26}$$

where $C_{p_{H_2O}}$ and $C_{p_{ice}}$ are the molar heat capacities at constant pressure. If ΔC_p is
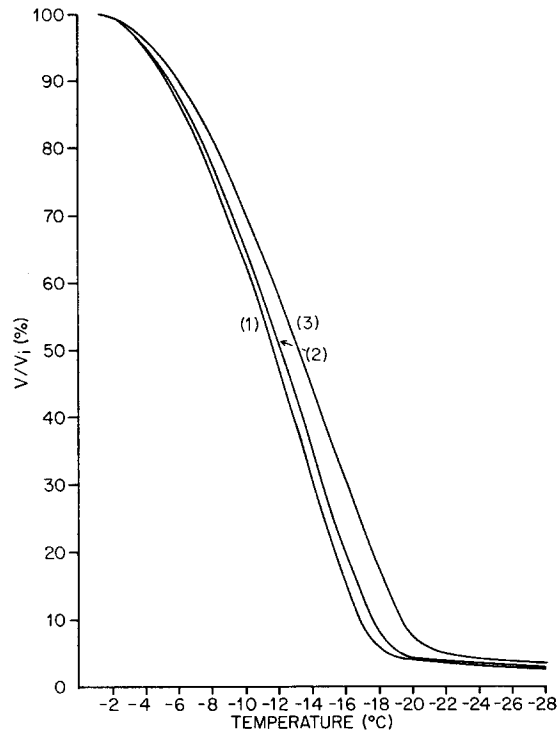

FIGURE 6. Effect of different values of L_f on calculated cell water content. Curves (1), (2), and (3) were calculated using the values of L_f at 0, −10, and −30°C (see text) and a cooling velocity of 200°C/min. $V_i = 88$, $k_g = 0.3$. The values of the remaining parameters are given in Table I.

considered constant with temperature, Equation 26 can be integrated to give

$$L_f = L_f^0 + \Delta C_p(T - 273) \tag{27}$$

where L_f^0 is the molar heat of fusion at 0°C.

Actually, ΔC_p is not constant, and for precise results, Equation 26 must be integrated without assuming ΔC_p to be constant. Using the equations given by Dorsey (1940, p. 562) for the relation between heat capacity and temperature, and integrating Equation 26 in the manner outlined by Glasstone (1946, p. 214), we obtain

$$L_f = L_f^0 + 9.080 \ T_{°C} - 0.02649 \ T^2_{°C} + 0.000216 \ T^3_{°C} \tag{28}$$

The values of L_f at -10, -20, and $-30°C$ turn out to be 1339, 1242, and 1146 cal/mole respectively using Equation 27 and 1342, 1242, and 1134 cal/mole using Equation 28. The value at $0°C$ is 1436 cal/mole. Thus, L_f changes some 0.6 to 0.7 per cent/degree C. To estimate the effect of the change, Equation 19 was solved using several values of L_f between 0 and $-30°C$ as a constant with the results shown in Fig. 6. The error is comparatively small. It could be eliminated by substituting Equation 27 or 28 in place of L_f in Equation 18, but in view of the other far greater sources of error, this refinement and its attendant complication of Equation 19 were not attempted.

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