FREEZING IN YEAST CELLS*

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INTRODUCTION

A vast literature deals with the effects of low temperatures and freezing on biological materials (see BĚLEHRÁDEK[1], LUYET AND GEHENIO[2], SMITH[3], and WOOD[4] for reviews). Much of this information is of a qualitative nature and is concerned with phenomena of an immediately practical nature such as frost hardiness of plants and insects, frostbite, preservation of tissues and foodstuffs, etc. Among the relatively few investigators who have approached the problem of cold injury (or protection) from a mechanistic point of view are MORAN[5,6], MENNIE[7], LUYET[8], LUYET AND GEHENIO[9], LOVELOCK[10,11], SCHOLANDER *et al.*[12], and MERYMAN and co-workers[13,14].

Perhaps the first step in the elucidation of a mechanism for cold injury is the quantification of the amount of cellular water frozen at various sub-freezing temperatures and the localization of this water when frozen, whether internal or external to the cell. It is the purpose of this paper to describe calorimetric measurements on populations of yeast cells that allow determinations to be made on the fraction of cellular water frozen as a function of temperature. From these and other measurements it is possible to speculate on the localization of at least a part of this frozen water.

METHODS

The primary measurements made were semi-microcalorimetric determinations of the heat required to thaw aliquots of yeast suspensions frozen to various temperatures. By usual calorimetric procedures this heat is associated with the thawing of a definite amount of ice, and hence the degree of cellular freezing can be computed if the thermal properties of all components of the system are known. The method of mixtures was used for the calorimetric determinations.

A cake (approximately 18 g) of fresh Fleischmann's baker's yeast was suspended in 75 ml of $M/15$ KH_2PO_4. Large particulate matter contained in the cake (about 7% of the cake by weight and presumably large starch granules) was removed by centrifugation at 500 *g* for 1 minute. The suspended cells were then spun down by centrifugation at 2,500 *g* for 5 minutes, resuspended, and recentrifuged. This washed centrifugate was accurately weighed and resuspended in a volume of $M/15$ KH_2PO_4 equal to 3/4's of the mass of the centrifugate. The final suspension was allowed to equilibrate while being gently agitated by shaking. The volume of equilibrated cells after this procedure was approximately ½ of the total volume of the suspension.

To determine accurately the ratio of the cellular mass to the total suspension mass, R, a modification of the method suggested by WHITE[15] was used. In this method a specially prepared peptone solution** which is not affected by equilibrated yeast cells is added to a yeast suspension

* This investigation was supported by a research grant (C-2390) from the National Cancer Institute, Public Health Service.

** This solution was prepared by mixing equal volumes of a 4% peptone solution prepared in $M/15$ KH_2PO_4 and a yeast suspension prepared as above. This mixture was shaken overnight and the diluted peptone solution minus any yeast-utilizable components was recovered and purified by repeated centrifugation at 2500 *g* until visually clear.

References p. 86/87.

whose R value is unknown. The peptone solution is diluted by the external water of the suspension but inappreciably affects the osmotic properties of the suspension. The cells are removed by gentle centrifugation and the new concentration of the recovered peptone solution is determined. Instead of the micro-Kjeldahl determinations used by WHITE for ascertaining the peptone concentrations we found it more convenient in these experiments to assay the concentrations of the peptone solutions by (1) measurements of the refractive index and (2) gravimetric determinations of the dry weight residues. In both methods calibration curves were constructed by diluting accurately the special peptone solution with supernatant from a standard yeast suspension. An Abbe-Spencer refractometer was used for the refractive index determinations; the reproducible accuracy of this instrument was $\frac{1}{2}$ part in the 4th decimal place. The dry-weight residues of the various peptone solutions were obtained by vacuum drying aliquots for 72 hours and weighing their residues to an accuracy of 10 μg. The R value was computed from the calibration curves and a knowledge of the density of the yeast cell[16]. For the standard yeast suspensions an R value of 0.50 $\pm$ 0.02 was computed by the refractive index method and a value of 0.480 $\pm$ 0.004 by the dry-weight method. The latter value will be used in all calculations.

Yeast suspension was pipetted into thin aluminum cups (diameter 1.5 cm and height 2.0 cm) of known weight; the weight of the suspension was determined by differential weighing. These cups and their contents were held for at least 20 minutes in a metal rig placed within a bath whose temperature was controlled by adding dry ice to different mixtures of water and methyl alcohol. Cups with their frozen contents were quickly removed from the temperature bath by means of fine threads attached to their sides and dropped into the calorimeter through a small hole in its lid. The time required for the entire transfer operation was recorded for each determination (approximately 10 seconds); control experiments were performed to ascertain the heat gained by the cups and their contents from the environment during the transfer process and corrections were made for this in all calculations.

The calorimeter was similar to that described by MILLER *et al.*[17]. It consists of a Dewar flask of volume 500 ml that is submerged to within 2 cm of its mouth in a water bath accurately controlled to $\pm$ 0.002° C. A lucite cover is used to cover the calorimeter vessel and holes are provided in this cover for a Beckman differential thermometer, a stirrer, a heating coil, and a drop-hole. The heat content of the calorimeter vessel and its associated apparatus was 19.0 $\pm$ 0.4 cal/C°. In all runs 225.0 ml of water were placed within the calorimeter thereby giving a total heat content of 244.0 $\pm$ 0.5 cal/deg. The stirrer was driven electrically and did not influence these determinations. The Beckman thermometer was used in the customary fashion and differential readings were accurate to within $\pm$ 0.002 °C. The heating coil was used to return the calorimeter and its contents to a standard temperature after a run. Normally 5 runs for each experimental condition were made, and, excluding occasional discrepancies, these showed agreement in the calculations for the desired parameter to within the computed limits.

To determine the fraction of yeast surviving various freezing treatments aliquots of frozen yeast in aluminum cups were thawed by dropping the cups and their contents into 200 ml of $M/15$ KH_2PO_4. After suitable dilutions, 0.5 ml aliquots were surface spread on nutrient agar (potato dextrose, Difco). Surviving fractions were determined from suitable controls. Incubation was at 30° C for 3 days.

Dry-weight fractions, w, of a standard yeast suspension were determined by (1) vacuum desiccation and by (2) evaporation at 105° C. The residual weights of samples attained a constant level after 4 hours with either method. The dry-weight fraction of the standard yeast suspension found by the two methods were 0.151 $\pm$ 0.001 and 0.150 $\pm$ 0.002, respectively. The dry-weight fraction of the equilibrated yeast cell, a, is computed from the relationship $a = w/R$. Using a value of 0.480 $\pm$ 0.004 for R and a value of 0.151 $\pm$ 0.001 for the dry weight fraction of the yeast, a is computed to be 0.313 $\pm$ 0.004.

The specific heat of the dry residue of the cell (hereafter designated as the non-aqueous component) was determined by standard calorimetric procedure. Pellets of vacuum-dried yeast compressed at 5000 lbs/in.^{2} were precooled to a known temperature in weighed aluminum cups and introduced into the calorimeter. The average specific heats for the non-aqueous component over the following temperature ranges were found:

0° to 25°: 0.26 $\pm$ 0.01 cal/g-C°
−22° to 25°: 0.23 $\pm$ 0.01 cal/g-C°
−45° to 25°: 0.25 $\pm$ 0.01 cal/g-C°
−72° to 25°: 0.23 $\pm$ 0.01 cal/g-C°

Since there is no apparent temperature dependence, an average value of 0.25 $\pm$ 0.01 cal/g-C° will be used.

References p. 86/87.

MODEL

When a cell suspended in salt solution is subjected to a temperature below the freezing point of the cell the cellular water will be affected in either or both of two ways: (1) a fraction of it may freeze, thereby increasing the osmotic pressure of the cellular interior until the freezing point is depressed to the temperature of the surrounding medium; or (2) a fraction of it may move across the cell membrane to the exterior where it freezes, thereby effectively increasing the internal osmotic pressure to a similar degree as in (1). This movement of water is brought about by the increased tonicity in the external medium due to the removal of pure water by external freezing.

Our primary purpose here is to determine the amount of frozen cellular water as a function of temperature and not the position of this water, whether within or without the cell. Thus it is desirable for these studies to formulate a model that is valid irrespective of the position of the frozen water.

In the system of calorimetry used the heat lost by the calorimeter is equal to the heat gained by the frozen yeast and the cup containing it, or,

$$H_{\text{calorimeter}} = H_{\text{yeast}} + H_{\text{cup}}$$

where $H_{\text{calorimeter}} = C\,\Delta T$ and $H_{\text{cup}} = M_c S_c^a (T_f - T_b)$; the notation used here and subsequently is listed in Table I. The heats required to raise the temperatures of the several components of the yeast suspension from T_b to T_f are listed below for the two alternate models already suggested (internal ice formation versus external ice formation):

I. *Frozen cellular water within cell*

A. Cell:

1. Non-aqueous component: $M_T\, R a\, S_s^a (T_f - T_b)$
2. Ice: $M_T\, R\, F\, (S_w^a T_f + L - S_i^a T_b)$
3. Water: $M_T (1 - a - F)\, S_w^a (T_f - T_b)$

B. External Medium:

1. Ice: $M_T (1 - R)\left(1 + \frac{kC_0}{T_b}\right)(S_w^a T_f + L - S_i^a T_b)$
2. Water: $M_T (1 - R)\left(-\frac{kC_0}{T_b}\right)(T_f - T_b)\, S_w^a$

II. *Frozen cellular water without cell*

A. Cell:

1. Non-aqueous component: $M_T\, R\, a\, S_s^a (T_f - T_b)$
2. Water: $M_T\, R\, (1 - a - F)\, S_w^a (T_f - T_b)$

B. External Medium:

1. Ice:
$$M_T (1 - R + RF)\left(1 + \frac{kC_0}{T_b} \cdot \frac{1 - R}{1 - R + RF}\right)(S_w^a T_f + L - S_i^a T_b)$$
$$= M_T \left[(1 - R)\left(1 + \frac{kC_0}{T_b}\right) + RF\right](S_w^a T_f + L - S_i^a T_b)$$
2. Water:
$$M_T (1 - R + RF)\left(-\frac{kC_0}{T_b}\right)\left(\frac{1 - R}{1 - R + RF}\right) S_w^a (T_f - T_b)$$

References p. 86/87.

TABLE I

DEFINITION OF SYMBOLS

F:	fraction by weight of equilibrated yeast that is frozen at temperature T_b;
C:	total heat capacity of calorimeter and enclosed water;
R:	fraction by weight of equilibrated yeast suspension that is yeast;
L^*:	heat of melting of ice;
kC_0:	freezing point for $M/15$ KH_2PO_4;
M_T:	total weight of yeast suspension sample that is added to calorimeter;
M_c:	weight of aluminum cup holding yeast sample;
$S_w^a, S_i^a, S_s^a, S_c^a$:	average specific heats over temperature range considered for water, ice, non-aqueous component of yeast, and sample cup, respectively;
ΔT:	temperature change (° C) in calorimeter when sample is added;
T_f:	final equilibrium temperature of calorimeter after sample is added;
T_b:	temperature of sample when dropped into calorimeter;
a:	fraction of yeast cell that is non-aqueous.

* Actually L is temperature-dependent and will vary depending on the initial freezing point of the cellular interior. Values for the internal osmotic strength of yeast vary from a maximum value of 0.2 (computed from [18]) to 0.06 [15]; these would correspond to freezing point depressions of approximately 0.3° and 0.1° C, respectively, which would change the heat of melting by less than 0.2 %. Accordingly, in the formulation of this model the freezing point of the cell is taken to be 0° C.

The total heat H_{yeast} required to raise the temperature of the yeast sample of mass M_T from a temperature T_b to a temperature T_f is the sum of the terms for either model. It can be seen that this sum does not depend on where the cellular water is frozen. Hence this formulation is valid under either hypothesis.

The above relationship can be solved for F, the fraction of the cell that is frozen water:

$$H_{\text{cal}} - H_{\text{cup}} - M_T(1 - R)\left[S_{ic}^a T_f - S_i^a T_b + L\left(1 - \frac{kC_0}{T_b}\right) + kC_0(S_w^a - S_i^a)\right] - M_T R(T_f - T_b)[S_w^a - a\,S_c^a - S_i^a]$$
$$M_T R[L + T_b(S_w^a - S_i^a)]$$

All quantities in the above are known or can be measured. The values of the various parameters with their associated uncertainties are listed in Table II. Also shown are the percent uncertainties introduced in F by the uncertainties in the individual parameters. The overall uncertainty in F will be the vector sum of the uncertainties due to the individual parameters since these are independent. These are approximately 3 and 4% respectively, at −5° and −72° C.

RESULTS

To test the overall accuracy of the technique, samples of distilled water were frozen at various temperatures and introduced into the calorimeter. A formulation very similar to that discussed in METHODS was used in which a quantity f, the fraction of the water frozen, was used as the unknown. At −10°, −22°, and −72° f was found to have values of 0.99 ± 0.01, 0.98 ± 0.02, and 0.99 ± 0.01, respectively. Thus this method and the parameters used for this determination are accurate and reproducible to within about 1%.

References p. 86/87.

TABLE II

SUMMARY OF ERRORS AND UNCERTAINTIES

Parameter	*Value used*	*Probable maximum uncertainty (same units as column 2)*	*Percent contributed uncertainty in F*		*Source*
			$-5°$	$-72°$	
C	244.0 cal/C°	0.5	1	2	Measurement
R	0.480	0.005	1	1	Measurement
L	79.7 cal/gram	0.1	$1/3$	$1/2$	Handbook[19]
kC_0	0.25 °C	0.1	2	1	Handbook[19]
M_T	—* grams	0.001	0	0	Measurement
M_c	— grams	0.001	0	0	Measurement
S_w^a	— cal/g-C°	0.005	$1/2$	1	Handbook
S_i^a	— cal/g-C°	0.005	1	1	Handbook
S_c^a	— cal/g-C°	0.01	0	0	Handbook
S_s^a	0.24 cal/g-C°	0.01	$1/5$	1	Measurement
ΔT	— °C	0.002	1	2	Measurement
T_f	— °C	0.1	$1/2$	1	Measurement
T_b	— °C	0.3	$1/2$	1	Measurement
a	0.313	0.003	$1/5$	1	Measurement

* Values not entered in this column were different in each experiment.

Experimental values for F, the fraction of the yeast cell frozen, for standard yeast suspension are shown in the curve A of Fig. 1. The standard errors of the mean of five determinations are indicated by the vertical lines. The shaded area associated with this curve represents the limits of uncertainty to be expected when this model is used with the values of the various parameters earlier mentioned and their associated uncertainties. For the lower temperatures the overall expected uncertainty is about 3% which increases to 4% for the $-72°$ point. The curve B and its points are obtained by the application of certain modifications to the simple theory that will be discussed later.

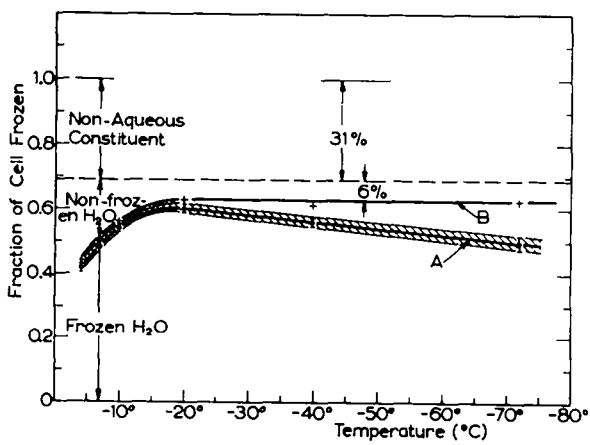

Fig. 1. Fraction of the normal yeast cell frozen as a function of temperature. The points of curve A are computed under the assumption that the specific heat of the non-freezable water is 1. The shaded region represents the uncertainties in this curve introduced by the uncertainties in the individual parameters. The points of curve B are computed under the assumptions that the fraction of frozen water should not decrease but should attain a constant value and that the specific heat of the non-freezable water need not be 1. The horizontal dashed line is the total water fraction of the cell.

In some cycling experiments the cups containing yeast suspension were precooled for 20 minutes at one temperature before being equilibrated at a second temperature

References p. 86/87.

prior to introduction into the calorimeter. These results and their controls are shown in Table III.

TABLE III

INFLUENCE OF CYCLING ON FREEZING FRACTIONS

Temperature at which sample frozen	*Temperature at which sample introduced into calorimeter* (T_b)	*Fraction of sample frozen* (F)
−22°	22	0.01 ± 0.01
−22°	2.5	0.44 ± 0.01
−2.5°	2.5	0.44 ± 0.01
−72°	−72°	0.51 ± 0.02
−72°	−22°	0.61 ± 0.01
−22°	−72°	0.52 ± 0.02
−22°	−22°	0.60 ± 0.01

As some of the interpretations might be influenced by the viability of the yeast cells, determinations were made of the live-cell count in standard yeast suspensions. A diluted portion (approximately 10^6 cells/ml) was spread on a flat surface of potato dextrose agar and division of individual cells was followed microscopically. At least 98% of the original cells were found capable of dividing at least to the 4-cell stage, and presumably they would ultimately form macrocolonies under proper growth conditions. Thus no more than 2% of the cells in the standard suspensions are non-viable.

Survival fractions were determined for yeast exposed to the freezing technique used in the calorimeter determinations; for 60-minute exposures at −10°, −22°, −45°, and −72° these were 1.06 ± 0.05, 0.94 ± 0.05, 0.85 ± 0.05, and 0.39 ± 0.03, respectively, when compared to unexposed samples.

DISCUSSION

Several other investigations have been carried out to determine the fraction of the water of an organism frozen at various subfreezing temperatures. Recent examples are KANWISHER's[20] investigations on several molluscs in which he found that as much as 76% of the total water of the organism may freeze apparently without harmful results and the studies of SCHOLANDER *et al.*[12] on a chironomus larva and masses of lichen in which over 95% of the total water was found to be frozen at −30° C. We know of no investigations that have been concerned with determining the fraction of cellular water that is frozen when unicellular organisms are exposed to freezing temperatures.

The shape of the experimental curve in the temperature region down to −22° is generally expected (Fig. 1). As the temperature is lowered and freezing occurs the equilibrium salt concentration is increased. One might expect that all of the cellular water (as measured by evaporation to dryness techniques) would not be available for freezing; that part strongly associated with the cellular components, the bound water fraction, would not be available for freezing[12,20,21]. Thus the F value would not be expected to reach the value of the overall water content of the cell (in this case $1 - a = 0.69$), even though the temperature is low enough to freeze all but

References p. 86/87.

a very small fraction of the unbound water. At any point on the curve, as indicated in the figure, the water is divided into three phases: (1) an ice phase, (2) a non-aqueous phase (the dry weight fraction), and (3) the non-frozen water phase. The non-frozen water phase can in turn be divided into two parts: the bound water part (incapable of being frozen under these conditions) and the free water part that is still liquid by virtue of its high salt concentration. As the temperature is lowered below $-22°$, one would expect the non-frozen water level to approach asymptotically a value such that the difference between this level and the total cellular water level (0.69) would be a measure of the bound water.

However, the F values that are computed do not give such a plateau or constant value for the non-frozen water, but instead, the amount of frozen water appears to decrease for temperatures lower than $-22°$. At the present we have two possible explanations for this. The first is that the bound water is not present in a fixed amount in the cell but changes with the conditions that obtain within the cell at different temperatures (*i.e.*, different internal salt concentrations). Variations in the bound water fraction have been reported[22]. At the present time we are not able to test this hypothesis further.

Another explanation, and one that we are able to treat in a somewhat quantitative fashion, is that the specific heat of the bound water is not that of water (*i.e.*, approximately 1 cal/g-C°). Our experimental curve for F has a peak value of 0.60 at approximately $-20°$. If we assume that this plateau value is associated only with bound water, it is possible to correct our results for any difference in the specific heats of bound water and ordinary water and to estimate the specific heat of bound water. This is done with our results by introducing two other parameters, g, the fraction of the cell that is bound water (assumed here not to change with temperature), and S^a_{bw}, the specific heat of this bound water. The corrected value F' can now be written as

$$F' = F + g \frac{(T_f - T_b)}{L + T_b} \frac{(S^a_w - S^a_{bw})}{(S^a_w - S^a_i)}$$

where F is the original computed value of the frozen fraction of the cell at a temperature T_b. By use of our first assumption that the F' values at $-22°$ and $-72°$ should be the same, and in the first approximation equal to 0.60, we can compute a value for S^a_{bw}. This value is then used to compute a new F' at $-22°$. Repeating this process twice, the results converge to the following values:

F'	(at $-22°$ and $-72°$ by method of procedure):	0.63 ± 0.01
g	(the bound water fraction of the cell):	0.061 ± 0.005
S^a_{bw}	(specific heat of the bound water):	0.26 ± 0.18 cal/gram C°

The curve corrected in this fashion is shown as curve B in Fig. 1. As a check, the values of g and S^a_{bw} obtained here were used to recalculate the value of F' at $-40°$. The recalculated value agrees with the assumed value of 0.63 within the experimental error.

The values of F' and g obtained above are not drastically different from those estimated from the uncorrected curve (0.60 and 0.09, respectively). The computed value of S^a_{bw}, even though there is considerable uncertainty in its value, is significantly different from that of normal water. The average specific heat of bound water in

References p. 86/87.

a 87.5% gelatin gel has been computed from the data of HAMPTON AND MENNIE[23] to be 0.56 over the temperature range from 25° to −78.5 °C. In view of the large uncertainties inherent in our calculation, our value is not to be considered different from the above. Certainly the practice of assigning a value of 1 to the specific heat of bound water ([24,25] and others) has little justification, either experimentally or theoretically. For example, PAULING has estimated that there is only a 15% increase in hydrogen bonding in the phase change from water to ice[26] in which the specific heat changes by a factor of 2. One would thus expect a decrease in the specific heat of bound water as a similar increase in hydrogen bonding is involved.

The above adjustment of data in which the assumption was made that the F' value plateaus at −22° is valid only if the total osmotic strength of the cellular interior is equal to or less than 0.16 molar; if this maximum value exists then no more than 1% of the cell would be salt solution at −22°. If ROTHSTEIN's data[18] of the composition of yeast is used and the assumption made that there is complete disassociation of all cellular constituents, a maximum value for the osmotic strength of 0.2 molar is obtained. Thus at temperature below −22° an insignificant portion of the cellular water is neither frozen nor bound.

The fact that the uncorrected F curve is lower at −72° than at −22° might lead one to believe that the conditions that exist at −72° are such as to change the cell irreversibly and somehow cause the observed difference. However, when suspensions were pre-cooled to −72° before being equilibrated in the bath at −22° prior to entry into the calorimeter, F values were obtained that were typical of the entry temperature and not of the preliminary treatment (Table III). Thus the cell has no memory of its previous treatment.

The additional hysteresis studies were done with cells at temperatures closer to 0° so as to negate the possibility that supercooling in a fraction of the population could influence the results obtained. Any role supercooling could play in these experiments is minimized by the results shown in Table III in which cells were cycled in various ways between −2.5° and −22°. It is interesting to note that the F values at −2.5° are considerably higher than one would deduce from Fig. 1. A possible explanation is that as freezing proceeds cellular constituents might be degraded into smaller components to such a degree that the osmotic pressure of the cell would be increased sufficiently to change the shape of the freezing fraction curve from that which would be expected if there were no degradation. Such a possibility has been discussed by CONWAY in connection with freezing curves obtained with mammalian cells[27].

Our results with the survival of yeast exposed to freezing temperatures invalidate the possibility that an abnormal degree of freezing in the non-viable cells might unduly affect these calculations. At −22°, 94% of the cells survive the freezing procedure while 91% of the total cellular water is frozen. Thus even if all of the cellular water in the 6% of the inactivated population were frozen, 90% of the cellular water in the surviving cells must be frozen. Thus it is possible for yeast cells to have at least 90% of their cellular water frozen and still survive. This does not mean, however, that this frozen water must be located within the cell.

Work to be published in detail elsewhere allows us to speculate on the position of the frozen cellular water. It has been shown[28] that the sensitivity of haploid yeast to X-rays in the temperature region from 0° to −30° C depends critically on the

References p. 86/87.

phase state of the cell suspension during irradiation, whether liquid (non-frozen) or solid (frozen); cells in the liquid phase are more easily inactivated presumably because of the ability of diffusible intermediates produced on the irradiation of the cellular water to move within the liquid phase cell and react with the radiosensitive cellular regions. WOOD AND TAYLOR[29] have found, however, that cells irradiated at $-72°$ in the frozen state have the same radiosensitivity as those irradiated in the liquid state and are approximately 3 times as sensitive as those irradiated at $-33°$ C, frozen. Furthermore, cells cooled first to $-33°$ and then irradiated at $-72°$ display the same sensitivity as those irradiated at $-33°$. Since the same degree of overall cellular water freezing exists in all these cases, the position of ice formation might be expected to be of importance. A plausible picture is that when the cells are originally frozen at $-72°$ the freezing rate is sufficiently high to cause internal ice formation, while at the slower freezing rates ($-33°$) the cell is effectively dehydrated and ice formation is external. This general dependence on freezing rates has been demonstrated by the studies of MERYMAN AND PLATT with liver tissue[13]. The difference in the radiation response can be explained by assuming that toxic materials are produced by radiations in frozen water and effectively trapped *in situ* by virtue of the probably low diffusion rates of the solid phase. When thawing occurs in the fast-frozen cells (ice formation interior) the toxic materials are spatially situated similarly to those produced by irradiation in the liquid phase. However, in slow-frozen cells (external ice formation), the toxic materials released on thawing are effectively diluted by the water of the external medium and the probability of their inactivating the cell is decreased. It is not possible to assess a value to the amount of internal and external ice formation other than by saying that a radiobiologically significant amount of water is internally frozen in fast-frozen cells.

SUMMARY

1. A model is formulated which allows a calculation to be made from calorimetric data of the fraction of the yeast cell that is frozen as a function of sub-freezing temperatures. This model is independent of the position of the frozen water, whether intracellular or extracellular.

2. Our normal yeast cell contains 69% total water. As the temperature is lowered below 0° C a progressive amount of this is frozen until at $-22°$ approximately 87% of this total water is frozen. In the region from $-22°$ to $-72°$ the total water frozen decreases to 74%. These calculations are based on the assumption that the specific heat of the non-freezable water (bound water) is the same as that of normal water.

3. It is possible to adjust the computed curve so that the frozen fraction of cellular water does not decrease with temperature below $-22°$ C by the assumption that the specific heat of the bound water component is not 1. With this assumption, the fraction of the cellular water that is frozen over the range from $-22°$ to $-72°$ is 0.91. Thus 9% of the total water is considered bound. The specific heat of bound water that emerges from these calculations is 0.26 ± 0.18 cal/gram C°.

4. Data on the survival of yeast under these freezing conditions show that cells can survive even though as much as 90% of their cellular water is frozen.

5. Certain radiobiological data indicate that at least a portion of this frozen water is located within the cell.

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Received February 5th, 1957

INFECTIVITY OF VIRAL NUCLEIC ACID*

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Methods have been described for the isolation of nucleic acid and protein from tobacco mosaic virus (TMV), yielding preparations which combined under suitable conditions to form particles resembling the original virus in physico-chemical and pathogenic respects[1]. Evidence has also been presented that "mixed" virus could be produced from protein and nucleic acid derived from different strains of TMV, and it has been shown that the progeny of such virus always resembles that strain which has supplied the nucleic acid, both in regard to symptomatology and chemical composition[2]. Thus the ribonucleic acid appeared to be the prime genetic determinant of a plant virus, just as the deoxyribonucleic acid is believed to be in bacterial viruses. Furthermore,

* Aided by a grant from the National Foundation for Infantile Paralysis and Research Grant No. C-2245 from the National Cancer Institute of the National Institutes of Health, Public Health Service.

References p. 96.