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THE ROLE OF INTRACELLULAR FREEZING IN THE DEATH OF CELLS COOLED AT SUPRAOPTIMAL RATES[1]

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I. INTRODUCTION

Cooling velocity is one of the major factors that determines whether viable cells can be frozen to temperatures that permit indefinite storage. Cooling either too slowly or too rapidly tends to be damaging. Figure 1 (F-1) shows examples from our laboratory of the existence of optimum cooling rates for mouse marrow stem cells, yeast, and human red cells. Others have also been published (e.g., Nag and Street, 1963; McGrath et al., and Thorpe et al., 1975). ~~McGrath et al.'s (——) data are shown in~~ (F-2) Although all ~~four~~ three curves are inverted "U's", they show large differe in the numerical value of the cooling rate that produces maximum su. namely, 1.6, 7, ~~20,~~ and 200°C/min in stem cells, yeast, ~~HeLa cells,~~ cells, respectively. These differences are of mechanistic significa But they are also of practical importance. Without knowledge of the curve of survival versus cooling rate. a finding of low survivals co mean that a particular cell has been cooled at a suboptimal rate or supraoptimal rate, and the remedies in the two cases are decidedly d

The existence of an optimum implies that the causes of injury at optimal rates are fundamentally different from the causes of injury suboptimal rates. I shall restrict my comments to the former.

II. PHYSICAL EVENTS DURING FREEZING

The physical events occurring in cells during freezing are depicted schematically in Figure 2. Down to about -5°C, the cells and their surrounding medium remain unfrozen both because of supercooling and because of the depression of the freezing point by the protective solutes that are present. Between -5° and about -15°C, ice forms in the external medium (either spontaneously, or as a result of seeding). But the cell contents remain unfrozen, and supercooled, presumably because the plasma membrane blocks the growth of ice crystals into the cell (Mazur, 1965). The supercooled water in the cells has by definition a higher chemical potential than that of water in partly frozen solution outside the cell, and in response to this difference in potential, water flows out of the cell and freezes externally. The subsequent physical events in the cell depend on cooling velocity. If cooling is sufficiently slow (upper right), the cell is able to lose water rapidly enough by exosmosis so that the resulting concentration of intracellular solutes eliminates supercooling and maintains the chemical potential of intracellular water in equilibrium with that of extracellular water. The result is that the cell dehydrates and does not freeze intracellularly. But if the cell is cooled too rapidly (bottom and center right), it is not able to lose water fast enough to maintain equilibrium; it becomes increasingly supercooled, and eventually freezes intracellularly.

(F-3)

Quantitative Theory

In 1963 ( ) I suggested that these qualitative statements could be couched in quantitative terms. More specifically, the suggestion was that the rate of exosmosis of water during freezing could be described by four simultaneous equations, namely,

$$dV/dt = (L_p ART \ln p_e/p_i)/v_1^o \qquad [1]$$

where V is the volume of cell water, t is time, L_p the permeability coefficient for water (hydraulic conductivity), A the cell surface area, R is the gas constant, T is temperature and v_1^o the molar volume of water. The symbols p_e and p_i refer to the vapor pressure of extracellular and intracellular water, where

$$d \ln(p_e/p_i)/dT = L_f/RT^2 - [n_2 v_1^o/(V + n_2 v_1^o)V]dV/dT \qquad [2]$$

~~T is temperature,~~ Here, n_2 is os moles of solute, and L_f is the latent heat of fusion. ~~R is the gas constant.~~

Time and temperature are related by the cooling rate, which, if linear, is given by

$$dT/dt = B \qquad [3]$$

and finally, L_p is related to temperature by the expression

$$L_p = L_p^g \exp[b(T - T^g)] \qquad [4]$$

where L_p^g is the permeability coefficient to water at a known temperature, T^g, and b is its temperature coefficient.

To avoid intracellular freezing, the water content of the cell given by Eqs. 1-4 must, before reaching the intracellular nucleation temperature, ~~be reduced to~~ have approached the equilibrium water content given by

$$\ln[V/(V + n_2 v_1^o)] = (L_f/R)(1/273 - 1/T). \qquad [5]$$

~~The analysis was based on a number of simplifying assumptions. Since then both the equations and a number of the assumptions have been examined by Mansoori (1975), Silvares et al. (1975), and Pushkar (19). Their refinements perturb the calculated results somewhat, as we shall see shortly, but not enough to alter their quantitative significance with respect to the response of cells during cooling.~~

The quantitative expressions permit one to calculate the extent of supercooling in cells as a function of cooling rate, provided one knows or

can estimate the permeability of the cell to water (L_p), the temperature coefficient of permeability (b), the O_s moles of solute in the cell initially, and the ratio of the cell surface to volume. The calculations are usually presented as plots of the relative water contents of cells as a function of temperature. Two previously published examples are shown in Figure 3 ~~6~~. The extent to which cells cooled at a given rate become supercooled is the extent to which a given curve departs from the equilibrium curve. For example, the curves for yeast cells cooled at 100°C/min indicate they become supercooled 15 degrees when the temperature has dropped to -16°C. 3 ~~4~~ (F-~~6~~)

These calculated curves permit one to estimate the probability of intracellular freezing. Cells which have dehydrated to equilibrium prior to reaching their nucleation temperature will have a zero probability of undergoing intracellular freezing. Cells which are still supercooled extensively when cooled to their nucleation temperature will have a probability approaching 1 of undergoing intracellular freezing. Figure 4 ~~5~~ ~~7~~ gives such estimated probabilities for yeast and human red cells, and compares them with the observed cell survival. The nucleation temperature was assumed to be -15°C (see later discussion). Clearly the drop in survival at supraoptimal rates coincides closely with an increase in the probability of intracellular freezing. 4 ~~5~~ (F-~~7~~)

Experimental Test of Predicted Intracellular Freezing and of Its Presumed Relation to Cell Death

The development of the freeze-cleaving technique for electron microscopy and the ~~recent~~ ~~construction~~ development of an optical microscope incorporating precise control of cooling rate (Diller and Cravalho, 1970) have

(Intracellular ice is identified by the response of the cleaved surface to etching and by the changes in cell morphology associated with warming [See below].)

recently permitted an experimental tests of the presumed relation between the loss in viability and the occurrence of intracellular freezing, and they have permitted an experimental test of the validity of the theoretical calculations. Figure 4 showed that both the loss in viability of yeast and the rise in the probability of intracellular freezing occur between cooling rates of 10° and 100°C/min. In Figure 5 we see electron microscopic (F-7) evidence for intracellular ice in yeast cooled at 55°C/min but no evidence for ice in yeast cooled at 6°C/min. Close correspondences between the cooling rates that produce loss in survival and the cooling rates that produce intracellular ice have also been observed in HeLa cells (Figure 6), (F-7) in human red cells in glycerol (Figures 7 and 8), (F-8) (F-9) and in hamster tissue culture cells (Bank, 1974; Mazur et al., 1972).

An especially elegant study on intracellular freezing versus cooling rate has been carried out recently with unfertilized mouse ova by Leibo, McGrath and Cravalho (1975) using the special low-temperature microscope developed at MIT. Figure 9 shows micrographs of ova cooled at 1.2, 2.4, (F-10) and 32°C/min. Intracellular freezing occurs at the latter two rates, but not at 1.2°C/min. The ovum cooled at 1.2°C/min can be seen to dehydrate markedly during cooling. Figure 10 compares the relation between the percentage of (F-11) unfertilized ova observed by Leibo et al. (1975) to undergo intracellular freezing at various cooling rates with the percentage of 1-cell fertilized ova that Whittingham et al. (1972) found to survive as a function of cooling rate. Again, the correspondence is close.

Table 1 summarizes data on the relation between cooling rate and (T-1) (1) predicted intracellular freezing, (2) observed intracellular freezing, and (3) observed cell killing. The major gaps in the table are for the calculated critical rates, and this stems chiefly from lack of information

on the permeability of cells to water (L_p) and its temperature coefficient (b).

The numerical values of the calculated and observed critical cooling rates for various cells vary over a range of several thousand. These differences are due chiefly to differences in L_p and b and to differences in cell volume or surface/volume ratio. The calculated effects of variations of these three parameters on cell water content versus temperature are illustrated in Figures ~~12~~ 11, ~~13~~ 12, and ~~14~~ 13.

(F-~~12~~ 11), (F-~~13~~ 12), (F-~~14~~ 13)

In general the correlation between the cooling rates that are predicted to produce intracellular freezing and those observed to do so, and the correlation between the cooling rates that produce intracellular ice and those that produce cell death, are good. The major discrepancy is for the human red cell frozen in saline: The cooling rate calculated to produce intracellular ice (5900°C/min) agrees well with the cooling rate observed to produce 50% killing (4500°C/min), but both exceed by about a factor of 6 the cooling rate observed by Diller (1975) to produce intracellular ice in half the cells. This discrepancy makes it desirable to examine more closely the assumptions underlying the theory, and the possibilities for error in the experimental measurements.

Further Consideration on the Theoretical Aspects of Intracellular Freezing

Validity of assumptions. The original analysis ( ) was based on a number of simplifying assumptions. Since then both the equations and some of the assumptions have been examined by Mansoori (1975), Silvares *et al.* (1975), and Pushkar (1976). The chief assumptions are listed in Table 2. Most appear to introduce little error. The parameters which have major influence on the kinetics of water loss, and which have the highest likelihood of being in error, are ~~the permeability of the cell to water~~ (L_p) and ~~its temperature coefficient~~ (b). The appropriate values at subzero

temperatures are not known, so we are still forced to rely on extrapolation from experimental data at above zero temperatures. Levin et al. (1975) have questioned the soundness of the extrapolation on theoretical grounds. They have developed a model based on absolute reaction rate theory, which indicates that it is unlikely that a single rate-limiting process dominates water transport in red cells as their temperature is lowered. Their model suggests that $\hat{Lp}$ at subzero temperatures will be lower than that predicted by extrapolation from data obtained above 0°C.

Another possible source of error in the numerical values of $\hat{Lp}$ could result from the changes in osmolality produced by freezing. The original analytical treatment was proposed before Rich et al. (1968) reported that $\hat{Lp}$ in red cells decreases with increasing osmolality. Silvares et al. (1975) have found that when this effect is incorporated into the kinetic equations, it produces an appreciable lessening of the extent of cell dehydration at a given cooling rate and leads to a critical cooling velocity for the human red cell that is about half of the value of 5900°C/min listed in Table 1. On the other hand, there is debate on the correctness of Rich et al.'s conclusions. Farmer and Macey (1970) and Outhred and Conlon (1973) find that $\hat{Lp}$ is independent of the osmolality of the suspending medium. But the latter's work in turn has been criticized by Forster (1971), who fully supports the findings of Rich et al.

Another inherent assumption in the theoretical treatments has been that the system of cells and external medium can be considered two homogeneous compartments separated by a semipermeable membrane. Pushkar et al. (1976) suggest that the assumption of homogeneity with respect to chemical potentials can introduce errors in cells like red cells with very high water permeabilities. They suggest that the neglect of concentration gradients and mass transport within the red cell also causes an overestimation

of the cooling rates required to introduce intracellular ice; however, they do not indicate the magnitude of the error.

Related to this point is the fact that cells contain many organelles which themselves are delimited by semipermeable membranes. It is quite conceivable, therefore, that diffusional lags can occur in cells and lead to intracellular freezing within certain organelles, but not within others. The existence of such lags can be demonstrated theoretically in systems of series of membranes, even those with relatively low permeabilities. One such simplified example is shown in Figure ~~15~~ 14 for a system of three concentric layers of membrane-delimited elements. There is microscopic evidence for differential intracellular freezing within cells, e.g., Nei (1976). (F-~~15~~ 14)

Despite the lack of information on water permeabilities at subzero temperatures and despite the underlying simplifying assumptions, the quantitative analyses seem fundamentally correct and seem reasonably capable of quantitatively predicting the extent to which cells will become supercooled during cooling. The next step is to translate the estimates of supercooling into estimates of the number of cells which will undergo intracellular freezing.

Estimation of the Probability of Intracellular Freezing

Qualitatively one can state that in order for intracellular freezing to occur the cell (1) must contain unbound (i.e., freezable) water, (2) that water must be supercooled, and (3) the temperature must be below that required for its nucleation. Figure ~~16~~ 15 shows the calculated relation between the probability of intracellular freezing (P) and the cooling rate for assumed nucleation temperatures (F-~~16~~ 15)

of -5, -7.5, -10, and -15°C. P has been assigned a value of 0 when the degree of supercooling (ΔT) is <2°C or when the fraction of the normal water content (V/V_i) drops below 0.15. P has been assigned a value of 1 when $\Delta T \gtrsim 2°$ and $V/V_i \gtrsim 0.15$. This last assumption about V/V_i has some experimental support, for approximately that fraction of the water in the few cells where it has been measured is incapable of freezing at any temperature (Wood and Rosenberg 1957; Souzu et al., 1961). Fortunately, the predictions as to the cooling rate dependence of the probability of intracellular freezing are rather insensitive to the values of ΔT and V/V_i selected. However, the probability of intracellular freezing is clearly sensitive to the value assumed for the nucleation temperature.

Nucleation of Supercooled Intracellular Water

A number of investigators had observed microscopically that, when precautions are taken to reduce or eliminate extracellular ice, cells undergo intracellular freezing at lower temperatures than when they are in contact with extracellular ice. On the basis of these observations, I made the suggestion (Mazur, 1965) that intracellular freezing results not from spontaneous crystallization within the cell but from seeding by extracellular ice crystals that have grown through aqueous channels in the cell membrane. The earlier microscope observations showed that, even in the presence of extracellular ice, intracellular nucleation generally occurred only below temperatures of -10°C or so, observations that suggested that the membrane was a barrier to the passage of ice crystals at temperatures above -10°C, but ceased to be a barrier at lower temperatures. The proposed explanation of these barrier properties was that only very small crystals can grow through the presumed ~~angstrom~~ nanometer-sized aqueous channels in the membrane. Small crystals have small radii of curvature,

and as is well known ice crystals with small radii of curvatures melt well below 0°C because of their high surface energies. Perhaps, then, only below about -10°C will extracellular crystals of suitably small size be stable enough to pass through the cell membrane and nucleate the supercooled cytoplasm within.

Additional information on nucleation has accumulated since that time. Mazur and Miller (1967, 1971) showed that yeast cells and various proteins are incapable of nucleating water supercooled to -20°C. Rasmussen et al. (1975) have gone considerably further. By preparing emulsions of water droplets containing yeast cells, they have been able to keep the water unfrozen to -40°C; and they have shown that, when the surrounding water is kept supercooled, the yeast cells also remain unfrozen and supercooled down to -40°C. Leibo et al. (1975) have shown that mouse ova in 1 M DMSO also freeze intracellularly only below about -40°C, and the nucleation temperature ~~appeared~~ was independent of cooling rate over the range of 2° to 30°C/min.

The temperature of -40°C is the homogeneous nucleation temperature for water (i.e., the temperature at which water freezes in the absence of any foreign, heterogeneous, nucleating agents) (Toscano et al., 1975). If the water in yeast and ova behaves like ordinary water, we can conclude that these cells are freezing by homogeneous nucleation and that they therefore contain no intracellular heterogeneous nucleating agents. If intracellular nucleating agents are absent in these two diverse cell types, there is no reason to suppose them to be present in other cells. Accordingly, if one observes intracellular freezing at temperatures well above -40°C ~~(which is the usual case)~~; it seems reasonable to conclude that it was produced by extracellular nucleating agents, the most likely of which is extracellular ice itself.*

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Observations on nucleation temperatures in cells are beginning to accumulate. As mentioned, older microscopic observations on cells frozen in water or dilute salt solutions generally show cell nucleation between -5° and -15°C when external ice is present. More recent observations are in agreement. Asahina and Emura (1966) report intracellular nucleation of ascites cells between -10° and -18°C; McGrath (1975), Luyet and Pribor (1965), and Gupta (1975) observe it above -20°C in HeLa cells and human red cells; and Sherman (1969) and Leibo (unpublished data, 1976) observe intracellular nucleation in mouse

ova at about -10°C when the ova are suspended in buffered saline. On the other hand, as was shown in the photographs in Figure ~~10~~ 9, Leibo et al. (1975) find that in 1 M DMSO the nucleation of ova does not occur until about -40°C! Why the presence of additive produces this dramatic drop in the nucleation temperature ~~of intracellular nucleation~~ remains an intriguing and significant question.

Regardless of the cause, differences in the nucleation temperature will cause major differences in the cooling rate required to produce intracellular freezing (Figure ~~16~~ 15). Leibo et al. (1975) have calculated, for example, that, if the nucleation temperature of sea urchin eggs is -40°C, the critical cooling velocity will be about 1.5°C/min; Mazur (1963) has calculated that if the nucleation temperature is -10°C the critical velocity will be a few tenths of a degree per minute.

As shown in Figure ~~16~~ 15, decreasing the assumed nucleation temperature of red cells from -15°C to -5°C causes the critical cooling velocity to drop by a factor of more than 5, from ~5000 to <1000°C/min. Such a drop would eliminate the discrepancy noted in Table 1 between the cooling rates calculated to produce intracellular ice and those observed to do so. On the other hand it would still not account for the discrepancy between the cooling rate observed to produce intracellular ice and the cooling rate observed to cause hemolysis. One explanation of this latter discrepancy might be that intracellular ice is not the cause of hemolysis, but I believe there are other more likely explanations. The intracellular freezing data and the survival data were obtained in different laboratories using sample containers with very different geometries. There is some indication that these sorts of differences may influence the relation between cooling rate and observed ice formation, for example, estimates of the cooling rates required for

intracellular ice formation that are derived from electron microscopy of freeze-substituted or freeze-cleaved red cells are generally above the 850°C/min reported by Diller (1975), namely, values of 1000 to 10,000°C/min (Rapatz et al., 1963; Mazur, 1966; Nei, 1976).

Another possible explanation of the discrepancy has to do with warming rate. There is growing evidence that rapid warming can "rescue" cells that contain intracellular ice. It apparently does so by reducing or preventing the recrystallization of intracellular ice.

The Contribution of Recrystallization to Injury from Intracellular Freezing

Ice crystals in rapidly cooled cells are small because crystal size is inversely related to cooling rate (Van Venrooij et al., 1975; Karrow and Shlafer, 1975). Small crystals, however, have higher surface energies than large crystals, and as a consequence they will grow or agglomerate if warming is slow enough to permit sufficient time for this to occur (Mazur, 1966). The agglomeration can be lethal. On the other hand, crystal growth does not occur if warming is sufficiently rapid because there is insufficient time for growth to occur before the melting point is reached. (Slow freezing also produces large crystals; but in that case the crystals are located outside the cell and, hence, are innocuous.)

The lethality of recrystallization has been demonstrated most clearly in yeast and higher plant cells. Yeast cooled at rates far above optimal will survive if warmed very rapidly, but they do not survive if warmed slowly (Mazur and Schmidt, 1968; Moor, 1964; Anderson et al., 1966; MacKenzie, 1970). The bottom half of Figure ~~17~~ 16 shows the results of warming the cells slowly to various subzero temperatures and then completing the warming and (F-~~17~~ 16)

thawing rapidly. Survival begins to drop when slow warming is allowed to progress above -40°C. The upper panel of the figure shows that it is also above -40°C when one first begins to observe the recrystallization of the intracellular ice that formed during rapid cooling.

Plant cells behave similarly. Sakai and Otsuka (1967) made an electron microscopic examination of mulberry parenchymal cells cooled at various rates and freeze-substituted, and demonstrated the presence of intracellular ice crystals when the cooling rates exceeded 10°C/min. (Mohr and Stein (1968) have reported a similar value for intracellular ice formation in tomato parenchyma.) Samples cooled very rapidly to -60°C or below showed no visible intracellular ice and were 100 percent viable after rapid warming. But intracellular ice was present, for when similar samples were warmed from -60° to -30°C for 10 min they showed evidence of large intracellular ice crystals. Parallel samples thawed from -30°C contained no viable cells.

The "Rescue" by Rapid Warming of Cells Containing Intracellular Ice

The rescue by rapid warming of cells that contain intracellular ice is not restricted to yeast and mulberry cells.

Figure ~~18~~ 17 shows that the optimum cooling rate for hamster cells shifts to higher values as the warming rate is increased. Bank (1974) reports on the basis of freeze cleaving studies that intracellular freezing in this strain of cells occurs at a cooling rate of $\simeq$ 50°C/min. This is close to the optimum for cells warmed at 27°C/min, but below the optimum for cells warmed at 500°C/min. Apparently, then, warming at 500°C/min reduced recrystallization sufficiently to permit the survival of the majority of cells even when they were frozen at $\gtrsim$ 100°C/min and presumably contained intracellular ice. Asahina and his colleagues (1966, 1968, 1970) have given other examples of the phenomenon of rescue in ascites cells.

Although direct microscopic evidence for a correlation between death and recrystallization during slow warming is available for only a few cells, there is considerable evidence that, when cells are cooled at supraoptimal rates, survivals improve dramatically with increasing warming rates. This is shown in Figures ~~19~~ 18 and ~~20~~ 19 for yeast and human red cells. Note that in these two cases survivals are relatively high and much less influenced by warming rate when the cells have been cooled at rates somewhat below optimum. This of course is consistent with the view that such cells contain little if any intracellular ice. Quite comparable results have been reported for mulberry cells by Sakai and Yoshida (1967). (F-~~19~~ 18) (F-~~20~~ 19)

Returning to human red cells, then, it may be that there is no true inconsistency between Diller's observation that intracellular freezing occurs at 850°C/min and Rapatz et al.'s (1968) observation that the drop in survival occurs at cooling rates above 3000°C/min. The cells cooled at rates of 800° to 3000°C/min may have been rescued by the rapid warming that Rapatz employed.

Interestingly, the discrepancy between the rates producing a drop in survival and those producing visible intracellular ice is much less evident in cells frozen in 1.5 M or 2 M glycerol (Figures 8, 9; Table 1) than in cells frozen in saline. But even in glycerol a comparison of Figures 8 (rapid warming) and 9 (slow warming) shows that rapid warming permits cells to survive higher cooling rates and supports the suggestion that it "rescues" some intracellularly frozen cells.

The mention of glycerol introduces the question of what, if any, is the influence of protective additives in intracellular freezing?

The Influence of Protective Additives in Intracellular Freezing

The main conclusion is that "protective" additives like glycerol and dimethyl sulfoxide do not in fact protect against intracellular freezing -- they protect only against slow-freezing injury. Their inability to protect rapidly cooled cells is illustrated in Figures ~~21~~ 20 and ~~22~~ 21, which show that the introduction of increasing concentrations of glycerol shifts the optimum cooling rate to lower values. The presumption is that, rather than protecting cells from intracellular freezing, glycerol either is without effect (Figure ~~21~~ 20) or it makes the cells susceptible to intracellular freezing at even lower rates (Figure ~~22~~ 21). One interpretation of Figure ~~21~~ 20 is shown schematically in Figure ~~23~~ 22. Increasing concentrations of glycerol are assumed to decrease the sensitivity to slow-freezing injury ("solution effects"), i.e., they shift the curves to the left -- but they are assumed to have no effect on the sensitivity of the cells to intracellular freezing.

(F-~~21~~ 20)
(F-~~22~~ 21)
(F-~~23~~ 22)

The suggestion that Figure ~~22~~ 21 represents a case in which the presence of additive makes the cells susceptible to intracellular freezing at even

lower cooling rates has been directly confirmed recently by Diller and colleagues (1975). They find that in the absence of glycerol intracellular freezing in human red cells occurs at cooling rates between 840° and 850°C/min, but in the presence of 1.5 M glycerol (see Figure 7) it occurs between 200° and 800°C/min. A similar downshift in the optimum cooling rate for lymphocytes with increasing concentration of DMSO has just been reported by Thorpe et al. (1976).

What could account for it? One possibility is that additives, perhaps simply by virtue of their viscosity, decrease the rate at which water can leave the cell during cooling. The slower the exosmosis of water, the slower must be the cooling to avoid intracellular freezing. On the other hand, if additives depress the nucleation temperature of cells, as suggested by the findings of Leibo et al. (1975) for mouse ova, one would expect a higher permissible cooling rate. This paradox remains to be resolved.

Influence of Supercooling of the Extracellular Medium on the Critical Cooling Rate

The theory discussed above assumes that the cells and surrounding medium are at their true freezing point prior to the calculation of the effects of subsequent cooling. This is equivalent to saying that the ice and water in the external medium and the water in the cells all have the same chemical potential at time zero. The fact that in practice the medium initially supercools causes no serious problem in the theoretical analysis. There is no difference in the chemical potential of water ($\Delta\mu_w$) across the cell membrane as long as both extra- and intracellular water are supercooled. When the external water finally begins to freeze, a transient $\Delta\mu_w$ is established across the membrane, but the gradient quickly vanishes as the released latent heat of fusion causes the temperature of both cells and medium to rebound quickly to near the true freezing point.

The amounts of water in the cell after the rebound and the chemical potentials of water in the cell and the medium are about the same as they would have been in the absence of supercooling.

Although this sort of supercooling introduces no serious conceptual problem, it can cause injury in cases where samples are immersed in a fluid bath that is progressively cooling. During the time the sample temperature has rebounded and is at its freezing plateau, the bath temperature continues to drop. Hence by the time the plateau is essentially completed, the now greater temperature differential between the sample and the bath causes the sample to cool rather rapidly back to the bath temperature. In some cases the cooling rate can be high enough to cause lethality, presumably as a result of the formation of intracellular ice (Mazur and Schmidt, 1968).

Diller (1975), however, has described another type of supercooling which can only be produced under highly specialized conditions in which heat can be withdrawn from the sample quickly enough to prevent thermal rebound when the external medium nucleates. Such conditions are met in the MIT-type microscope. The result with that instrument is that a system of supercooled cells in supercooled medium at, say, -10°C, where $\Delta\mu_w = 0$, is nearly instantly transformed isothermally into a system consisting of fully supercooled cells in frozen medium at -10°C, ~~and~~ a system in which $\Delta\mu_w$ becomes the difference between the chemical potentials of ice and supercooled water at -10°C.

Diller (1975) has shown that this special type of supercooling produces a major decrease in the cooling rates observed to produce intracellular ice in human red cells; i.e., with supercoolings of 0, -5, and -12°C, the critical cooling rates are 845, 800, and 11°C/min. I would suggest, however, that the phenomenon fundamentally has little to do with cooling rate <u>per se</u>. It

has more to do with how close the cell is to its intracellular nucleation temperature at the instant the isothermal freezing of the external medium occurs. As the fall in temperature resumes, the fate of the cell will depend on whether it can dehydrate to equilibrium before reaching its nucleation temperature or whether it reaches its nucleation temperature before it has fully dehydrated. The lower the temperature of the "isothermal" transition, the less time would be required for cells to cool to their nucleation temperature (which is > -20°C for red cells) and the slower would have to be the cooling to permit dehydration before that point, and thereby avoid intracellular ice formation. In fact, one might predict that if the "isothermal" transition were effected below the cell nucleation temperature, intracellular freezing will occur even if the cooling rate is zero or even if the sample actually warms. The following data support this view. When yeast cells are immersed in a bath at -15°C, 97% survive when the system remains supercooled; but only 27% survive when the external medium freezes at -15°C, even though the freezing in this case causes an immediate temperature rebound to near 0°C (Mazur, 1961). On the other hand, if supercooling is kept to a minimum by seeding near 0°C and the yeast are cooled slowly enough to -15°C to preclude intracellular ice, some 80% survive (Mazur and Schmidt, 1968).

Conclusions on Intracellular Ice as the Cause of Injury in Rapidly Cooled Cells

In summary, although only a few cell types have been studied in detail, it seems increasingly certain that death in cells cooled at supraoptimal rates is the result of the formation of intracellular ice crystals during cooling and their growth by recrystallization during warming. The conclusion is no different than that reached over a decade ago (Mazur, 1963, 1965). What is different is that it is now supported by quantitative experimental data.

To state that the killing of rapidly cooled cells is due to intracellular ice is not the same as stating that intracellular ice formation is invariably lethal. I have already given several examples where it is not lethal (e.g., Figure 18). Others have been reported by Asahina and his colleagues (Asahina et al., 1968, 1970; Shimada and Asahina, 1975) by Sakai (1971), and Sherman and Kim (1967).

Two-Step Cooling

Luyet and Keane (1955), Taylor (1960), and Sakai (1960, 1965) noted that a variety of cells would survive rapid freezing to -70° or below if the rapid freezing were carried out in two steps separated by a brief holding period at an intermediate temperature. The cells included bull sperm, chick skin, and plant parenchyma. The two-step freezing of mammalian cells (chiefly hamster cells and lymphocytes) has now been investigated in considerable detail by Farrant and his colleagues (Farrant et al., 1974; Walter et al., 1975; McGann and Farrant, 1976; Farrant, 1976). The intermediate holding temperature is critical. ~~As shown in Figure 24,~~ Death results if the temperature is too high or too low.

Sakai seems to have been the first to propose a partial explanation of the phenomenon. He suggested that the initial cooling to -30°C, for example, in conjunction with the brief holding period provides sufficient time to permit the cells to equilibrate by dehydration and extracellular freezing rather than by intracellular freezing; and he proposed that -30°C is sufficiently low for nearly all freezable water to be removed from the cells by exosmosis. As a result, no intracellular freezing can occur

during the subsequent rapid cool to -196°C. There are indications, however, that the cooling rate in the first cooling step may often be above the value that would have been required to produce intracellular ice if cooling had been carried to still lower temperatures (Walter et al., 1976; Farrant, 1976). The other requirement for success, therefore, may be that the intermediate holding temperature be above the intracellular nucleation temperature. According to this view, depicted schematically in Figure ~~25~~ 23, most of the dehydration of the cell would occur during the holding period at the intermediate temperature. If that period were long enough, the cells would have lost all freezable water prior to the second rapid cool to < -70°C. Walters et al. (1975) and Farrant (1976) have electron microscope evidence on freeze-substituted cells which directly supports this view. (F-~~25~~ 23)

Mechanism of Injury from Intracellular Ice

The demonstration that when death occurs it is due to intracellular ice says nothing about the mechanism by which intracellular ice injures. Although it is generally agreed that injury from extracellular ice is not a result of direct physical effects of the ice on cells, I have suggested that injury from intracellular ice and its growth by recrystallization is a direct physical consequence of the ice, and have suggested that the effect is on intracellular membranes (Mazur, 1965, 1966). These suggestions remain speculative. Farrant et al. (1976) have recently proposed an alternative speculation, namely, that injury from intracellular ice results from osmotic events, especially when water appears during thawing. Assessment of this view will have to await its being formulated in terms permitting quantitative analysis or experimental test.

Whatever its cause, injury from intracellular freezing can be eliminated by the simple expedient of cooling cells slowly enough to eliminate the formation of internal ice in the first place. The problem is that cooling rates that are slow enough to eliminate intracellular ice may also be slow enough to injure cells by events that have nothing to do with the location of the ice -- events associated with the major alterations in extra- and intracellular solutions produced by the removal of liquid water during freezing, and which, therefore, have been termed "solution effects". Understanding the nature of these solution effects and their role in freezing injury now represents the major challenge in modern cryobiology.

FOOTNOTE

*This point of view is somewhat at odds with that proposed recently by Toscano et al. (1975). They argue on theoretical grounds that in red cells cooled at rates of less than 10,000°C/min intracellular homogeneous nucleation would be expected to occur at about -60°C, not -40°C. If the nucleation occurs heterogeneously, their calculations indicate nucleation temperatures ranging from -50° to -3°C depending on the contact angle between the catalyst and water, the radius of the catalyst, and the cooling rate. Only the third is known. They suggest, further, that intracellular proteins (e.g., hemoglobin) could be the intracellular nucleating agents.

A number of comments are in order:

1. They indicate that the cause of the 20° discrepancy between the homogeneous nucleation temperature of red cells and that of pure water is due "to the formation of a solid eutectic solution" [in the cells?], below -21°C. But, first, cytoplasm contains solutes like Ca^{++} and Mg^{++} which would be expected to suppress the eutectic points considerably. Indeed Sussman and Chin (1966) and Wikefeldt (1971) have shown by NMR that liquid water is present in muscle and blood down to -70°C. Second, it is not clear why the presence of a eutectic mixture would reduce the homogeneous nucleation temperature, since a eutectic by definition contains ice. Third, their arguments ought not to apply to yeast and mouse ova, since the cells in both cases underwent little or no dehydration at the temperature (-40°C) at which intracellular freezing was observed.

2. They propose that heterogeneous nucleation at -3° to -50°C could be initiated by either intracellular protein or extracellular ice. But as already pointed out, neither whole cells nor selected soluble proteins can nucleate supercooled water above -20°C, and apparently not above -40°C. Hence, it continues to appear more likely that the nucleating agent is extracellular ice *per se*. My suggestion that the ability of membranes to block such nucleation above about -10°C is due to the small radius of curvature of water filled channels remains just that -- a suggestion. However, Banin and Anderson (1975) have shown that the equation assumed to control ice crystal growth through small pores in membranes is nearly identical to the equation experimentally demonstrated to control the thickness of the unfrozen water layer at various subzero temperatures on a variety of powdered minerals.

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Table 1

Calculated and Observed Effects of Cooling Rates on Intracellular Freezing and on Cell Survival

Cell Type	Additive	Cooling Rates (°C/min) Yielding: 50% Intracellular Freezing (Calculated)	50% Intracellular Freezing (Observed)	50% Survival* (Observed)	References
1-Cell mouse ova	1 M DMSO	-	2.2	1.2	Whittingham et al. ( )
					Leibo et al. ( )
Yeast	None	30	6-55	20	Mazur ( )
					Bank and Mazur ( )
HeLa	None	-	55	70	McGrath et al. ( )
Hamster tissue culture	1.25 M Glyc.	-	≈55	~60	Bank ( )
					Mazur ( )
Human RBC	None	5,900	845	4,500	Diller ( )
					Rapatz et al. ( )
	1.5 to 2 M Glyc.	-	400	500	Diller et al. ( )
					Miller and Mazur ( )
					Rapatz et al. ( )

*50% of maximum at optimum rate.

Table 2

Assumptions Underlying Analysis of Kinetics of Water Loss during Freezing

Assumptions in Original Analysis ( )	Likely Magnitude of Error Introduced	Reference
1. Intracellular and extracellular solutions are ideal (obey Raoults Law)	Small	Mazur ( ); Mansoori ( ); Silvares *et al*. ( )
2. Ice and extracellular solution remain in equilibrium	Negligible	Mazur ( )
3. L_f is constant	Negligible	Mazur ( ); Silvares *et al* ( )
4. W_2 is constant	Small	Mazur ( ); Mazur *et al*. (1974)
5. Values of Lp and b can be extrapolated to subzero temperatures	Major	Mazur ( ); Silvares *et al*. ( ); Levin et al.
6. All mass transfer is due to water (i.e., reflection coeff., $\sigma_2 \approx 1$)	Small	Mazur ( ); Mazur *et al*. (1974)
7. Cell and medium are in thermal equilibrium	Negligible	Mazur ( ); Mansoori ( )
8. Constancy of A*	Small	Mazur ( ); Mansoori ( )
9. $L\hat{p}$ is independent of osmolality	Moderate	Silvares *et al*. ( )
10. Lack of intracellular gradients of $\hat{\mu w}$	Small to Moderate	Pushkar ( )

*Mansoori (1975) has questioned the ~~original~~ assumption that the cell surface area remains constant during osmotic shrinkage; however, allowing A to vary as the 2/3 power of volume has relatively little effect on the overall calculated kinetics of water loss. Further-e, the little evidence available favors the ~~former~~ assumption of constant area. This

sumption implies that a membrane will develop folds or pleats during osmotic shrinkage. Pertinent to this view is a recent study by Knutton et al (1976) on cultured mammalian cells which shows that isotonic ~~lettuce~~ cells contain folds in the form of microvilli, and that these microvilli disappear when the cells swell in hyposmotic media. Their calculations indicate that the surface area of the microvilli is sufficient to account for the observed increase in cell volume without any increase in overall surface area.

FIGURE LEGENDS

wing No.

22126-1
25430
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Figure 1. Survival of frozen-thawed mouse marrow stem cells (A), yeast (B), and human red cells (C) as a function of the cooling rate. The cells were suspended in water (yeast) or solutions of glycerol in saline (stem cells and red cells), frozen at controlled rates to ~ -75°C, transferred to liquid nitrogen (-196°C), and thawed rapidly at 500 to 1,000°C/min. Curves A, B, and C are redrawn from Leibo *et al.* (1970), Mazur and Schmidt (1968), and Miller and Mazur (1976).

31731

Figure 2. Survival of HeLa cells as a function of the cooling rate to -20°C. Redrawn from McGrath *et al.* (1975).

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Figure 3. Schematic of physical events in cells during freezing.

Figure 4. Calculated fraction of intracellular water remaining in (A) yeast cells and (B) human red cells as they cool to various temperatures at the indicated rates. From Mazur (1970).

31736

Figure 5. Effect of cooling rate on the survival and on the calculated probability of intracellular freezing in yeast and human red cells. The

survival data for yeast and red cells are from Mazur and Schmidt (1968) and Rapatz et al. (1968), respectively.

Figure 5 ~~8~~. Appearance of freeze-cleaved yeast cells after freezing
31743 to -196°C at 6°/min or 55°C/min. These cells were suspended in water. The cell labelled "standard freeze cleave" was suspended in 20% (w/v) glycerol and frozen at ~ 75,000°C/min. From Bank and Mazur (1973).

Figure 6 ~~9~~. Survival and intracellular freezing of HeLa cells as a function of the cooling rate to -20°C. Redrawn from data of McGrath et al. (1975).

Figure 7 ~~10~~. Effect of cooling rate on survival and on observed intracellular ice formation in glycerolated human red cells. Warming was rapid in the survival experiments. The data on survival are for cells equilibrated with 2 M glycerol and are from Rapatz et al. (1968), O, and from Miller and Mazur (1976), ●; the data on intracellular ice formation were for cells in 1.5 M glycerol and are from Diller et al. (1975).

Figure 8 ~~11~~. As in Figure 10, but warming was slow (1°C/min). Survival
31738 data are from Miller and Mazur (1976).

Figure 9 ~~12~~. Intracellular freezing in mouse ova in 1 M DMSO versus cooling rate. Photomicrographs from Leibo et al. (1975) and Leibo (1976).

Figure 10 ~~13~~. Effect of cooling rate on the survival of mouse ova and on the fraction of ova undergoing intracellular freezing. Survival data are from Whittingham et al. (1972). Intracellular freezing data are from Leibo et al. (1975) and Leibo (1976).

Figure 11. Calculated percentage of cell water remaining in a 6 μm diameter spherical cell as a function of temperature and of the permeability coefficient for water. The calculation assumed a cooling rate of 100°C/min and a temperature coefficient b of 0.0325 (Q_{10} = 1.4). From Mazur (1965).

Figure 12. Calculated percentage of cell water remaining in a 6 μm diameter spherical cell as a function of temperature and the temperature coefficient (Q_{10}) of the water permeability ($L\hat{p}$). [Note $b = 0.1 \ln Q_{10}$] The calculation assumed a cooling rate of 100°C/min and a value of $L\hat{p}^{g}$ = 0.15. E is the activation energy for permeation. From Mazur (1965).

Figure 13. Calculated probability of intracellular freezing in spherical cells of the indicated diameter. The probability is related to the extent to which the cell interior becomes supercooled relative to external ice during cooling. The parameters involved in the calculations are the osmolality of the isotonic cell (M), the permeability of the cell to water at a given temperature ($L\hat{p}^{g}$), the temperature coefficient (b) of $L\hat{p}$. The curves for the three smaller diameters are for hypothetical cells, where M = 0.5, $L\hat{p}^{g}$ = 0.1, and b = 0.0325. The curve for the largest cell is based on published values for the eggs of the sea urchin Strongylocentrotus: M = 0.98, $L\hat{p}^{g}$ = 0.38, b = 0.065. Details on these parameters and on the underlying theory and equations are given by Mazur (1963).

Figure 14. Calculated percentages of intracellular water remaining at the indicated temperatures in the three layers of a three-layered sphere surrounded by ice. The curves labeled α, β, and γ refer to the outer, middle, and center layer, respectively, each layer being 6 μm thick. The cooling rate is 10°C per min. The values of $L\hat{p}^{g}$ and b are 0.3 and 0.065, respectively.

The intracellular osmolality is assumed to be 0.3. From Mazur (1966).

Figure ~~16~~ 15. Probability of intracellular freezing in human red cells as functions of the assumed nucleation temperature of the cell and of the cooling rate.

Figure ~~17~~ 16. Effect of slow warming to various temperatures on survival and on the recrystallization of intracellular ice in rapidly frozen yeast. The yeast were rapidly frozen to -196°C. In the survival studies (Mackenzie, 1970), the cells were warmed slowly to the indicated temperatures and then thawed rapidly. In the recrystallization studies (Bank, 1973), the cells were warmed slowly to -50°, -35°, and -20°C, held 24 hr, 30 min, and 5 min, respectively, recooled to < -100°C and freeze cleaved.

Figure ~~18~~ 17. Interactions of cooling rate and warming rate on the survival of frozen-thawed Chinese hamster tissue culture cells. The cells were suspended in 0.5 M DMSO, frozen to -196°C, and warmed at the rates indicated. From Mazur et al. (1969).

Figure ~~19~~ 18. Survival of slowly cooled (upper curve) and rapidly cooled (lower curve) yeast cells as a function of the warming rate. The cells were suspended in water and cooled to -196°C prior to warming. From data of Mazur and Schmidt (1968).

Figure ~~20~~ 19. Survival of frozen red cells as a function of warming rate. The cells were equilibrated in 2 M glycerol, frozen to -70°C at the indicated rates, transferred to -196°C, and warmed. From Miller and Mazur (1976).

Figure ~~21~~ 20. Survival of mouse marrow stem cells suspended in Tyrode's solution containing 0, 0.4, 0.8, or 1.25 M glycerol after cooling to -196°C at various rates and rapid warming. Redrawn from Leibo et al. (1970).

Figure ~~22~~ 21. Survival of human red cells in 0, 0.8, and 1.1 M glycerol as a function of cooling rate. Calculated by Leibo (1976) from data of Rapatz and Luyet (1968).

Figure 22 ~~24~~. Hypothetical relation between survival, cooling velocity, and additive concentration on the basis of two injurious factors. A. Separate contributions of intracellular ice formation (curve AB) and solution effects (curves CD) to injury. The three CD curves show the hypothetical effects of three different concentrations of a protective additive. B. Calculated survival as a result of the contribution of both intracellular freezing and solution effects. From Mazur et al. (1970).

Figure 24 ~~25~~. Effect of the intermediate temperature on the survival of bull sperm subjected to two-step cooling to -196°C. The sperm, suspended in 7% glycerol, was placed in baths at the indicated temperatures, held five minutes, and then transferred to liquid nitrogen. Courtesy of S. P. Leibo from data of Luyet and Keane (1955).

Figure 23 ~~26~~. Comparison between events occurring during progressive cooling and those occurring during two-step freezing.

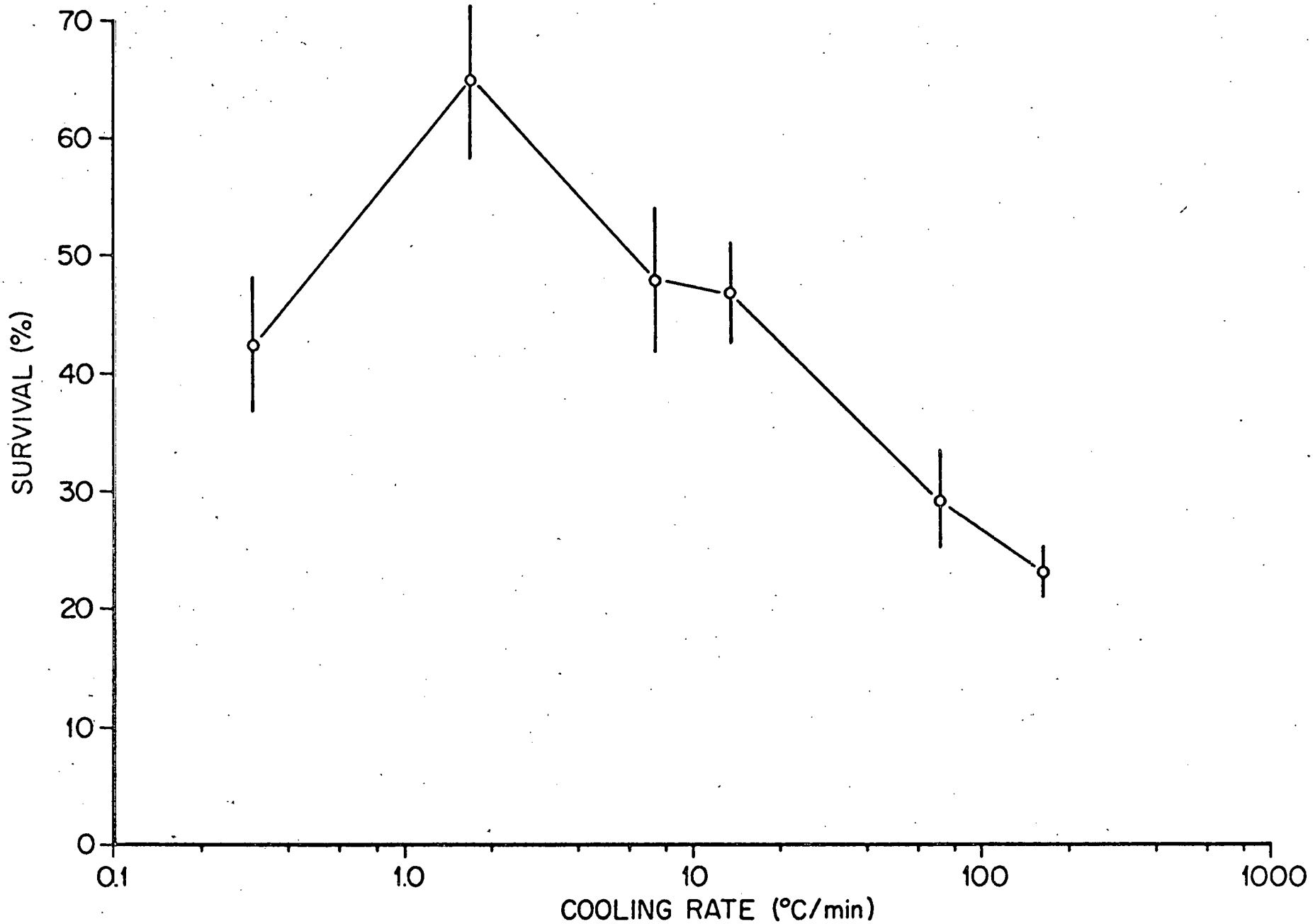

70
60
50
40
30
20
10
0
SURVIVAL (%)
0.1
1.0
10
100
1000
COOLING RATE (°C/min)

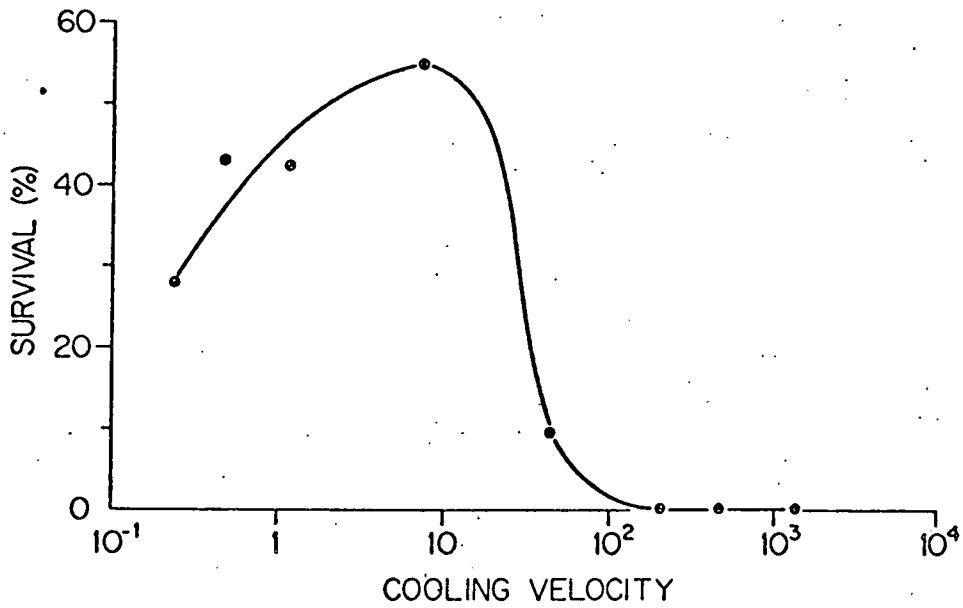
Fig. 1

Fig 16

OK, but data points not checked

Use Portions of new drawing attached

25430



~~Fig. 16~~

~~FIGURE 16~~ Survival of yeast cells suspended in distilled water as a function of cooling rate.

Bank.

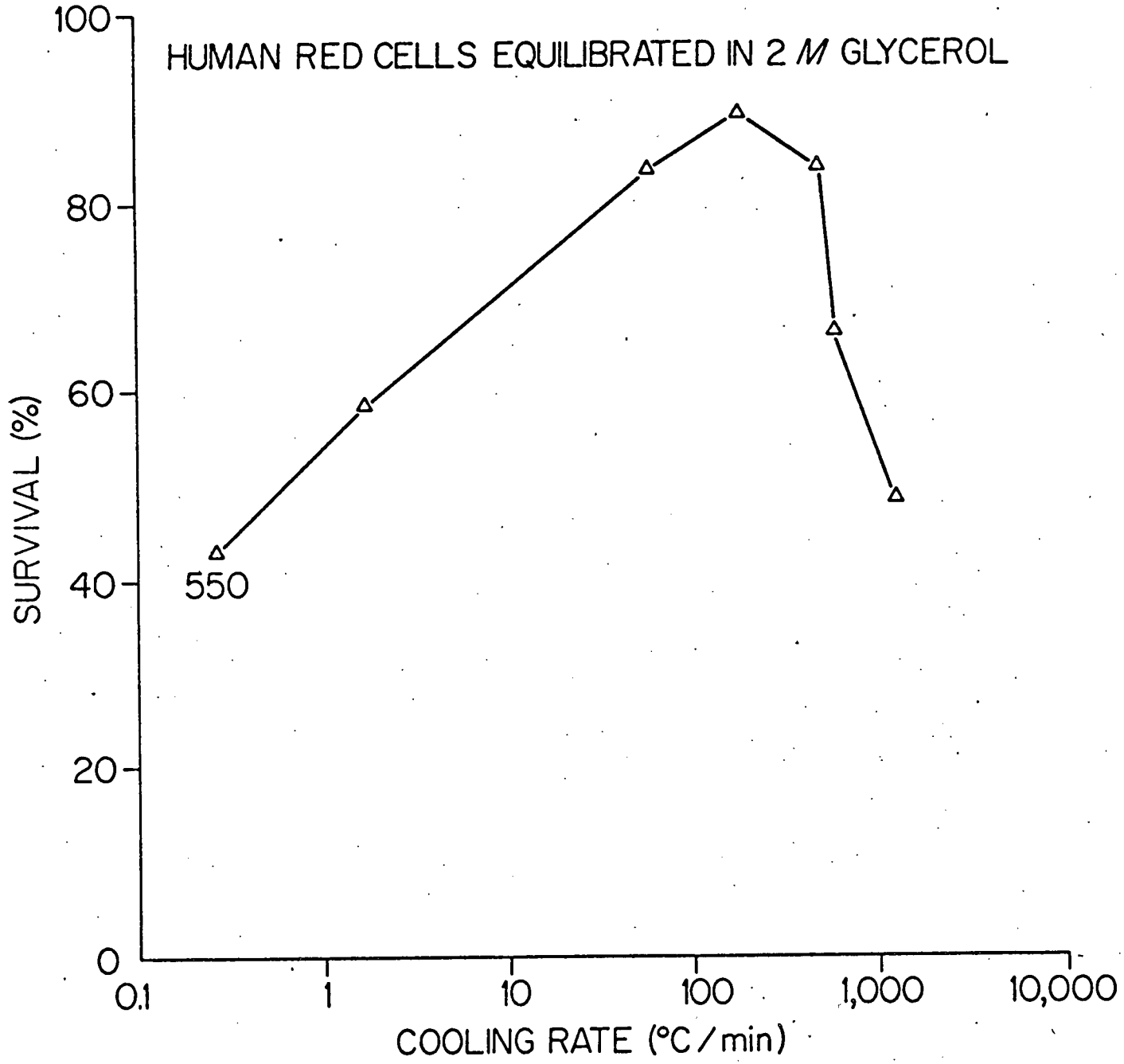
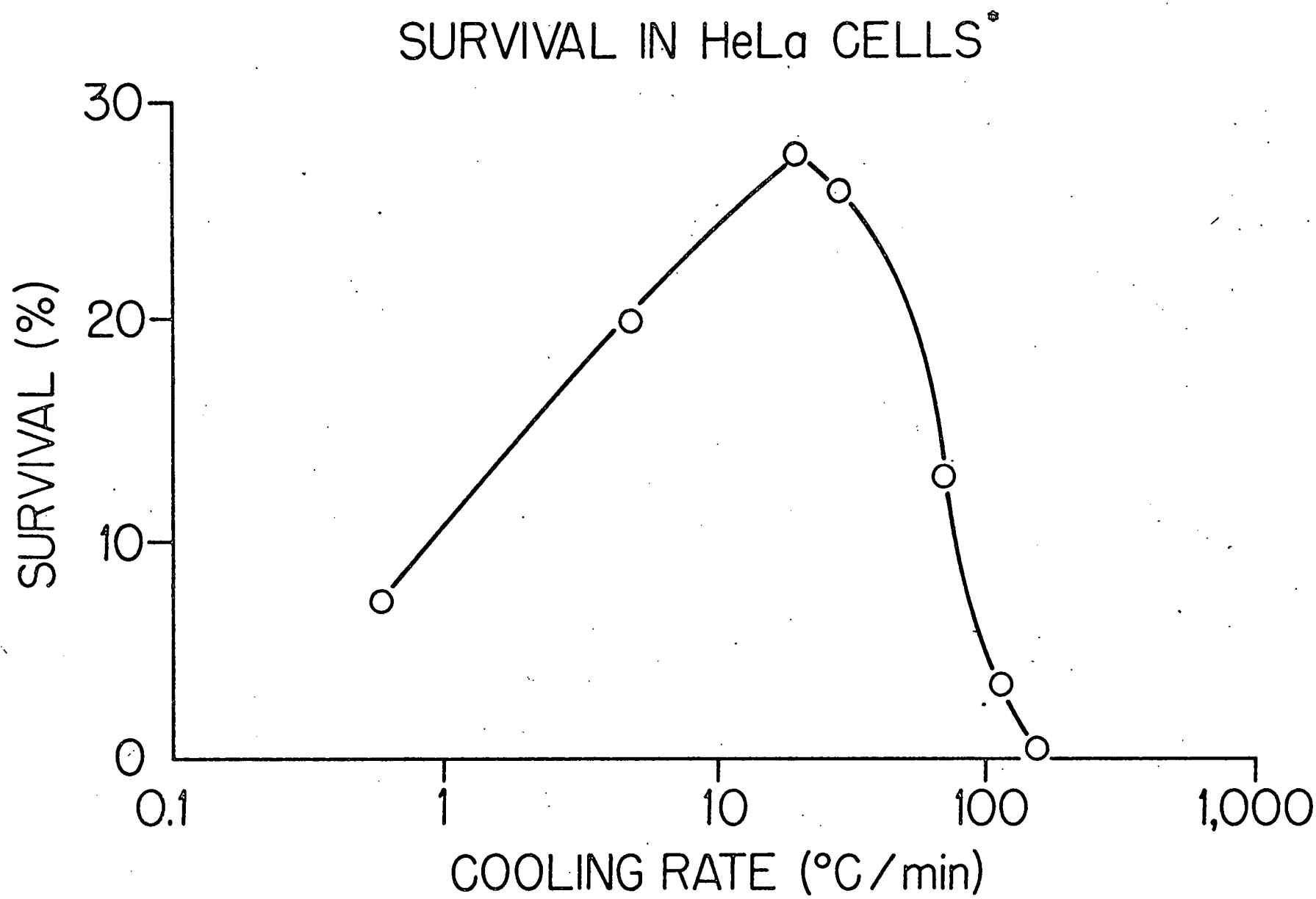

HUMAN RED CELLS EQUILIBRATED IN 2 M GLYCEROL
SURVIVAL (%)
100
80
60
40
20
0
550
0.1
1
10
100
1,000
10,000
COOLING RATE (°C / min)

Fig. 7-C
31,745

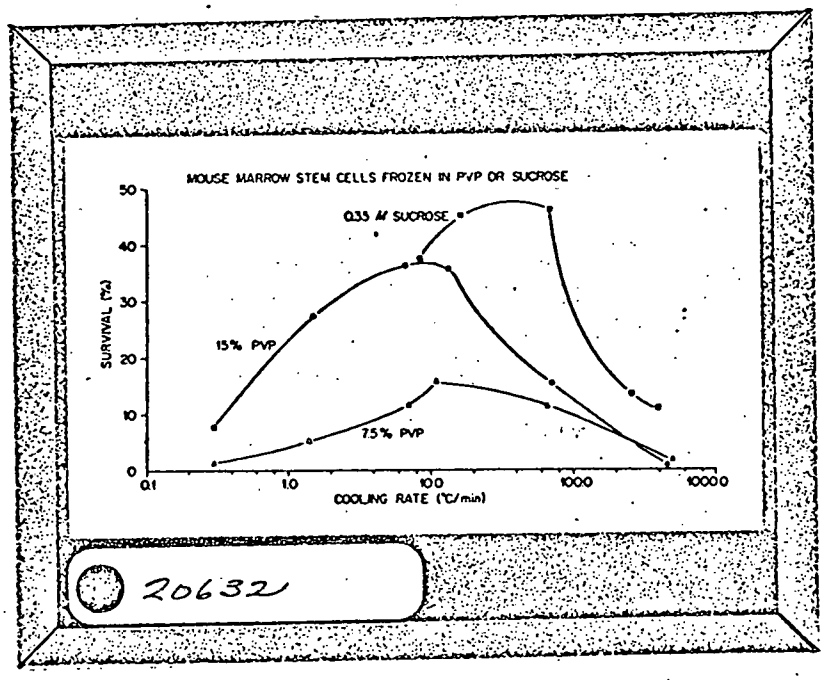


*REDRAWN FROM MCGRATH ET AL., CRYOBIOLOGY 12, 540 (1975).

Fig. 2
3173I

20632 (3)
2x2 SLIDE
(OH)

Another example?



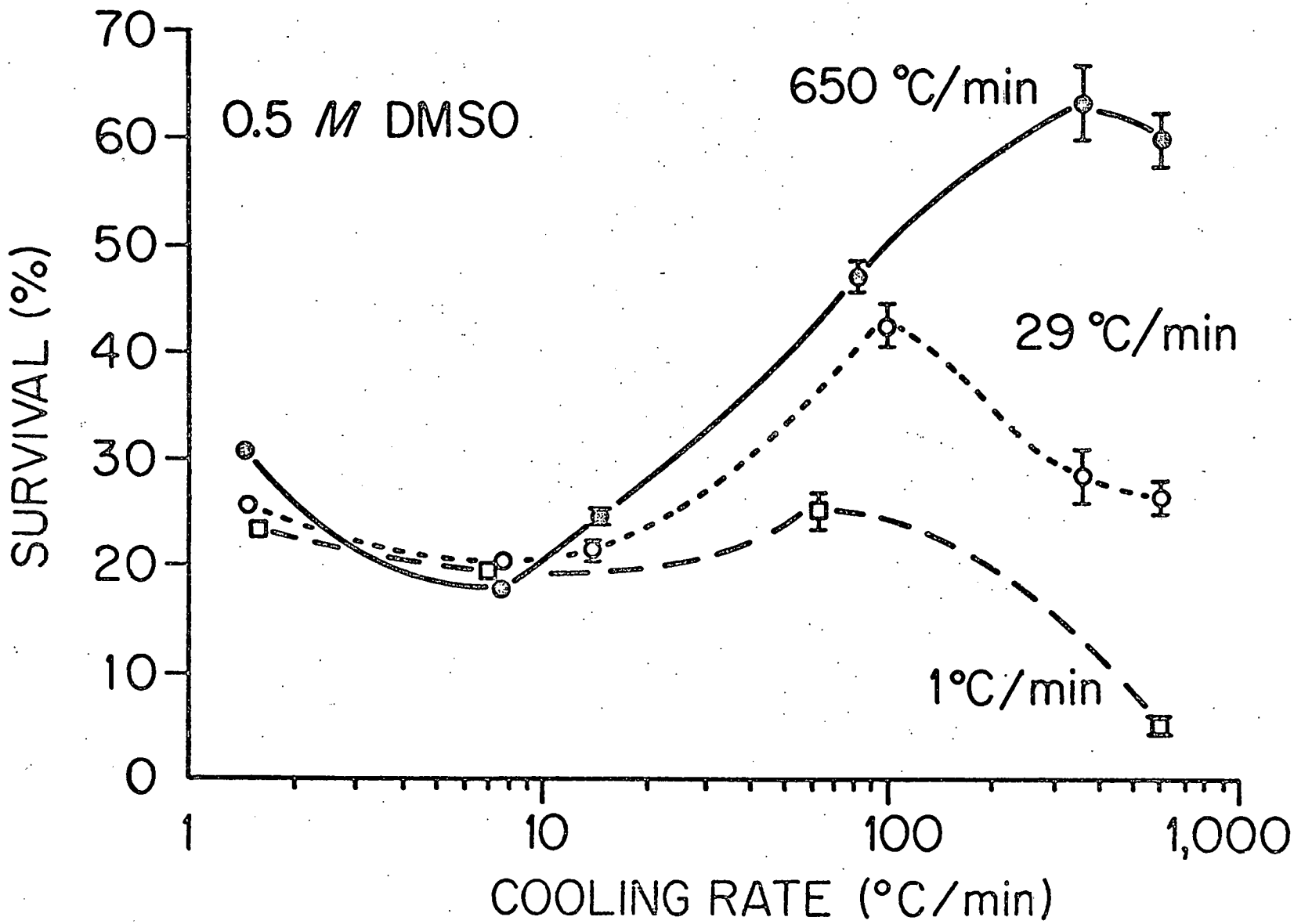
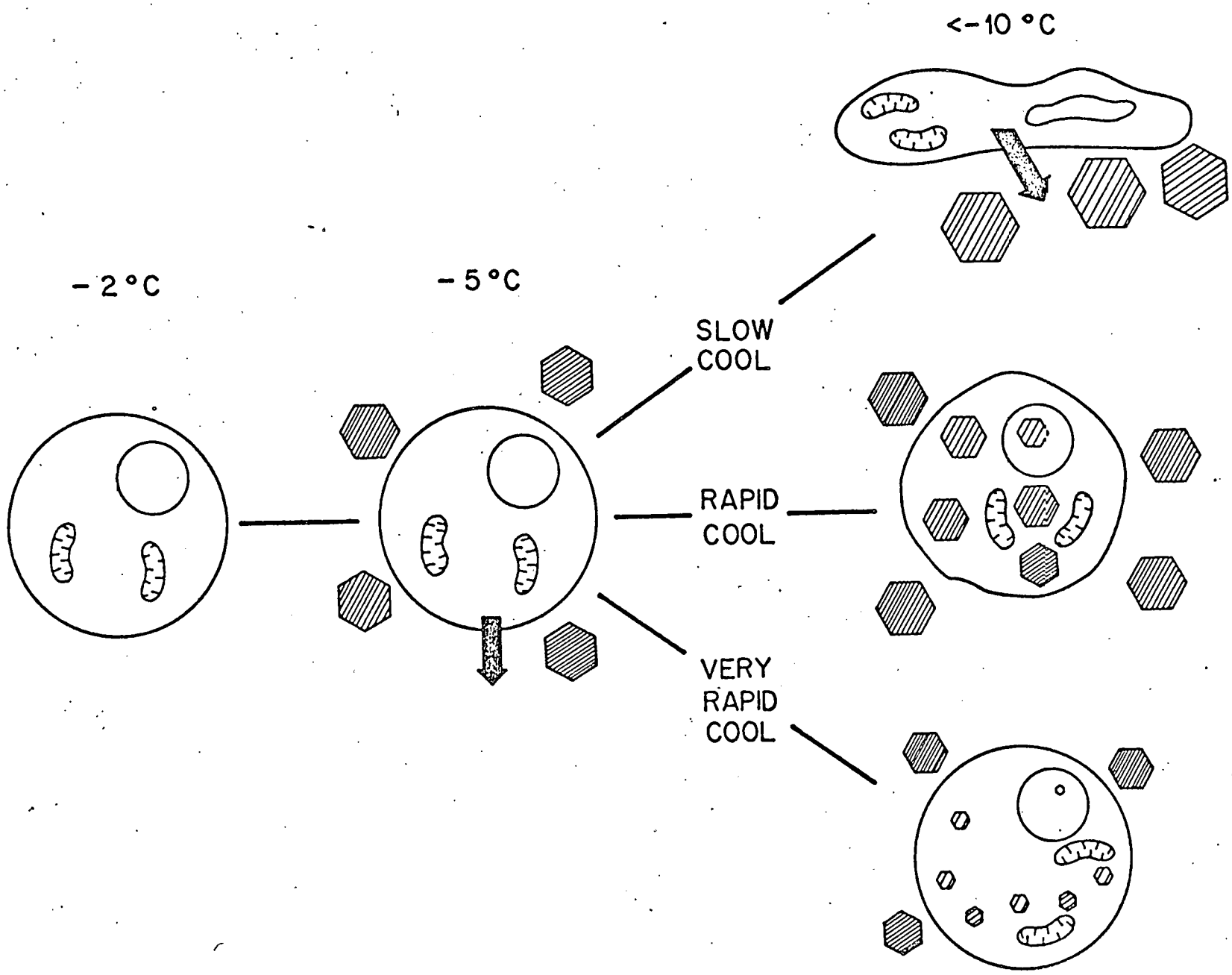

0.5 M DMSO
650 °C/min
29 °C/min
1°C/min
SURVIVAL (%)
70
60
50
40
30
20
10
0
1
10
100
1,000
COOLING RATE (°C/min)

Fig. 4
24870-1

2 ?737-1

PHYSICAL EVENTS IN CELLS DURING FREEZING



2x2 (5)
3¼x4
28737 (Ok)

22379

Science, 197

3 1/4 x 4 SL.

2 x 2

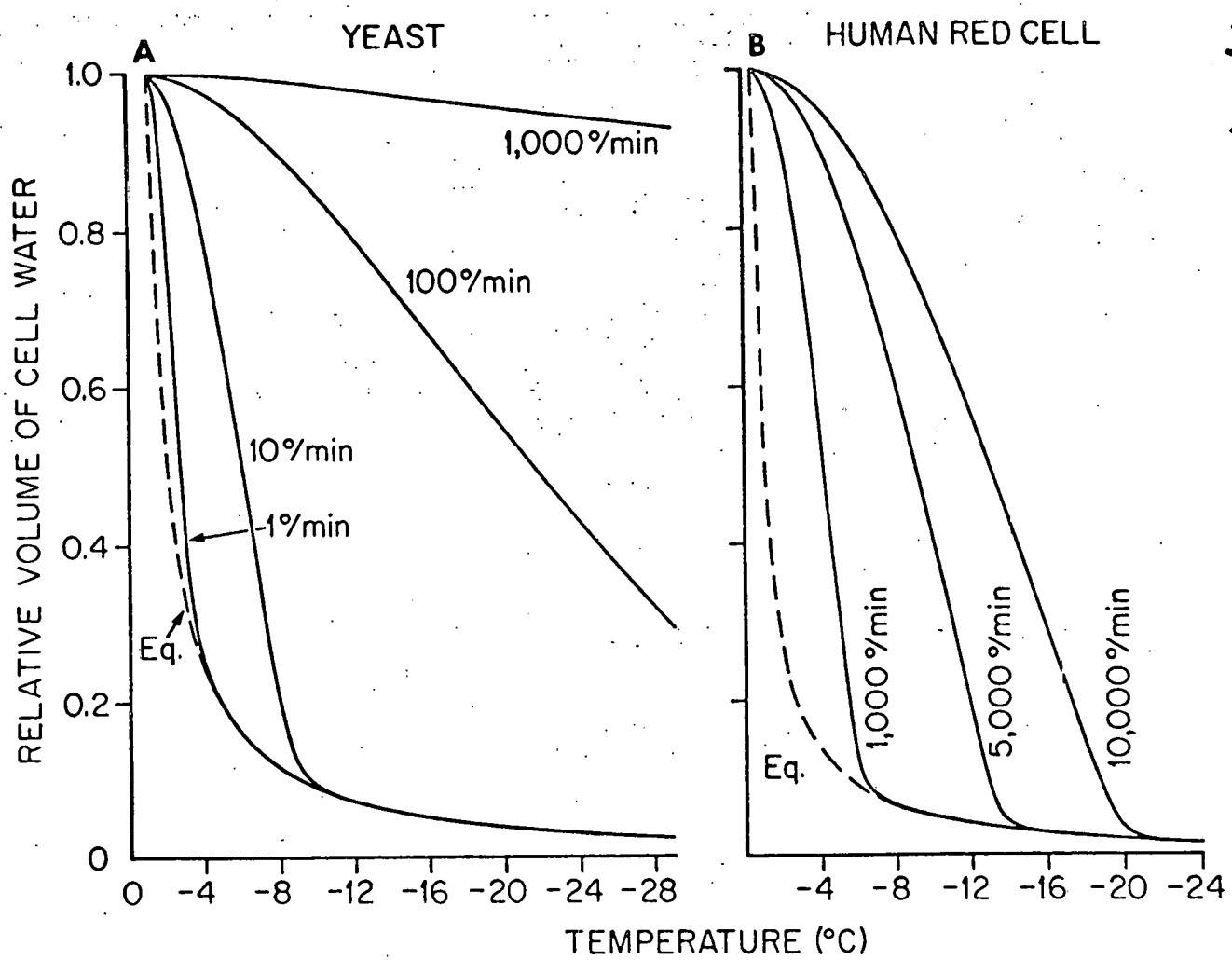
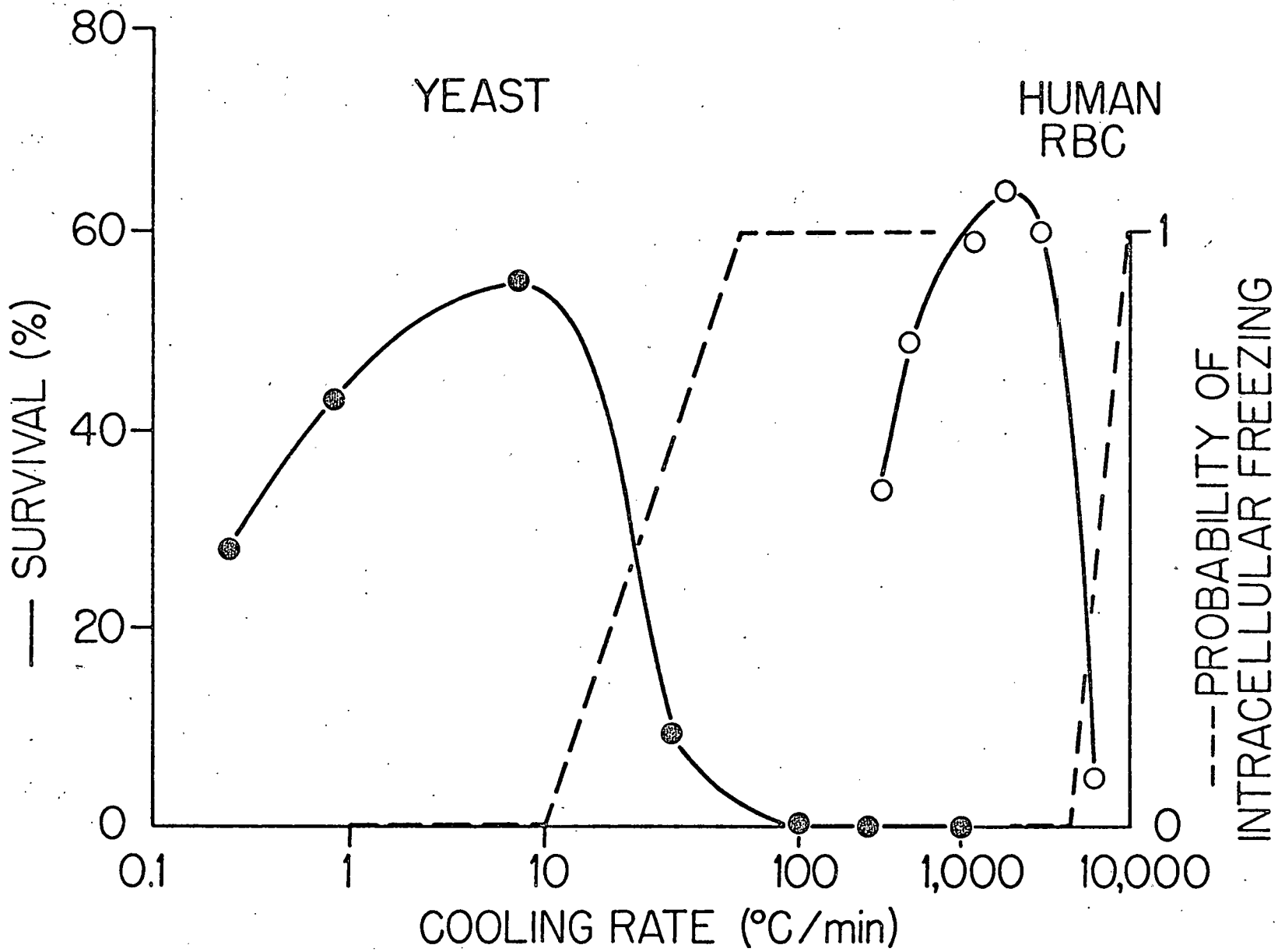


Fig. 1

Fig. 6

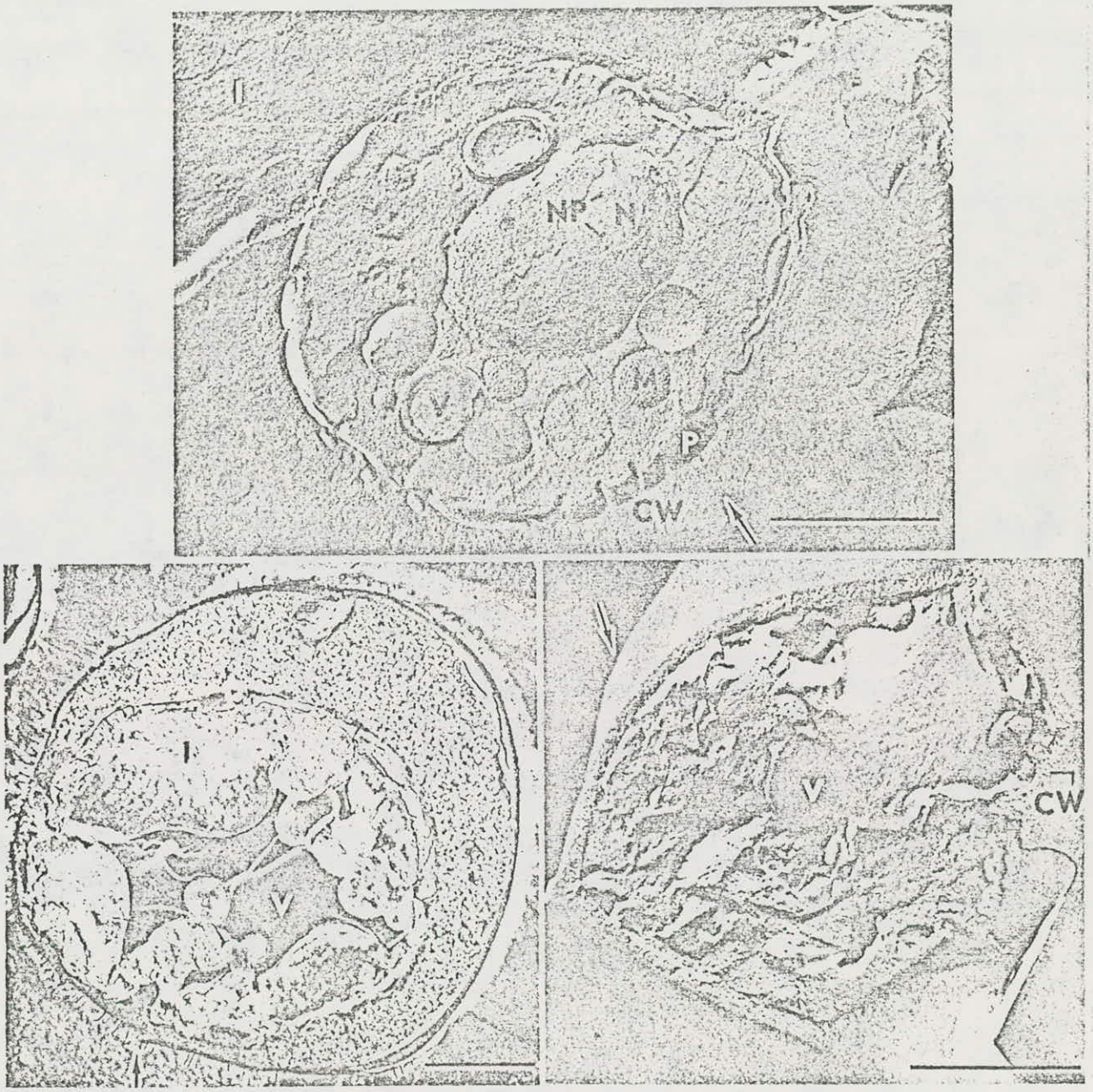


●, MAZUR AND SCHMIDT, CRYOBIOLOGY 5, 1–17 (1968);
O, RAPATZ ET AL., CRYOBIOLOGY 5, 18 (1968).

Fig. 17
31736

Fig. 8
31743

STANDARD FREEZE-CLEAVAGE



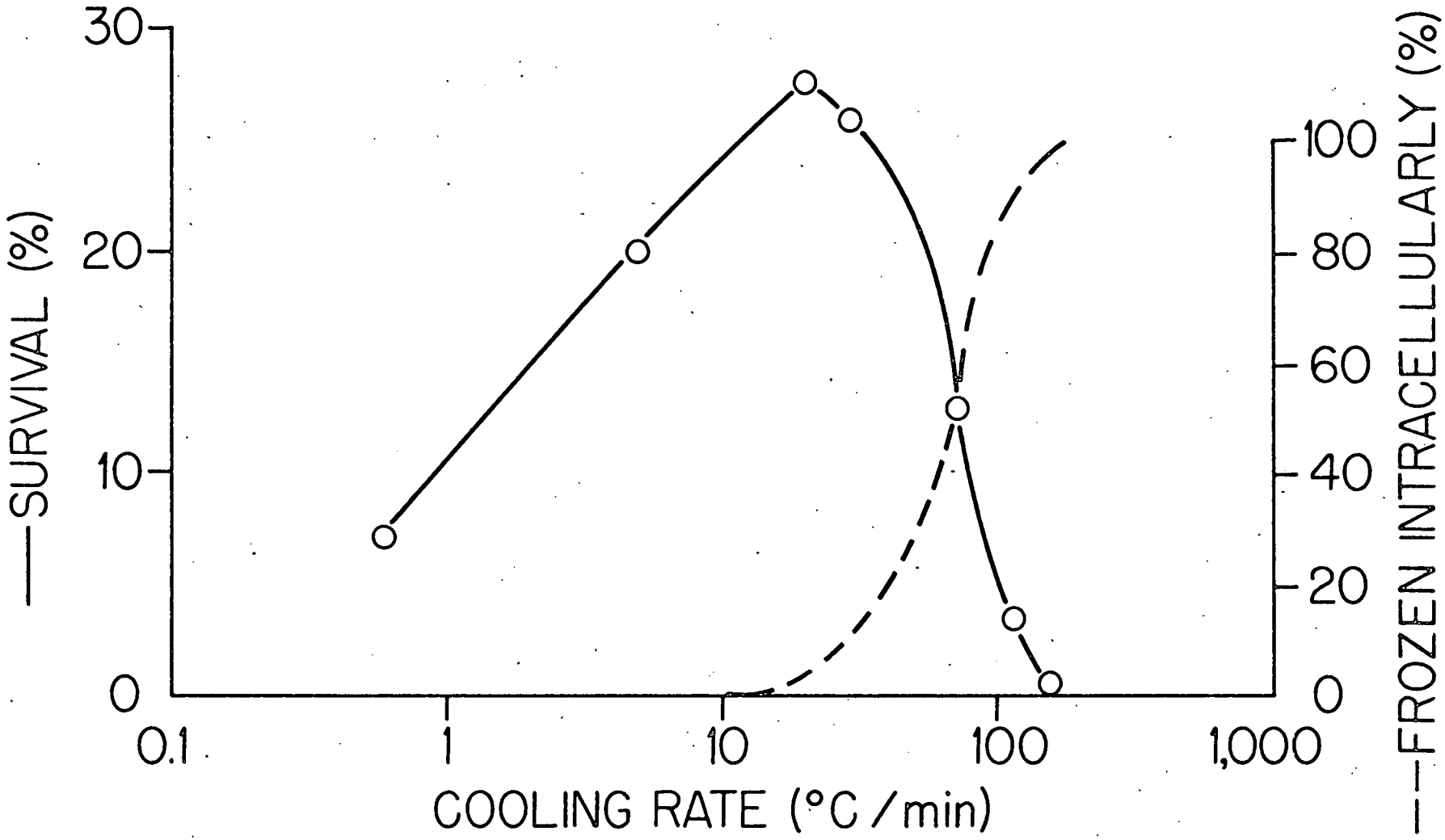
CR = 55 °C / min.

CR = 6 °C / min.

Fig. 9.

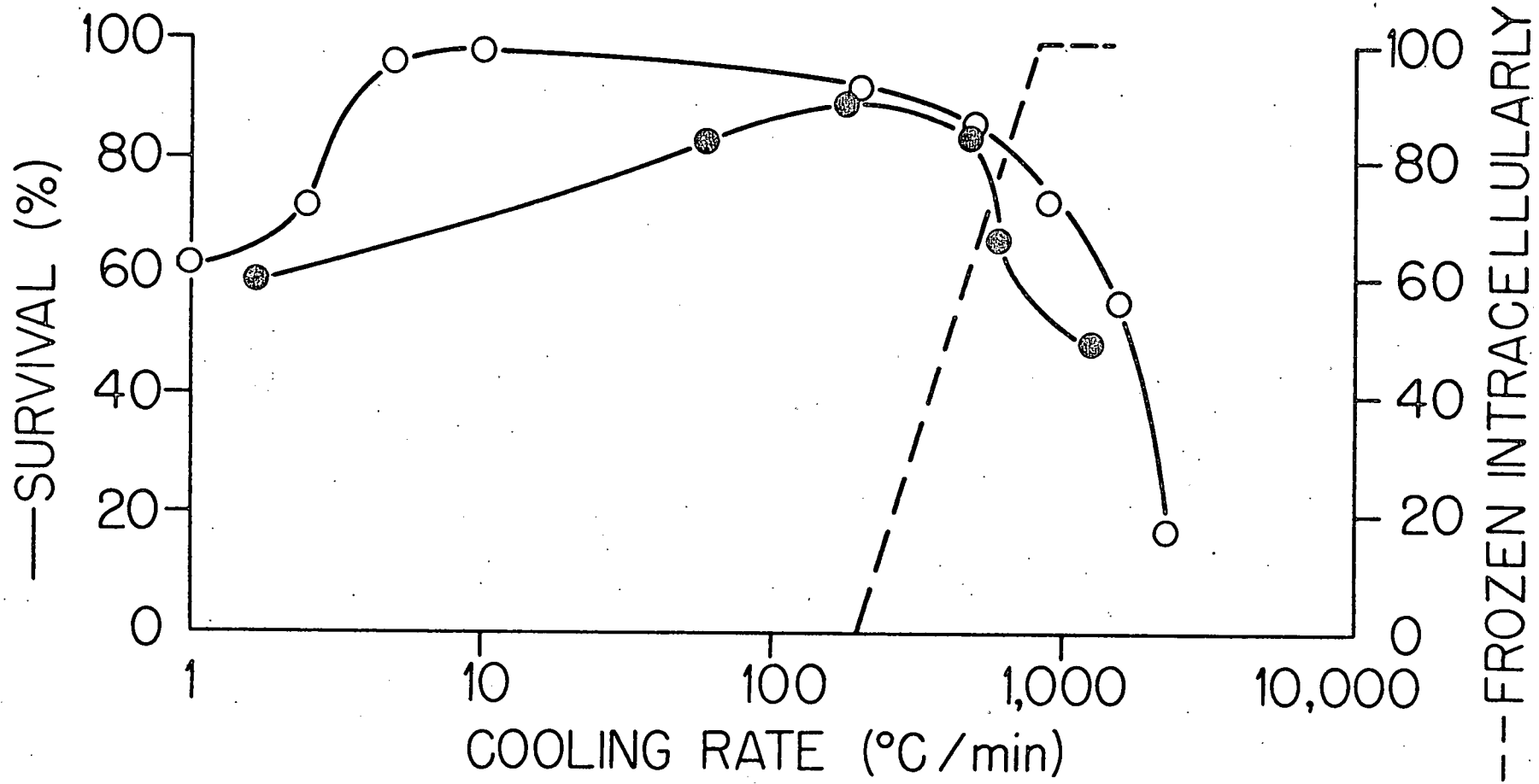
3/732

SURVIVAL (—) vs INTRACELLULAR FREEZING (--) IN HeLa CELLS*



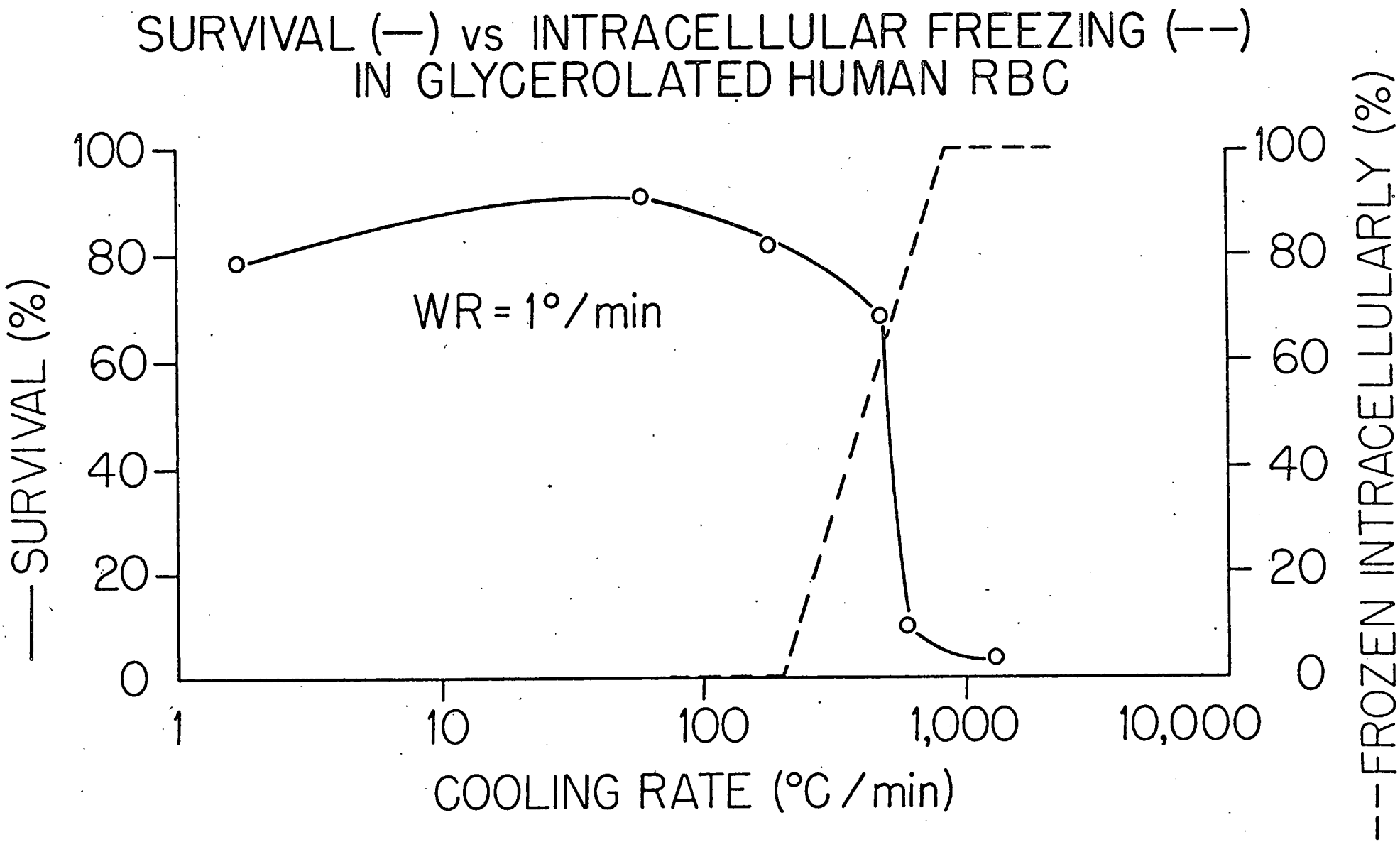
*REDRAWN FROM MCGRATH ET AL., CRYOBIOLOGY 12, 540 (1975).

SURVIVAL (—) vs INTRACELLULAR FREEZING (--) IN GLYCEROLATED HUMAN RBC



O, RAPATZ ET AL., CRYOBIOLOGY 5, 18 (1968); ●, MILLER AND MAZUR, CRYOBIOLOGY, IN PRESS; --, DILLER, CRYOBIOLOGY 12, 480 (1975).

Fig. 10

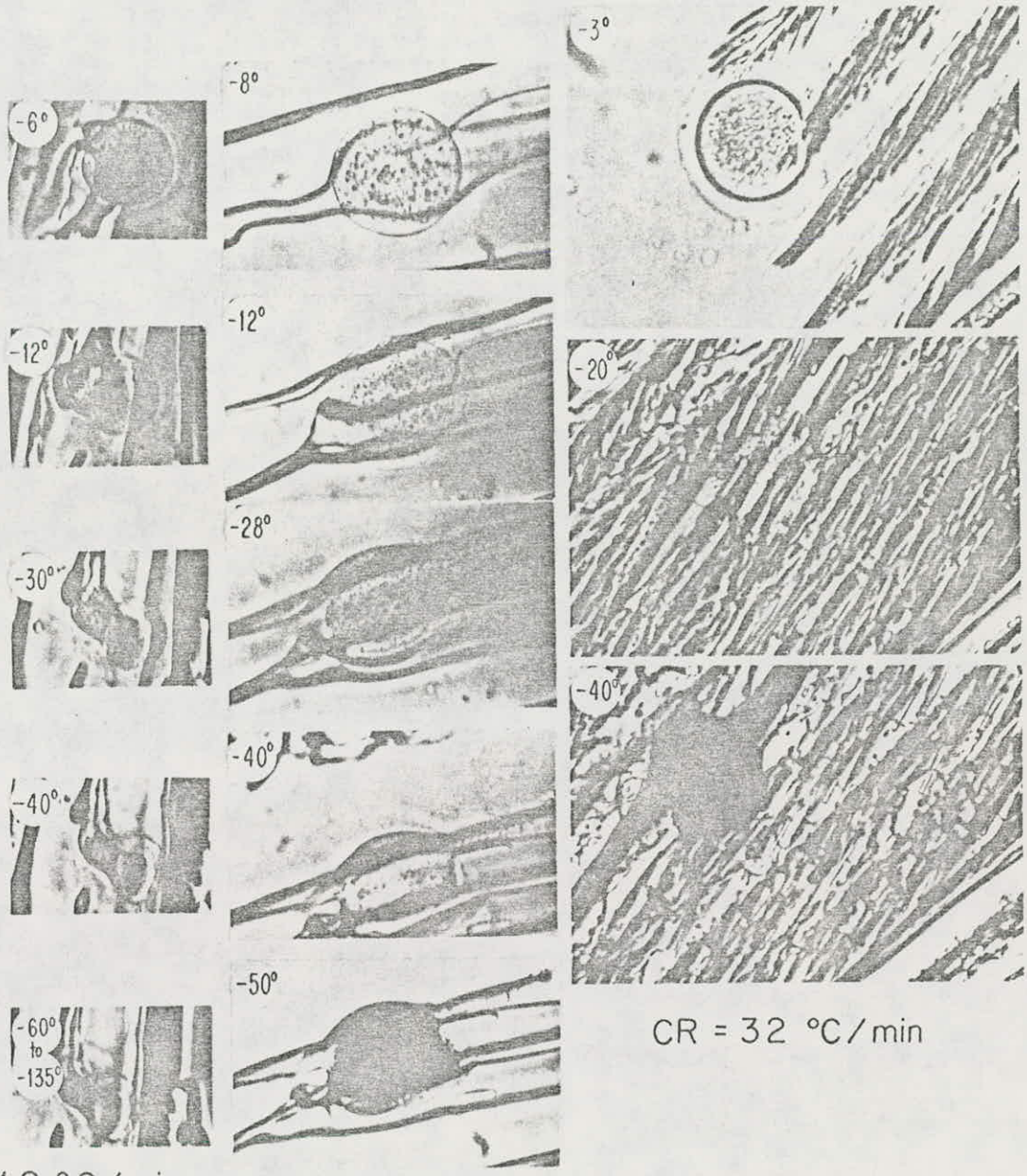


●, 2 M GLYCINE (MILLER AND MAZUR, CRYOBIOLOGY, IN PRESS);
--, 1-5 M GLYCINE [DILLER ET AL., CRYOBIOLOGY 12, 580 (1975)].

3738-1
Fig. 11

31740
Fig. 12

MOUSE OVA-INTRACELLULAR FREEZING vs COOLING RATE*



CR = 1.2 °C/min

CR = 2.4 °C/min.

CR = 32 °C/min

*LEIBO ET AL. (1976).

31352-1

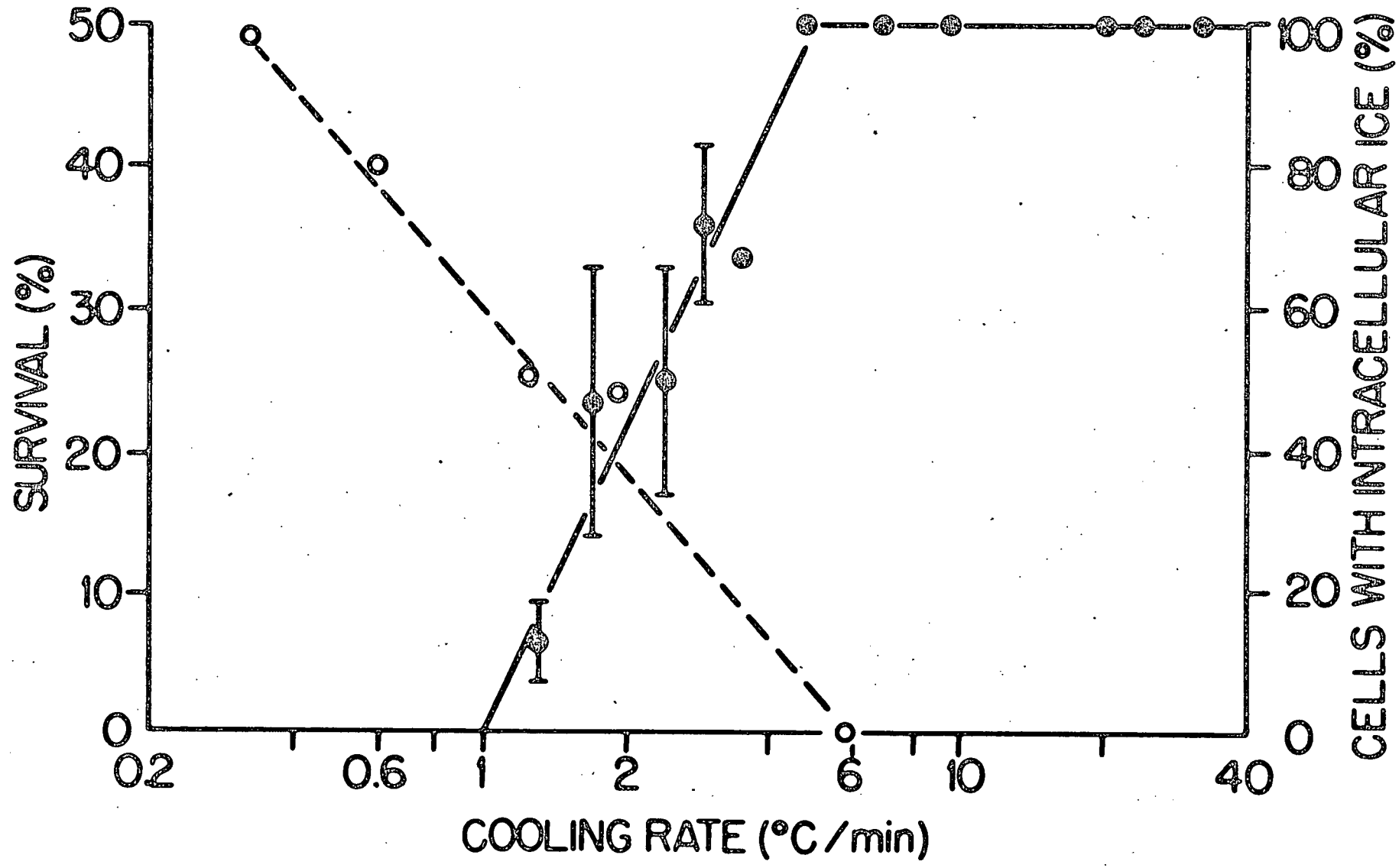


Fig 13

Annal. N.Y. Acad. Sci. 125, 1965
Cryobiol. 2, 1966
3¼ x 4" Slide UC 19
2" x 2" slide

Fig 14

13,572

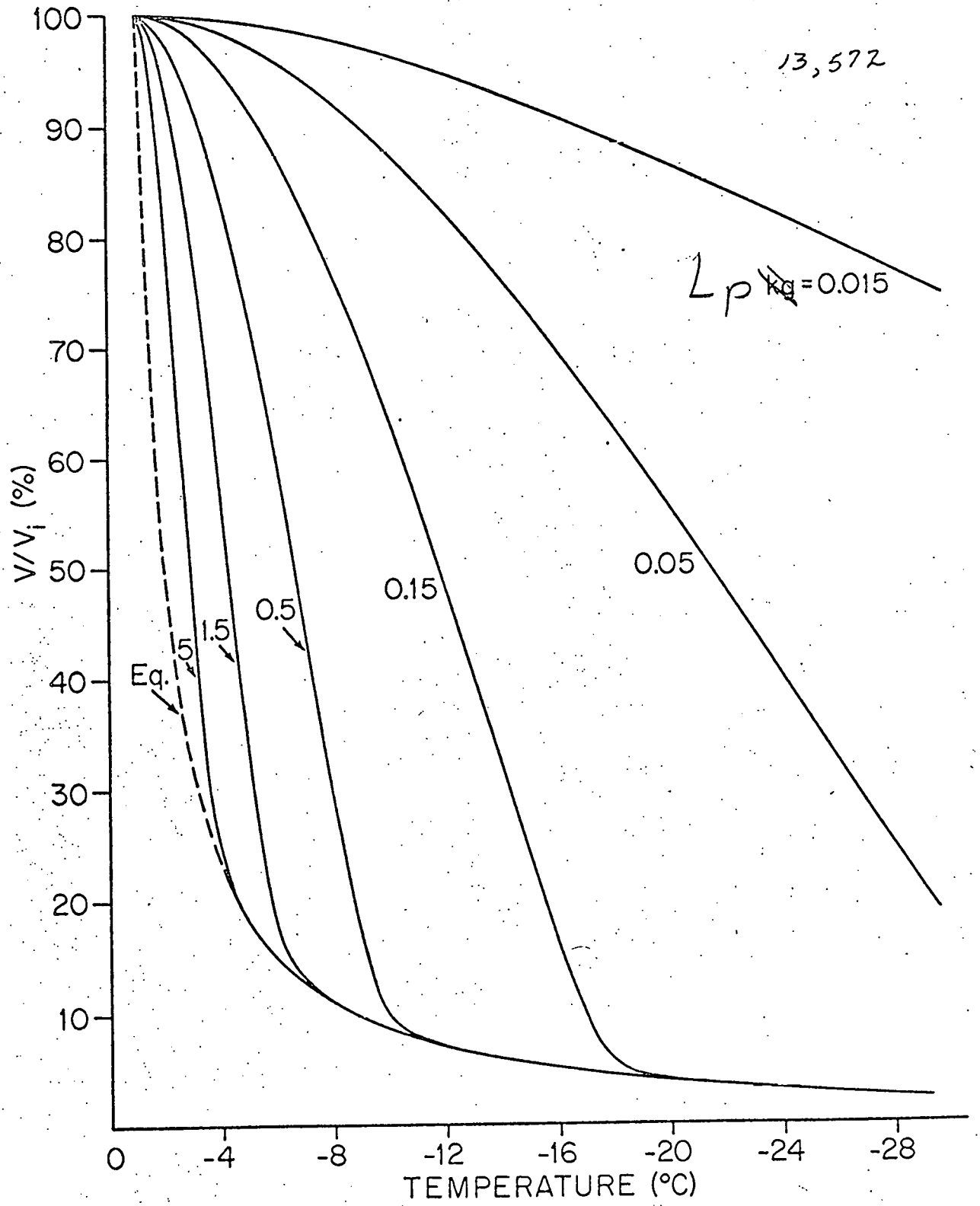


FIG. 5

6 μ cell
Q10 = 1.4
Cooling rate = 100°C/min.

Annal. N.Y. Acad. Sci. 125, 1965

3¼ x 4" R-38

Fig. 15

13,571

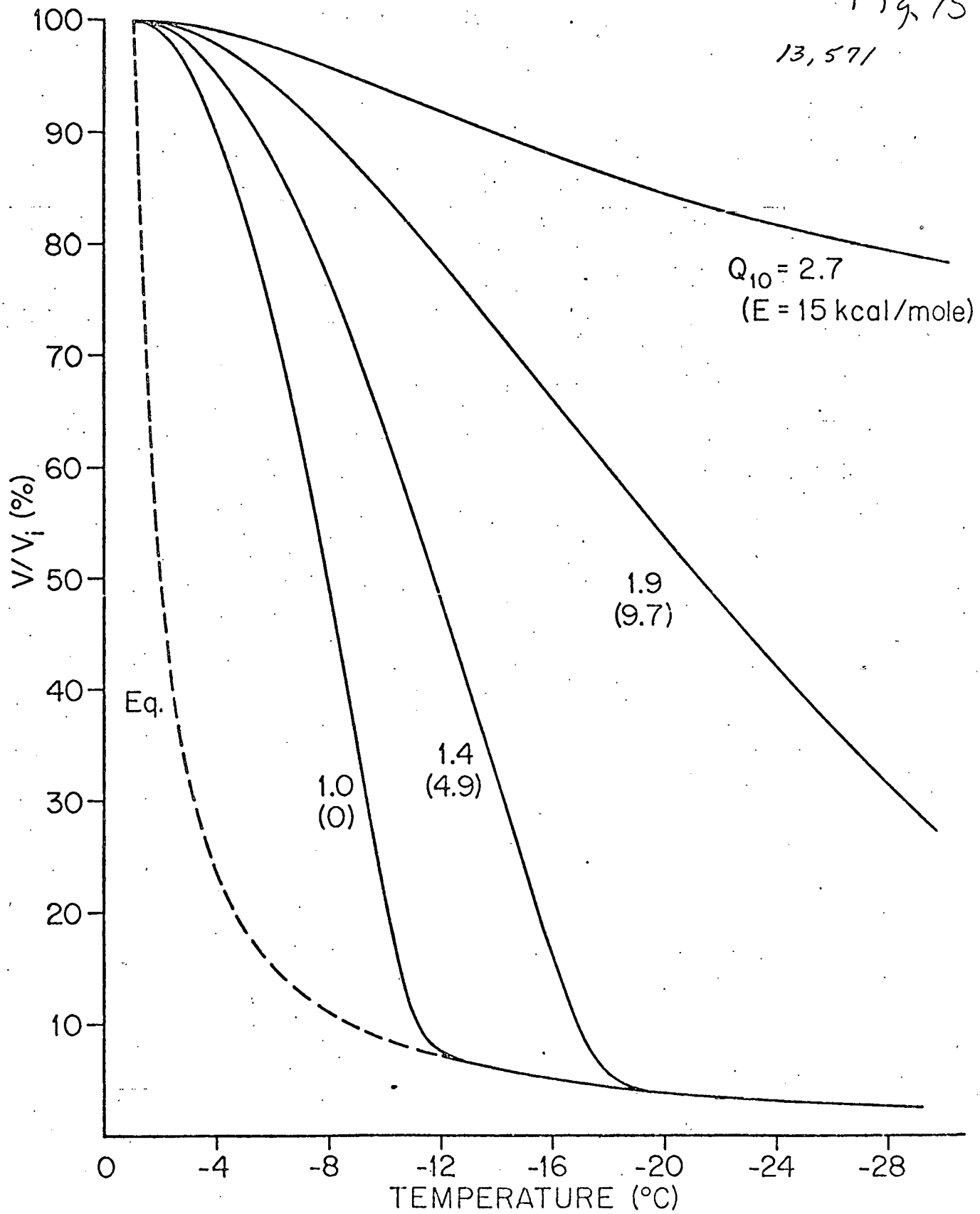


Fig. 4

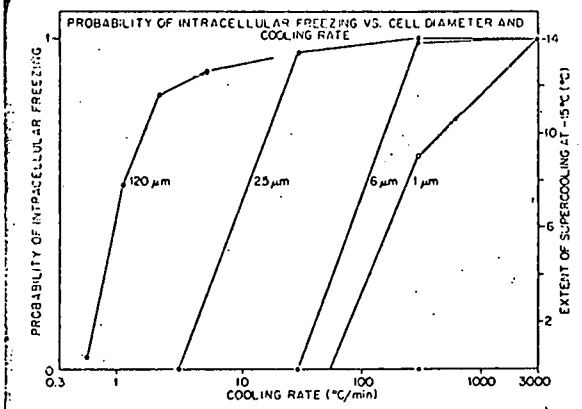
cell diameter = 6 μ
Kg = 0.15
cooling rate = 100°C/min.

F-16

2" x 2"

OAK RIDGE NATIONAL LABORATORY

2730L



12,709

Fig 17

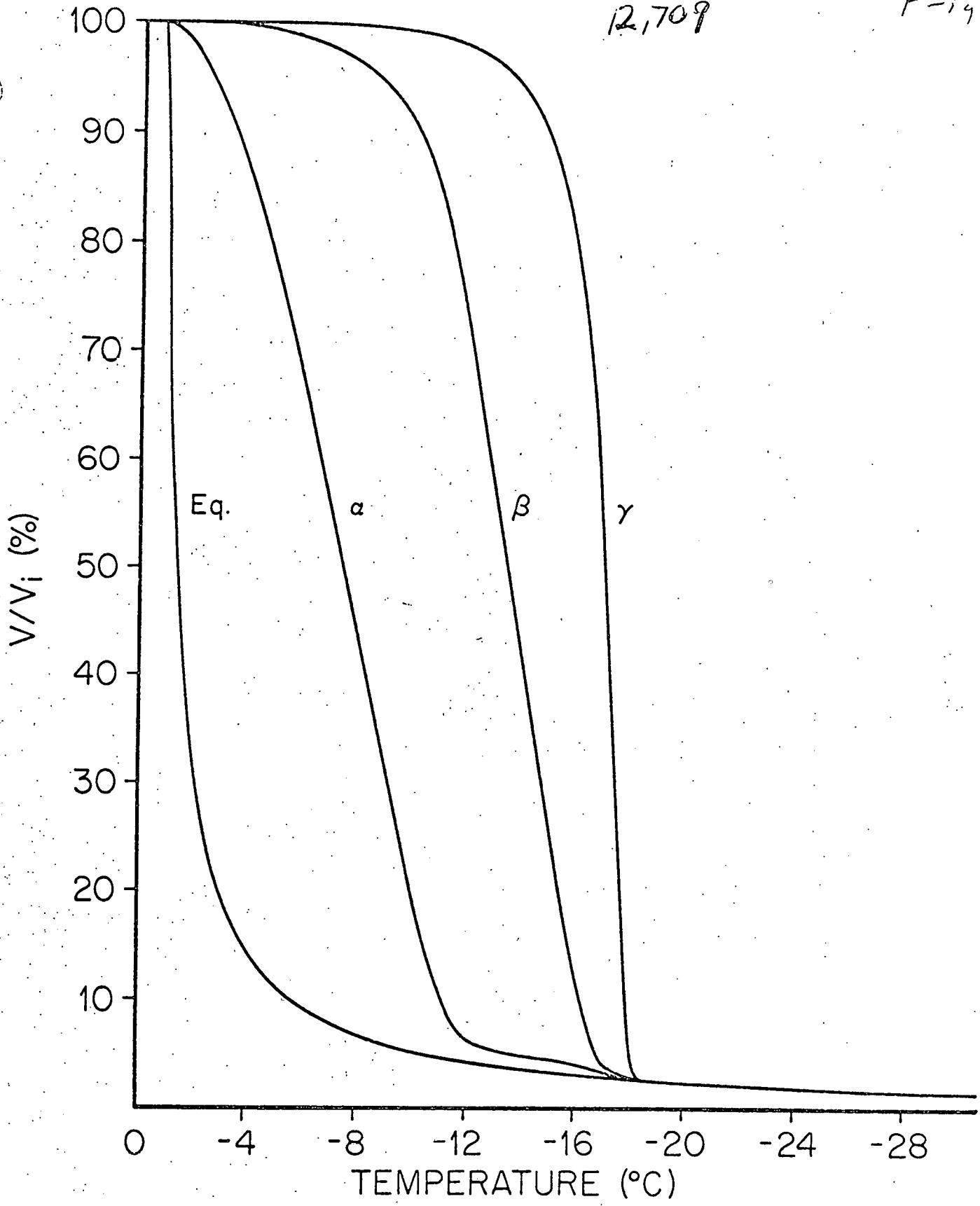


Fig. 8

[cooling rate = 10°c/min.
[illegible]
Kg = 0.3]

NO. 4-10 SEMI-LOGARITHMIC
1 10 INCH CYCLE X 10 DIV. PER INCH (70 DIV.)
SPAULDING-MOSS COMPANY
BOSTON 10, MASS.
MADE IN U. S. A.

Probability of Intracellular Freezing vs Human RBC Vs Nucleation Temp. and Cooling Rate

(Assumptions: P = 0 if $\Delta T < 2°$ or $V/V_i < .15$ at nucl. temp
P = 1 if $\Delta T \geq 2$ and $V/V_i \geq .15$ " " "

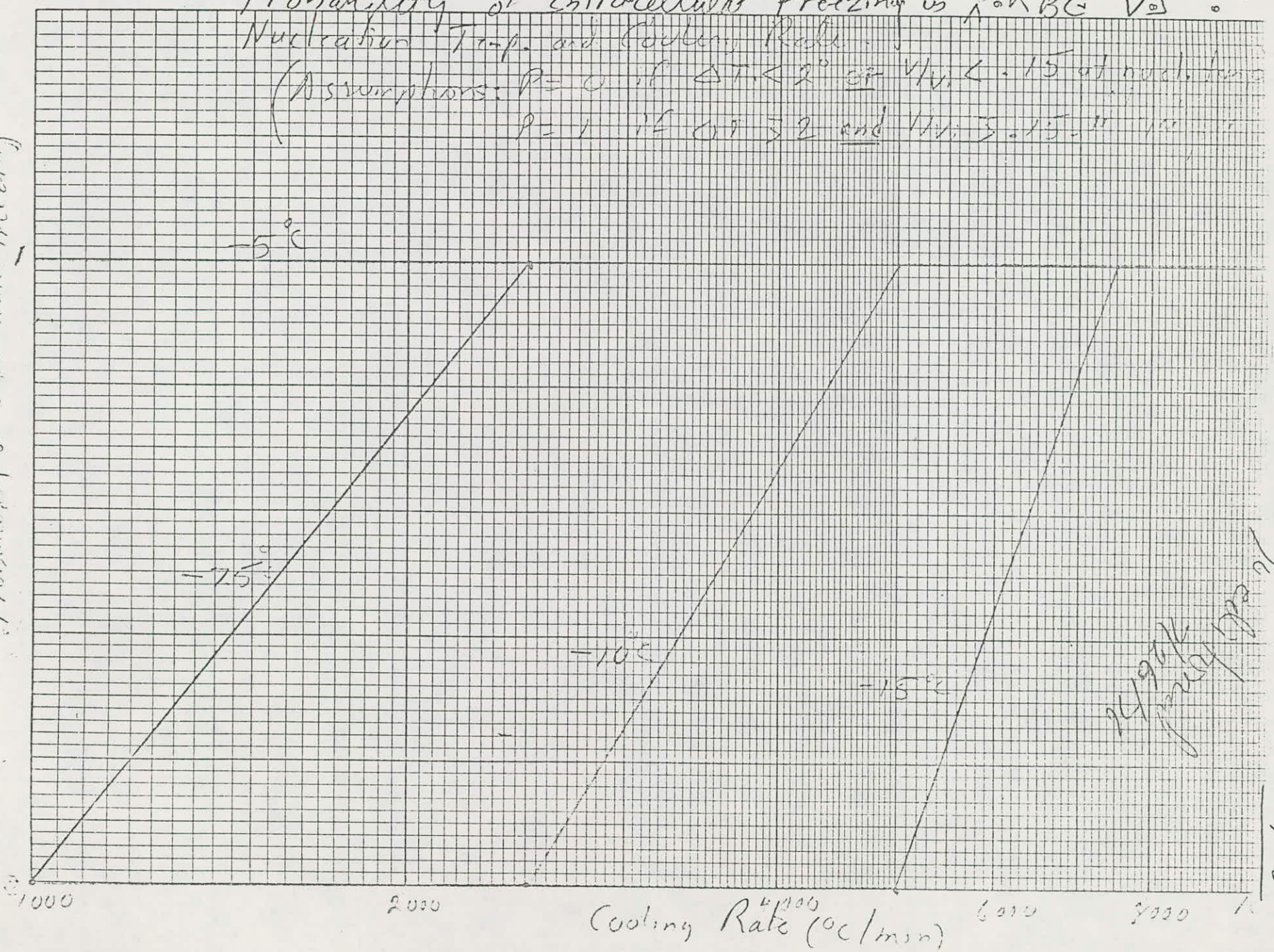


Fig. 18

Probability of Intracellular Freezing as HRBC vs Nucleation Temp + C.R. 3rd-Assumption 4/7

Temp	1000 ΔT, V/Vi	P	2500 ΔT V/Vi	P	5000 ΔT V/Vi	P	7500 ΔT V/Vi	P	10000 ΔT V/Vi	P
−5	3, .3	1	4, .7	1	4, .8	1	4, .9	1	4, .9,	1
−7.5	0, .07	0	6, 34	1	6.5, .65	1	7, 8	1	7, .8	1
−10	0, .05	0	6, .06	0	8.6, .04	1	9, .6	1	9, .7	1
−14	0, .04	0	0, .04	0	1, .04	0	12, .25	1	13, .43	1
−15	0, .04	0	0, .04	0	0, .04	1	12, .17	1	14, .29,	1
−20	0	0	0	0	0	0	0	0	3.3,	

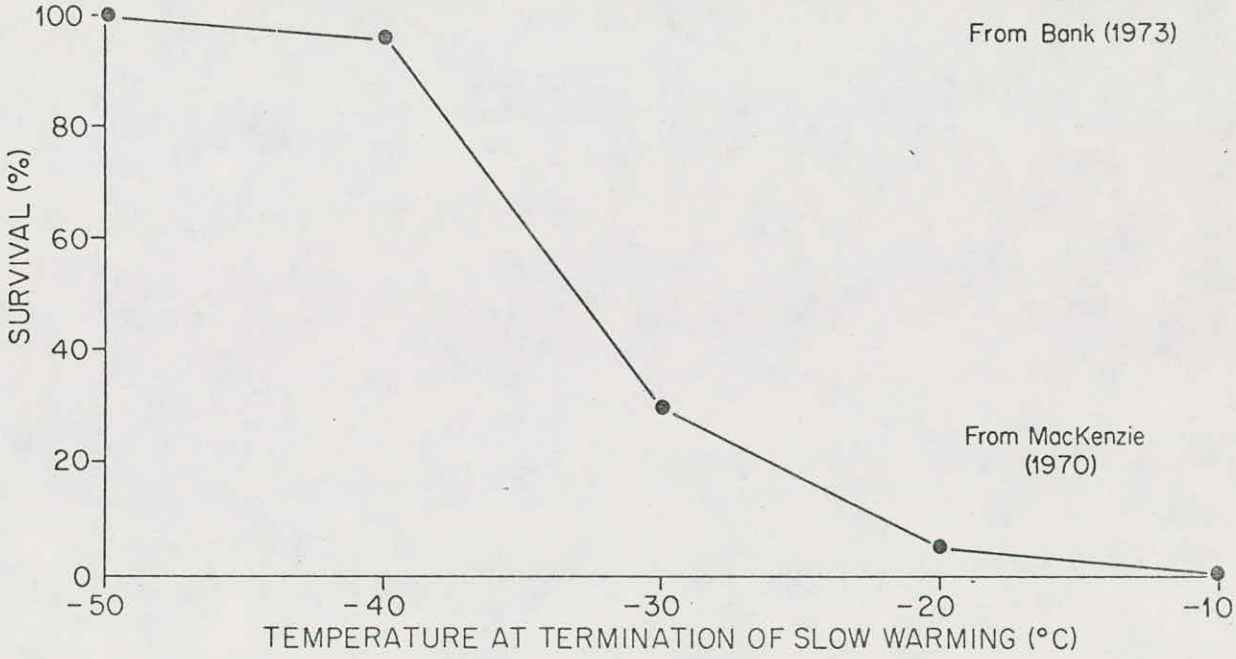
Assumption P = 0 if (a) ΔT < 2°
or V/Vi < .15

P = 1 if ΔT ≥ 2
and V/Vi ≥ .15

Fig. 18

25593-1

SURVIVAL AND RECRYSTALIZATION IN YEAST



31741

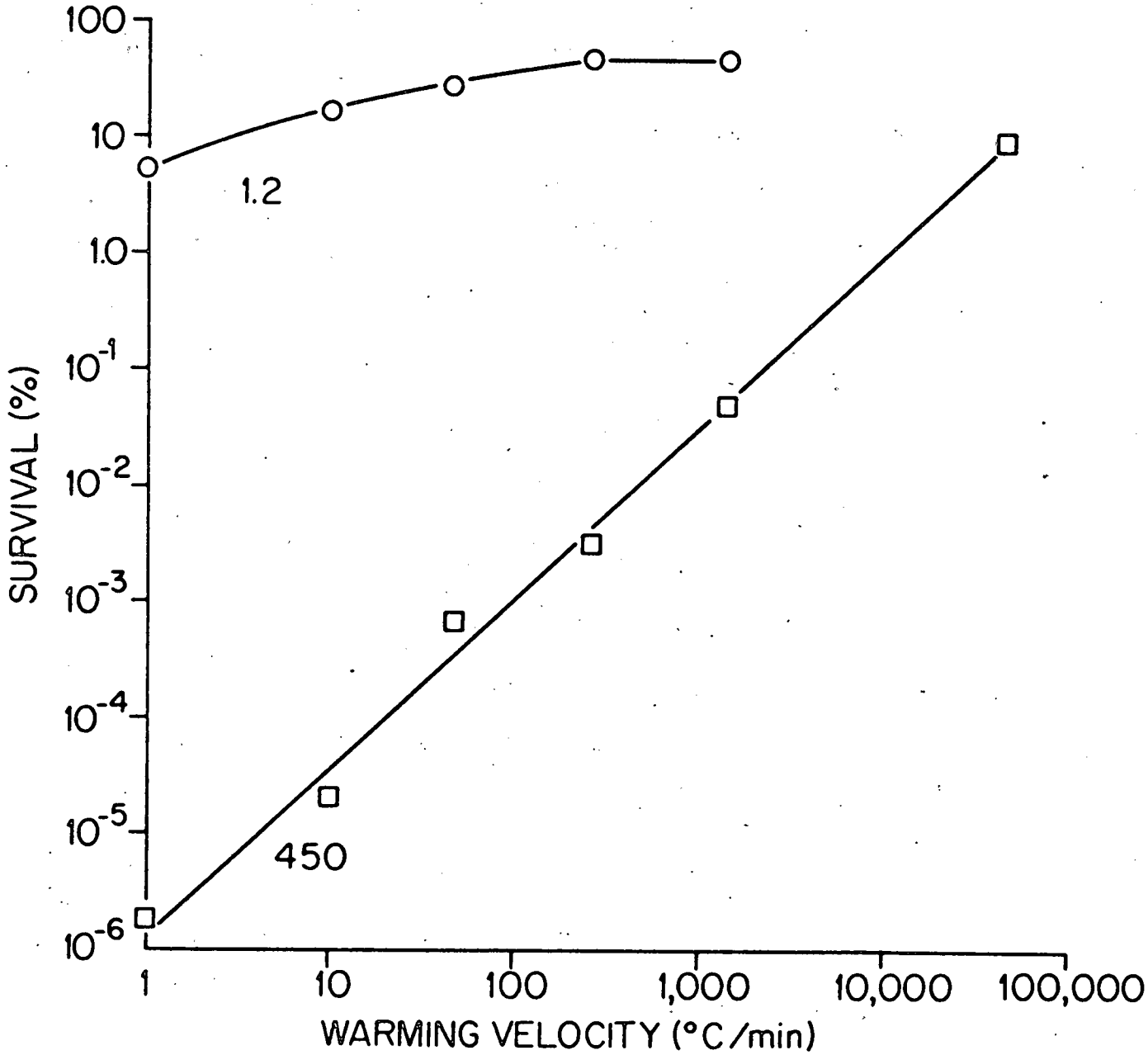

100
10
1.0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
SURVIVAL (%)
1.2
450
1
10
100
1,000
10,000
100,000
WARMING VELOCITY (°C/min)

Fig. 28

2,108-3

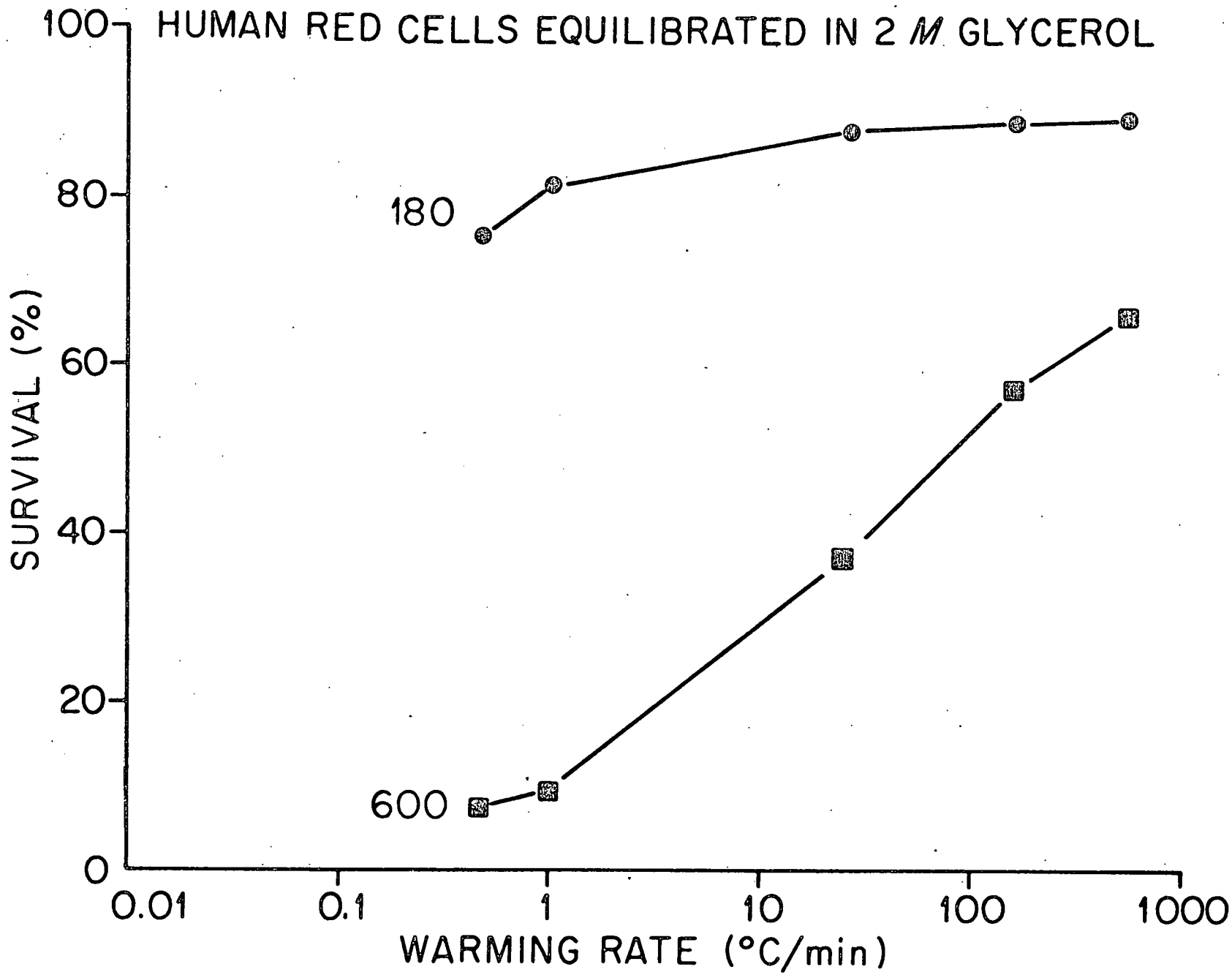


Fig. 20
21

22125

CRYOBIOLOGY
2X2 "SLIDE

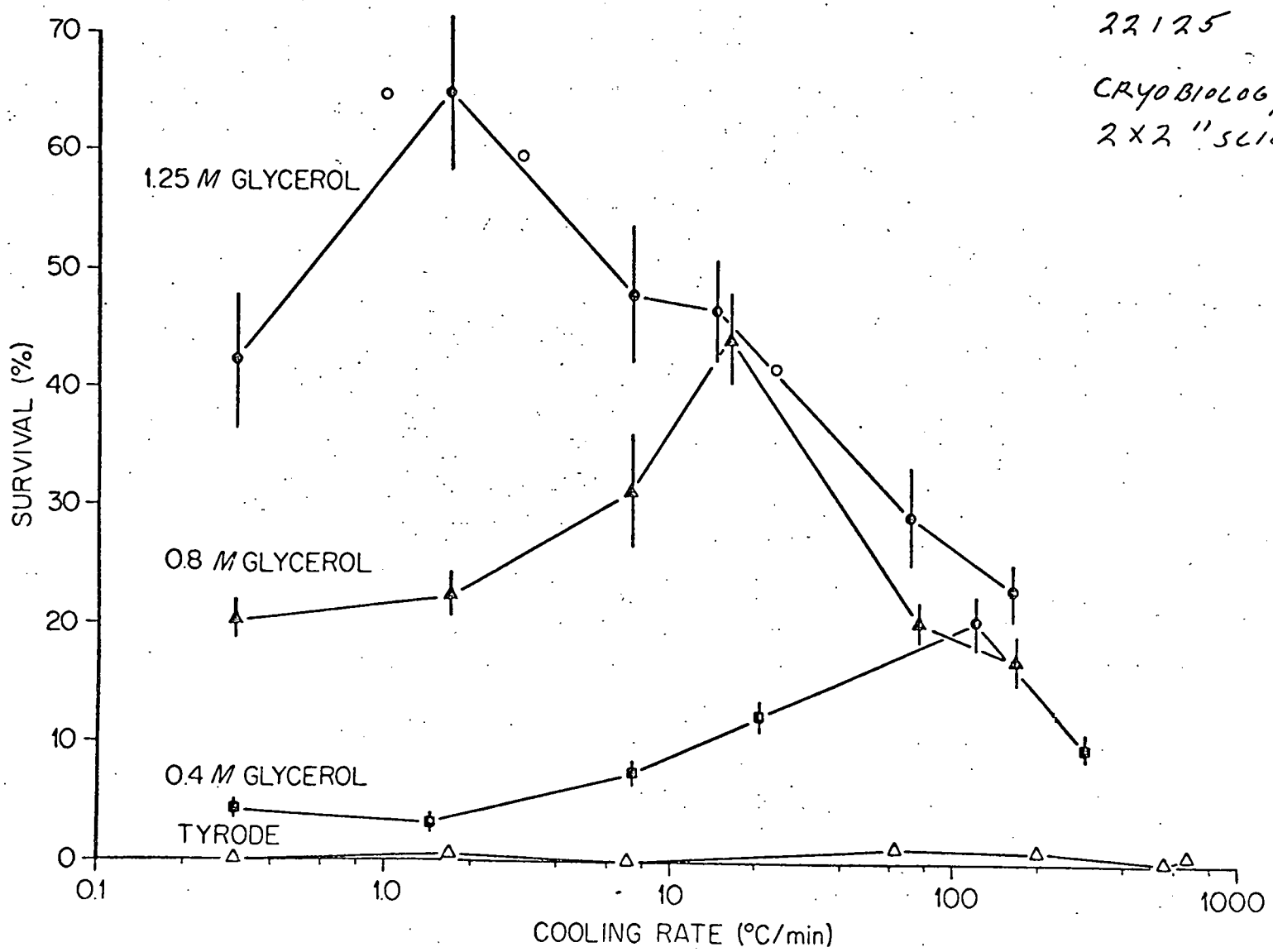
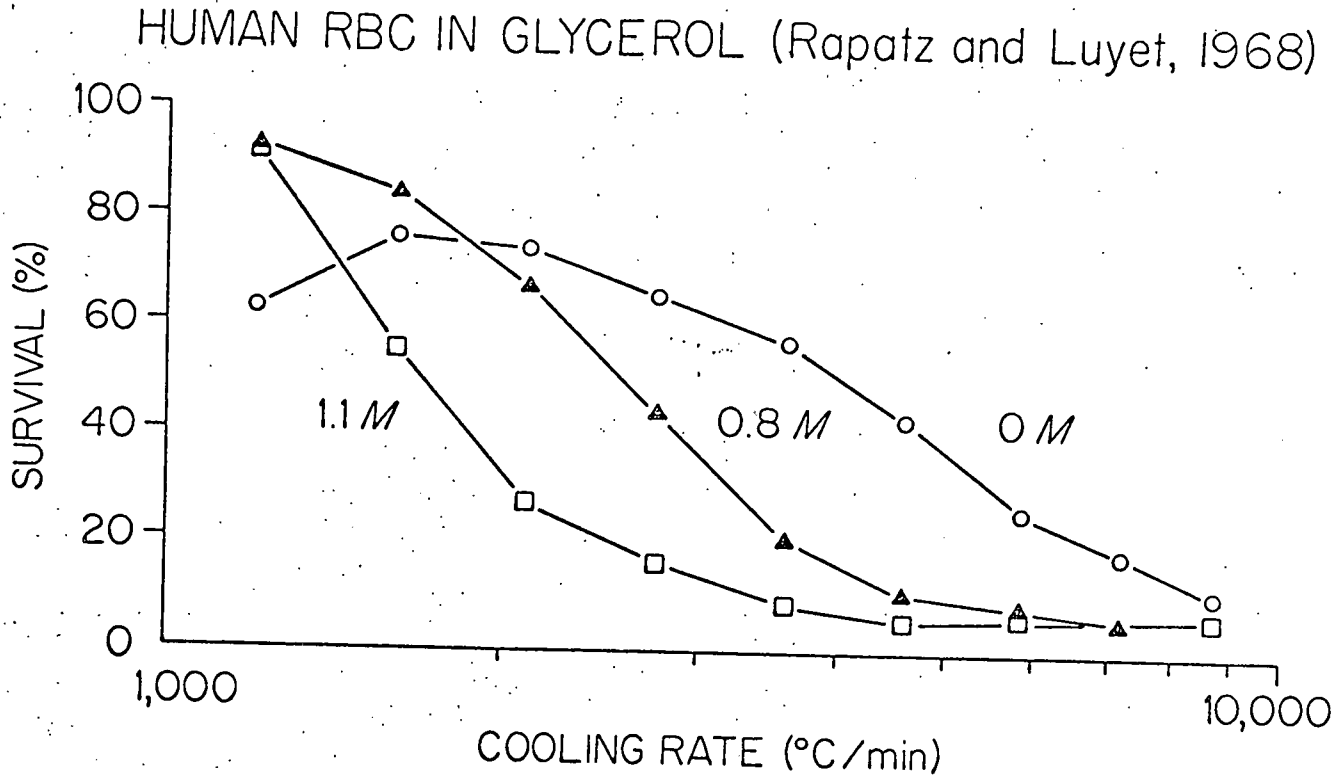


Fig. 22

31810

Fig. 23


HUMAN RBC IN GLYCEROL (Rapatz and Luyet, 1968)
SURVIVAL (%)
100
80
60
40
20
0
1.1 M
0.8 M
0 M
1,000
10,000
COOLING RATE (°C/min)

3¼"x4", 2"x2

Fig. 2

21456-1

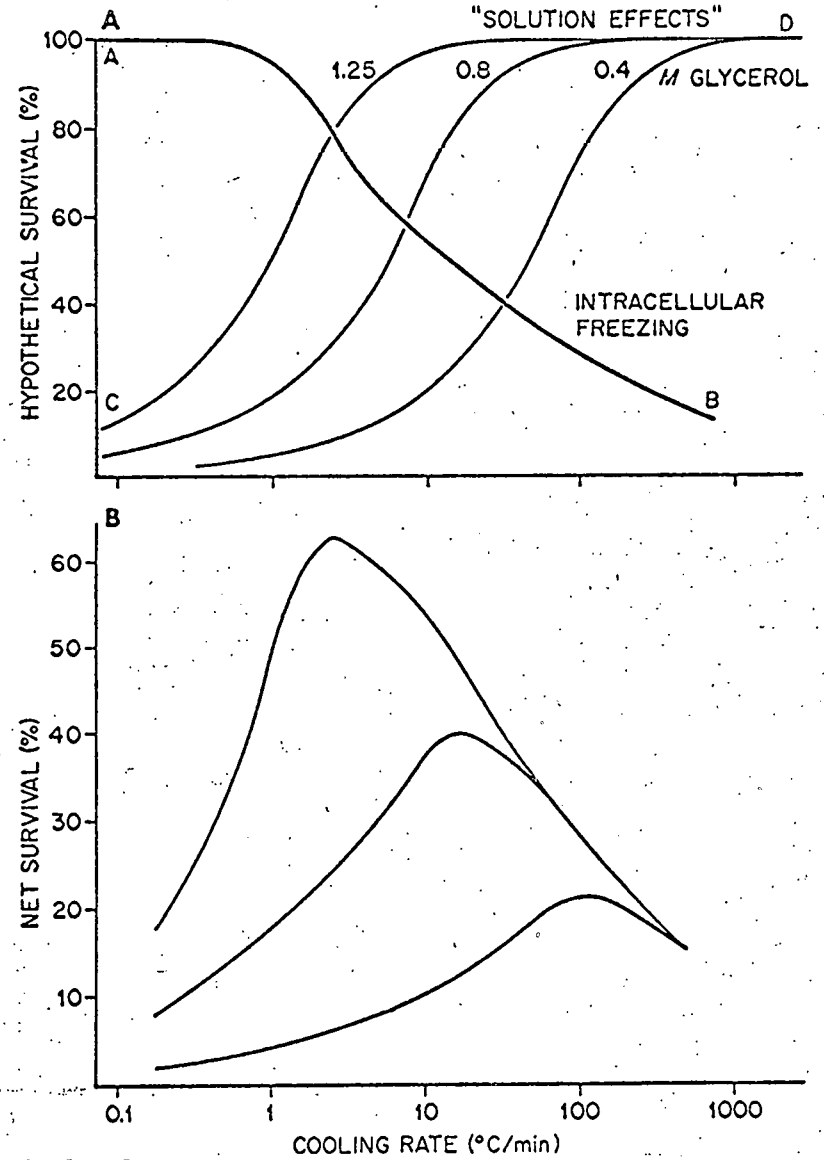


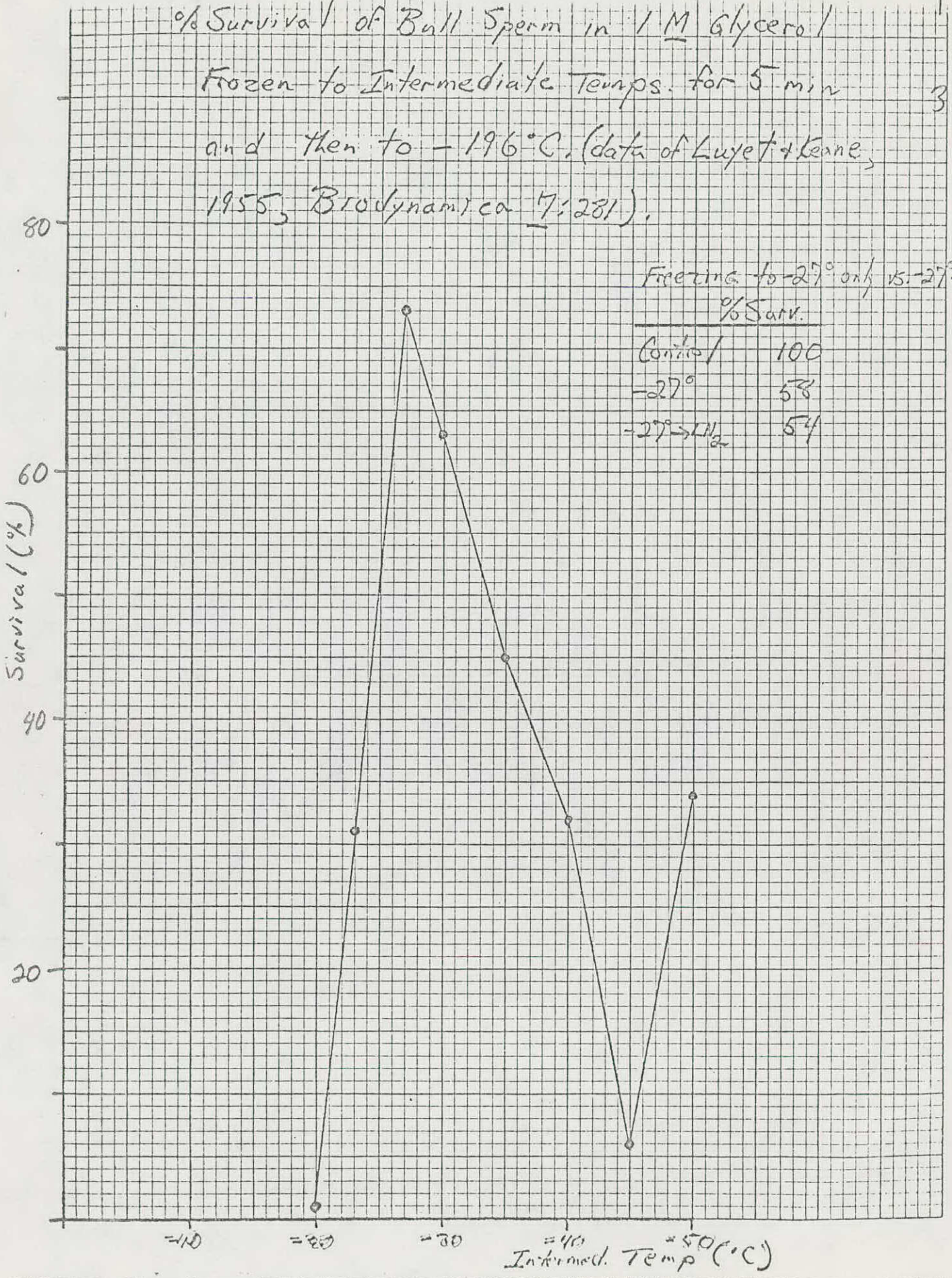
Fig. 3

Fig. 3. Hypothetical relation between survival, cooling velocity, and additive concentration on the basis of two injurious factors. A. Separate contributions of intracellular ice formation (curve AB) and solution effects (curves CD) to injury. The three CD curves show the hypothetical effects of three different concentrations of a protective additive. B. Calculated survival as a result of the interaction of both intracellular freezing and solution effects.

Fig. 24

25

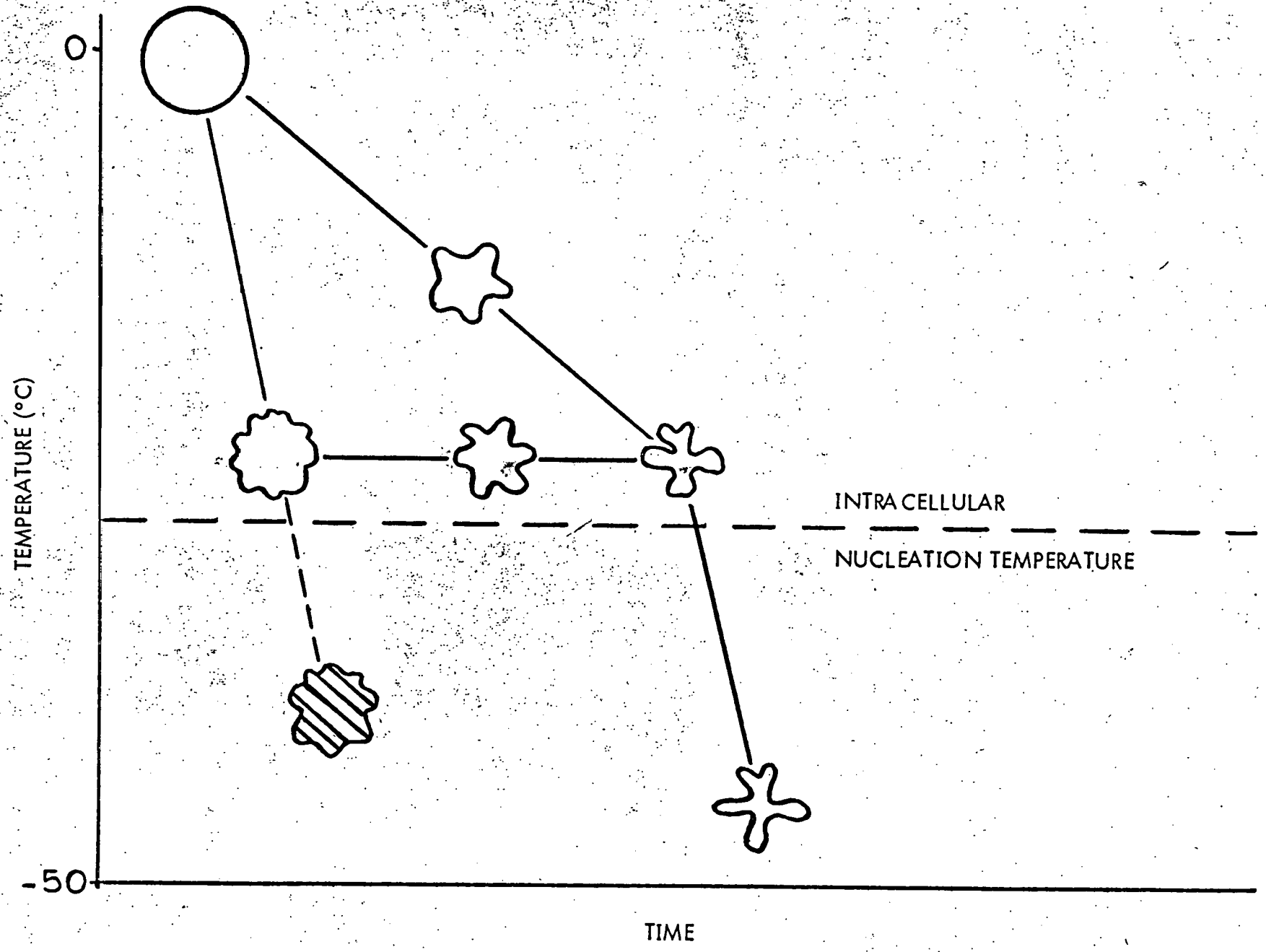
31103



9-19-75

Data of Luyet & Keane 1955 Biodynamica 7:281

$\bar{x}$ % Surv. of Bull sperm in 7% Glc. Frozen to LN_2 after 5 min at inter. temp

	Bull #	Control	-20°	-23°	-27°	-30°	-35°	-40°	-45°	-50°
Ms. %	1	85	0	27	46	48				
Ml. %		100	0	32	54	56				
	2	56	.3	7	46	47				
		100	.5	13	82	84				
	3	70	.3	9	48	48				
		100	.4	13	69	69				
	4	83	3	32	62	61				
		100	4	39	75	73				
	5	88	1	51	74	69				
		100	1	58	84	78				
	6	60				30	21	12	1	
		100				50	35	20	2	
	7	85				53	43	29		16
		100				62	51	34		19
	8	85				48	46	35		39
		100				56	54	41		46
	9	75				22	19	15	7	
		100				29	25	20	9	
	10	53				39	32	24		20
		100				74	60	45		38
	$\bar{x}$	100	1	31	73	63	45	32	6	34

TWO-STEP COOLING VS. PROGRESSIVE COOLING