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PRESERVATION OF MAMMALIAN GERM PLASM BY FREEZING

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DISCLAIMER

I. GENERAL COMMENTS ON THE FREEZING OF CELLS

Mammalian spermatozoa were first frozen successfully to "dry-ice" temperatures in 1950 (Smith and Polge, 1950), a feat that some consider to mark the beginning of modern cryobiology. The accomplishment quickly led to the successful freezing of other mammalian cells, and it quickly produced major repercussions in the cattle industry by virtue of the logistical advantages of frozen sperm in artificial insemination. The combination of AI and freezing has permitted the widespread distribution of sperm from superior sires, and has thereby been a powerful force in the development of strains of cattle with superior agricultural characteristics.

The successful freezing of bovine sperm in 1950 led immediately to attempts to freeze mammalian ova, but nearly a quarter of a century had to pass before the attempts were successful. Success was first reported by Whittingham et al. (1972) for mouse embryos, and shortly thereafter independently by Wilmut (1972). The successful freezing of cattle embryos was reported four years later by Willadsen et al. (1976).

The explanation of the 25-year lag provides an interesting example of interplay between basic and applied research. The procedures that led to the initial success in freezing sperm were partly empirical (for little was known of the mechanisms of freezing injury) and partly due to good fortune (the striking protective effect of glycerol was discovered somewhat serendipitously). But empiricism and good fortune were not sufficient for the freezing of the female genome. Success for the latter followed the evolution of some understanding of the fundamentals of cryobiological processes, an evolution that began in the early 50's, accelerated in the 60's, and is continuing now.

Contrary to the usual impression, the challenge to cells during freezing is not their ability to endure the very low temperatures required for long

storage, it is the lethality of an intermediate zone of temperature (~ -15° to -50°C) that a cell must traverse twice -- once during cooling and once during warming. No ordinary, thermally driven, reactions in aqueous systems occur at liquid nitrogen temperatures (-196°C). The only reactions that can occur are direct ionization from background radiation. It can be calculated that some 5,000 to 20,000 years would have to elapse to accumulate enough damage to kill half the stored cells (Mazur, 1976; Ashwood-Smith and Friedman, 1977). Experimental confirmation of that prediction is lacking, but there is no confirmed case of cell death ascribable to storage at -196° for some 2 to 15 years, even when cells are exposed to levels of ionizing radiation 100 times background (Lyon et al., 1977).

The problem, then, is to discover how cells can be cooled to -196°C and returned therefrom without being killed. It is now clear that they face a sequential series of challenges, any one of which can be lethal. The two most important of these challenges occur during cooling, and are critically dependent on the rate of cooling. The two are intracellular freezing and solution-effect injury, respectively (Mazur, 1970). Cells subjected to freezing are essentially subjected to enormous changes in solute concentrations and osmotic pressures, and the cells respond in classical osmometric terms. Solute permeation at temperatures below 0°C is so much slower than water permeation that osmotic response can be satisfactorily explained solely in terms of the movement of water out of the cell during cooling and into the cell during warming. Briefly, the sequence during cooling is the following: Ice initially forms in the external medium. But, because it cannot pass through the cell membrane at temperatures above about -10°C, the cell interior remains unfrozen and becomes increasingly supercooled. As cooling below 0°C progresses, more and more of the external medium becomes converted to ice, and the solute concentration in the residual unfrozen puddles

rises progressively and dramatically to multi-molar values. High solute concentrations are equivalent to low chemical potentials of water, and consequently a large difference in chemical potential tends to develop between the supercooled solution inside the cell and the progressively concentrating solution outside the cell. There are two ways that this difference in chemical potential can be eliminated: Water can flow out of the cell and freeze externally, thereby concentrating the intracellular solutes. Or intracellular water can freeze *in situ*, also thereby concentrating the intracellular solutes. Which of these two routes prevails is critical to whether or not the cell survives, and which of these two routes prevails depends critically on the cooling rate. If cooling is sufficiently slow, equilibration is achieved through water efflux. If cooling is not sufficiently slow, the cell is unable to lose water fast enough to attain chemical potential equilibrium; it becomes increasingly supercooled; and at some temperature below about -10°C it freezes intracellularly and is usually damaged lethally (Fig. 1). The two most important determiners of "sufficiently slow" are the inherent permeability of the cell to water and the temperature coefficient of that permeability (Mazur, 1977a). With a few special exceptions, a necessary condition for cell survival is that cells be cooled slowly enough to avoid intracellular freezing. "Slow enough" ranges from about $\leq$ 1°C/min or less for mammalian embryos to about 1,000°C/min for the human red cell.

(F-1

Although slow cooling is necessary for survival, it is not sufficient in the case of mammalian cells. Cooling rates slow enough to prevent intracellular freezing result in death from solution effects. Which aspects of solution effects are responsible for damage are complex and are vigorously debated. One theory is that the lethal factor is the concentration of solutes, especially electrolytes, produced by the conversion of water to ice (Lovelock, 1953a). A

second theory is that the lethal factor is the osmotic shrinkage of cells to a critical minimum volume (Meryman, 1977).

It is with respect to solution-effect injury that protective additives like glycerol exert their effect. The preponderance of evidence is that the protection is colligative -- i.e., the additives reduce the electrolyte concentration at any subzero temperature in proportion to the mole ratio of additive to electrolyte present prior to freezing (Lovelock, 1953b; Mazur, 1977b). This physical-chemical effect is dramatic (Fig. 2). Thus, to protect, an additive must be present in high molar concentrations. The concentration dependence is nicely illustrated in recent work on the freezing of fetal rat pancreases (Fig. 3). (F-2) (F-3)

For mammalian cells, then, two requirements for successful freezing are (1) a sufficiently low cooling rate to prevent intracellular ice, and (2) a sufficiently high molar concentration of a protective additive to suppress the electrolyte concentration. Two corollaries to point (2) are that the additive must be a highly soluble, relatively low molecular weight compound and it must be nontoxic in high concentration. The two compounds that to date best meet these corollary conditions are glycerol and dimethyl sulfoxide (Me_2SO).

But once again these two necessary requirements of slow cooling and high concentrations of additive may not be sufficient for survival. Success in the freezing of mouse embryos came about because of the discovery that for these cells a third requirement is that warming be relatively slow (Whittingham et al., 1972; Leibo et al., 1974). Before 1972, it had been a near universal belief that the higher the warming rate the better. Since 1972, other examples of a detrimental effect of too high a warming rate have appeared (Miller and Mazur, 1976). The explanation of the damaging effect of rapid warming is still unclear, but the simplest working hypothesis is that the damage is essentially osmotic.

It possibly arises by excess glycerol being driven into the cell during slow cooling, and then being unable to leave the cell sufficiently rapidly during rapid warming. Damage from too high a rate of warming may be just one example of osmotic traumas associated with the presence of high concentration of additives inside cells, additives that have rather low permeability coefficients. Another step in the freeze-thaw sequence where osmotic trauma is likely is during the return of cells from the high molarity freezing medium to physiological saline. There is increasing evidence that this transfer must be carried out slowly and sometimes with considerable precision to minimize damage (Valeri, 1976; Strong et al., 1974; Bank and Maurer, 1974; Mazur and Miller, 1976; Thorpe et al., 1976).

II. POTENTIALS OF FROZEN ANIMAL CELLS AND ESPECIALLY OF FROZEN GERM PLASM

The potentials of the ability to store viable cells at -196°C derive from the ability to block nearly all biological activity and change for periods of up to hundreds of years.

Genetics and Evolutionary Biology. Induced or spontaneous mutations arise in the course of laboratory and agricultural experimentation. In many instances only small percentages of these mutants can be maintained because of limitations of space, personnel, and money. The maintenance of variants in the form of reproducing colonies often puts major demands on all three. Even in cases where the heavy use of a particular mutant or strain favors its being maintained in the form of a breeding colony, the strain or variant could be lost by disease or catastrophe, and it almost certainly will become slowly altered by genetic drift. Low-temperature storage of germ plasm would ameliorate or eliminate these problems. Equally important, it can provide a powerful research tool for studying genetic drift by providing a nearly immutable standard against which to assess the magnitude of the drift.

Reproductive Physiology, Aging, and Immunology. The ability to preserve germ plasm or somatic cells opens approaches to separating time and animal age or time and generation, especially in allogeneic animals. One can, for example, collect cells (e.g., lymphocytes) from an animal when it is young and transplant them into the same animal when it is older, thereby obtaining information about such phenomena as the weakening of the immunological systems with age. Or one ought to be able to collect two-cell embryos, separate the blastomeres, freeze one of them, and allow the other to develop in a foster mother; then, when it is a mature animal, allow it to serve as the foster mother for its identical twin, which had been preserved as a blastomere in liquid nitrogen.

Freezing can also provide a method for reducing sample-to-sample and experiment-to-experiment variability by the storing of pooled frozen samples or by the pooling of samples after thawing. The pooling of frozen samples has markedly reduced experimental variability in studies on monocytes (Holden et al., 1977), and there is no reason to expect it not to be equally efficacious for mammalian ova and embryos. The ability to pool material might be especially helpful in cases where the availability of sufficient quantities of a cellular or subcellular component is limiting. This could be the case, for example, of the reactants in some enzymatic processes or the case of material from exotic animals in zoos and in the wild.

In addition, as I've already inferred, freezing would permit ova and embryonic cells to be collected at one stage of the reproductive cycle or one stage of development, and then be transferred back into the very same individual at a later cycle or at a later stage in development. Edwards and Steptoe (1977), for example, are pursuing the idea that the ability to freeze human ova may aid in providing a method for women with blocked fallopian tubes to bear children. The approach would be to collect ova from one cycle, store them in the frozen

state until the next cycle, then thaw them, carry out *in vitro* fertilization with the husband's sperm, and transfer the fertilized embryo back into the woman's oviduct. Human ova have not yet been successfully frozen, but the probability is high that success will be achieved soon.

III. STATUS OF FREEZING OF OVA AND EMBRYOS OF VARIOUS TAXONOMIC GROUPS

The success to date of freezing ova and embryos of various animal groups is surveyed briefly in a recent NAS report (Russell et al., 1978), and in the case of mammals it is discussed in detail in the proceedings of a recent Ciba Symposium (Elliott and Whelan, 1977). Embryos of mice, rats, rabbits, sheep, goats, and cattle have now been frozen successfully. "Successful" freezing means both that high percentages of the frozen-thawed embryos are able to develop in culture (where culture techniques are available), and that they are able to develop to apparently normal offspring when transferred to foster mothers. But the situation with respect to ova and embryos of oviparous and ovoviviparous animals is quite the opposite. Few, whether vertebrate or invertebrate, have been frozen successfully. This may in part reflect the fact that many attempts were made before the fundamentals of cryobiology began to evolve. But it probably reflects more the basic morphological and physiological differences between these two types of ova and ova from viviparous animals. Two obvious and probably pertinent differences are size and permeability to water and solutes.

Although there is precedent for dramatic species differences in freezing sensitivity (e.g., sperm of pig versus man), there is no inherent reason to expect great difficulties in the freezing of ova and embryos from a wide variety of mammalian species. In contrast, the successful freezing of ova and embryos from invertebrates and nonmammalian vertebrates will likely be challenging.

This brief discussion has been restricted to ova and embryos. The status of freezing of sperm from various taxonomic groups is discussed in this volume by Crabo (1978).

IV. FREEZING METHODS

As shown by the chronology of attempts to freeze mammalian embryos, the evolution of methods for the successful freezing of cells and cell aggregates can be difficult, and in some cases it could even be impossible. But once the correct values for the several critical cryobiological parameters have been uncovered, the methodology for the successful freezing of that particular cell becomes relatively straightforward.

Procedures for the freezing of mouse embryos have been described in some detail just recently (Leibo and Mazur, 1978). And the procedures used for the freezing of ova and embryos of other mammalian species are quite similar. Accordingly, the discussion here will be restricted to a synopsis of the essentials. The essential requirements are that the embryos be (a) suspended in a protective additive of sufficiently high concentration to avoid damage from solution effects, (b) frozen at rates slow enough to preclude intracellular ice, (c) frozen to temperatures low enough to permit long-term storage in an unchaged state, (d) thawed sufficiently slowly, and (e) transferred sufficiently slowly back to physiological media to minimize damage from osmotic shock.

A. Protective Additive

Embryos are collected from superovulated animals by standard procedures and placed in a balanced salt solution. To that solution is added sufficient protective additive to make its concentration 1 to 1.5 M. The additive that investigators have used most is Me_2SO, but several reports indicate

that glycerol can be equally effective, provided it is permitted to permeate, and provided that considerable care is taken to minimize osmotic shock during dilution (cf Section E below).

The question of whether additives must permeate cells to protect is a matter of basic importance and some controversy. The survival of some cells requires permeation, but the survival of others clearly does not (Mazur, 1977). Mouse embryos are intermediate: Permeation of additive does not appear to be essential but permeation does improve the percentage survival (Jackowski and Leibo, 1976; Jackowski, 1977). With Me_2SO, the question for embryos is somewhat moot in practice, since the commonly used prefreezing incubation of about 30 min at 0°C produces nearly complete permeation.

B. Cooling Rate

Cooling rates must be below about 2°C/min to prevent intracellular freezing (Leibo, 1977; Leibo et al., 1978), and survivals are generally highest when cooling is 0.5 to 1°C/min. These rates of cooling can be obtained with apparatus ranging from large double-walled tubes placed in liquid nitrogen and containing ~ 0.5 to 1 liter of ethanol (cost approximately $25) to sophisticated controllable liquid nitrogen and mechanical refrigerators (cost > $2,500). The former produce precise reproducible cooling. Their only limitation is the number of samples that can be treated.

Extensive supercooling of samples prior to ice formation will markedly change the cooling rates, and can cause all embryos in a sample to be killed by intracellular freezing (Leibo and Mazur, 1978). Extensive supercooling can be avoided rather simply by "seeding" samples with small ice crystals. Such seeding, therefore, is desirable in all cases and may be mandatory in some.

C. Final Temperature

To avoid intracellular ice, slow cooling must continue to at least -50°C, and indications are that it is desirable to continue it to about -70°C. Cooling to still lower temperatures, however, can be abrupt, and cooling to still lower temperatures is essential for long-term storage (e.g., > ~ 7 days). There are many documented cases of biological death occurring at dry-ice temperatures (-75° to -78°C). But there are no confirmed cases of biological death in liquid nitrogen (-196°). The temperatures produced in multi-stage mechanical refrigerators and in the nitrogen vapor over liquid nitrogen are below -100°C and appear satisfactory. At -196°C, embryos and ova should remain viable for decades or centuries.

D. Warming and Thawing

As mentioned, a major contributor to success in freezing mouse embryos was the discovery that rather low rates of warming are required. The exact warming rate, however, is not critical, and rates of 2° to 40°C/min appear equally satisfactory (Leibo et al., 1974). As with cooling, the procedures for achieving these rates can vary from the simple (hanging frozen samples in room temperature air) to the sophisticated and complex (Leibo and Mazur, 1978).

E. The Return to Physiological Media

The criticality of the procedures in this step depends on the protective additive present (the requirements are more critical with glycerol than with Me_2SO) and with the species (e.g., they appear more critical with rabbit than mouse [Bank and Maurer, 1974]). The chief problem is to dilute at an appropriate temperature in such a way and at such a rate that intracellular additive can flow out of the cell without the cell undergoing osmotic swelling to a deleterious extent. Temperature is important through its influence on permeability coefficients. Two approaches to dilution are discussed by Leibo and Mazur (1978).

While the requirements are not overly stringent or technically difficult in any individual step, all of the steps must be carried out appropriately if one is to obtain high percentage survivals. When the overall procedure is carried out appropriately, embryo viability will usually exceed 90%.

Embryo viability can be assessed in several ways. The assay most relevant to most of the eventual uses of frozen embryos is the ability of thawed embryos to develop to term after transfer to foster mothers. A faster, cheaper, and more quantitative assay of function is cleavage of embryos in culture, especially when culture conditions exist that permit in vitro development to the blastocyst stage. Other assays that have been used are fluorescence (i.e., the ability of a cell to reduce fluorescein diacetate) (Jackowski, 1977) and morphological appearance (Whittingham et al., 1972). In the mouse, all four assays correlate exceedingly well. In the cow, our experience has been that morphological appearance does not correlate well with in vitro development (Leibo and Mazur, unpublished).

In conclusion, embryos of several mammalian species can be frozen to -196°C (or below) by procedures that result in the thawed embryos being indistinguishable from their unfrozen counterparts. The survival often exceeds 90%, and in liquid nitrogen it should remain at that high level for centuries. Sublethal biochemical changes are also precluded at -196°C.

Radiation-induced ionization can occur, but theoretical arguments indicate that they will result in very small and probably immeasurable numbers of mutations even after decades of storage. No developmental abnormalities have been detected in mouse offspring derived from frozen-thawed embryos (Maurer et al., 1977), and, since all the manipulations are carried out on the preimplantation stages, none would be expected (Austin, 1973). The ability to

maintain mammalian ova and embryos in an unchanged state for days to decades has potential uses in genetics, reproductive physiology, biochemistry, and developmental biology. These potentials do not as yet exist for non-mammalian ova and embryos, since very few have as yet been successfully frozen.

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FIGURE LEGENDS

Figure 1. Intracellular freezing of unfertilized mouse ova in 1 M Me_2SO versus the cooling rate. Intracellular freezing is indicated by the sudden darkening at ~ -40°C of the ova cooled at 2.4 and 32°C/min. No intracellular freezing is observed in ova cooled at 1.2°C/min. Photomicrographs from Leibo, 1977, and Leibo et al., 1978 (reprinted by permission).

Figure 2. Concentration of salts produced in the unfrozen portions of aqueous solutions of glycerol in buffered saline as a function of temperature. The buffered saline consisted of 0.148 M NaCl and 0.01 M phosphate buffer. (From Rall et al., 1978, by permission of the Biophysical Journal.)

Figure 3. Survival of frozen-thawed 16-1/2- to 17-1/2-day fetal rat pancreases as a function of the Me_2SO concentration in the suspending medium. (From Mazur et al., 1976, by permission of the National Academy of Sciences.)

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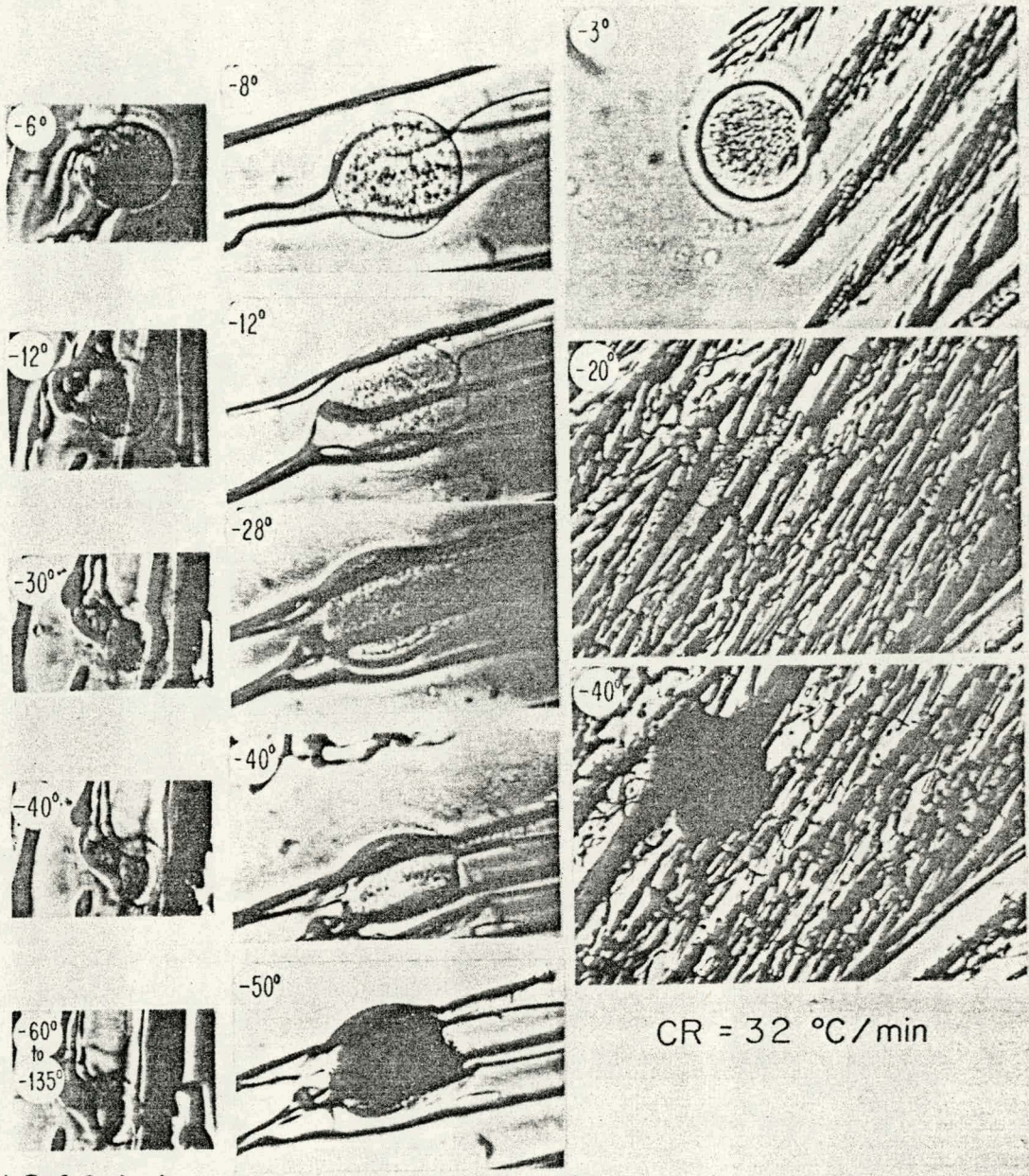
MOUSE OVA–INTRACELLULAR FREEZING vs COOLING RATE*



CR = 1.2 °C/min

CR = 2.4 °C/min.

CR = 32 °C/min

*LEIBO ET AL. (1976).

Modified from 31366, 31368, 31370

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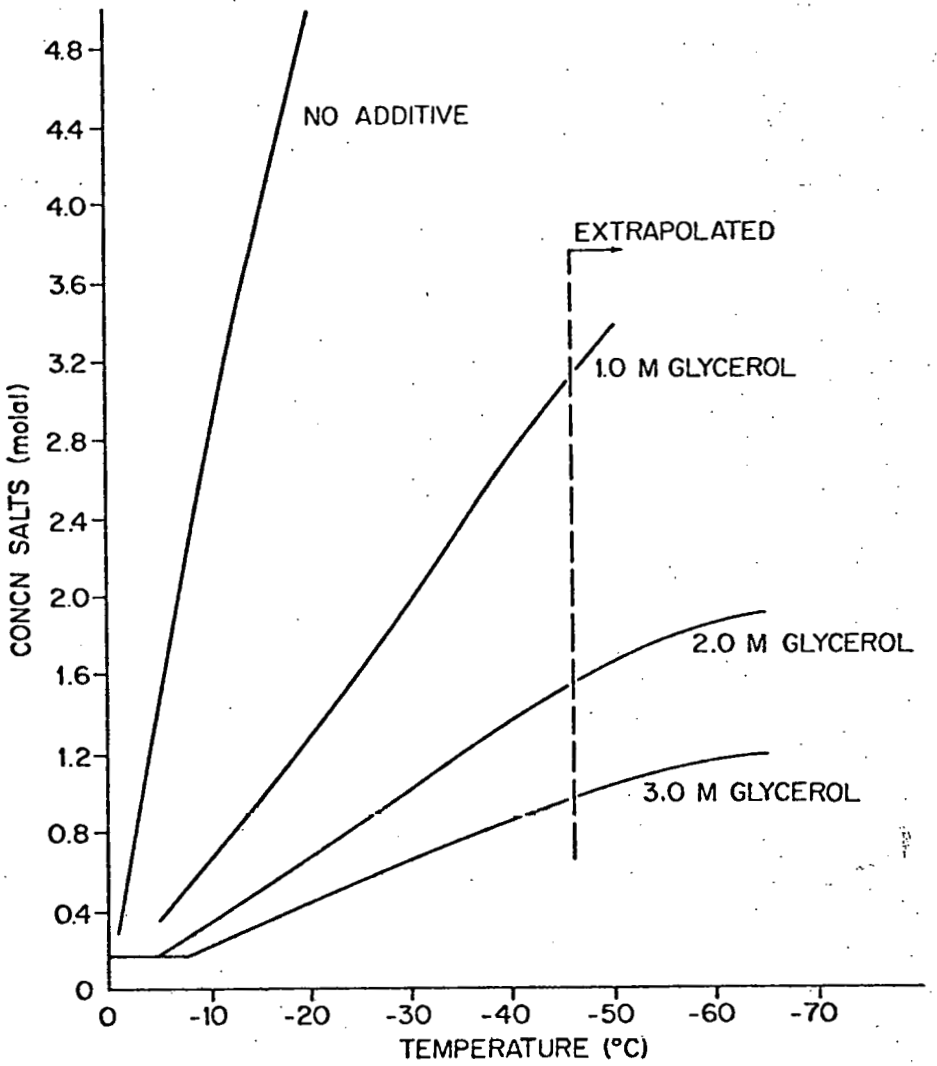

fig 6

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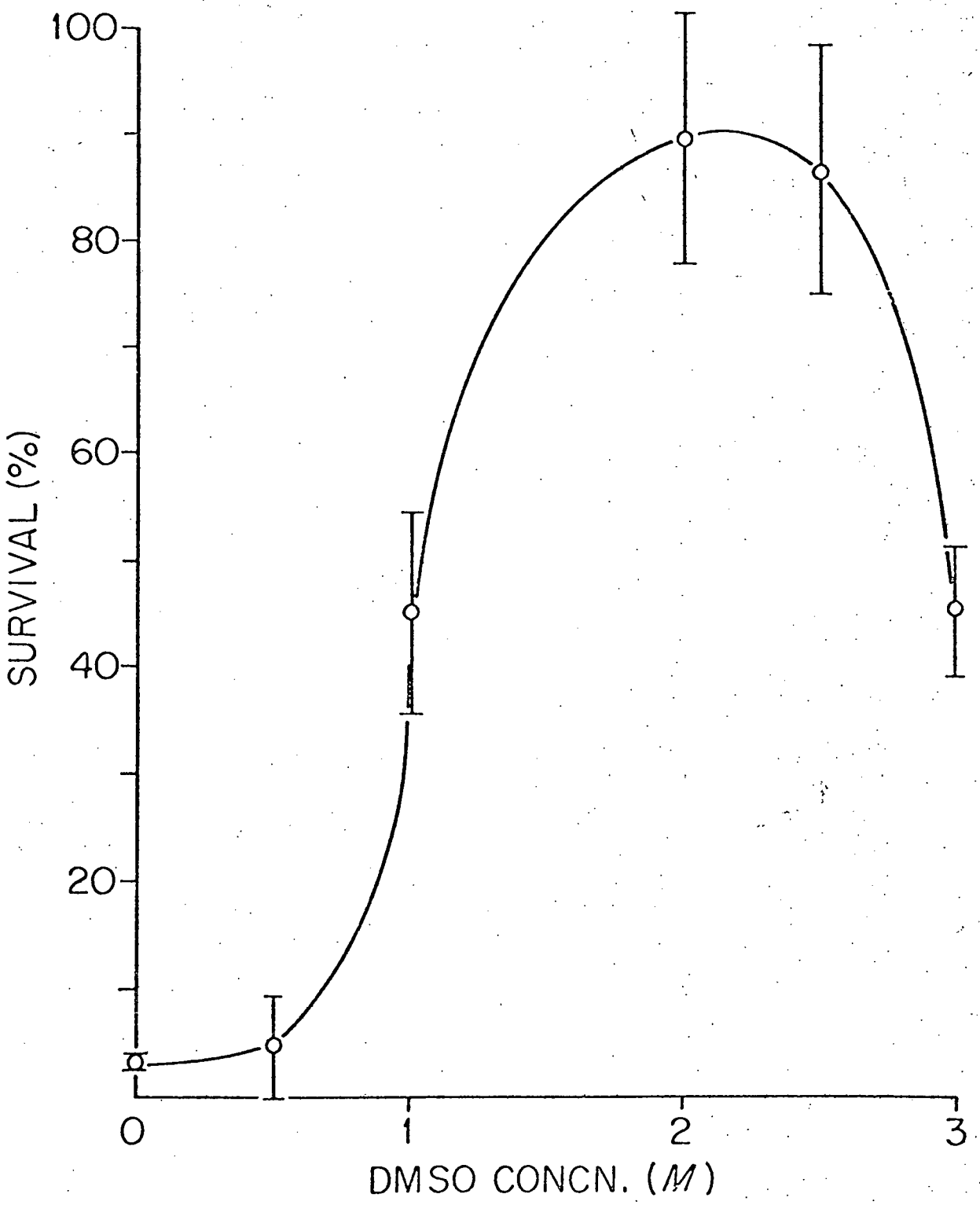
FREEZING OF FETAL RAT PANCREAS



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