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Contributions of Cooling and Warming Rate and Developmental Stage to the Survival of *Drosophila* Embryos Cooled to −205°C[1,2]

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Because of their high susceptibility to chilling injury, permeabilized *Drosophila* embryos can not be cryobiologically preserved by slow freezing at rates low enough to prevent the formation of intraembryonic ice. Calculations indicated that to outrun the chilling injury they must be cooled and warmed rapidly at an estimated 20,000°C/min or faster. Ordinarily, such cooling rates would inevitably produce lethal intracellular ice. To prevent this, embryos must contain and be surrounded by sufficiently high concentrations of glass-promoting solutes to induce vitrification on cooling and prevent devitrification on warming. Like Steponkus *et al.* (*Nature* **345**, 170, 1990) we have used ethylene glycol as the solute and have exposed permeabilized 12-h embryos to it in two steps. (Permeabilization was effected by exposing dechorionated embryos to a mixture of 0.3% 1-butanol in *n*-heptane for 90 or 110 s.) The two steps were (i) a 30-min exposure to 2 *M* ethylene glycol at 23°C and (ii) a 5-min exposure to 8.5 *M* ethylene glycol [±10% polyvinylpyrrolidone (PVP)] at 5°C. The volumetric response to the first step indicates that full permeation of the 2 *M* glycol has been approached by 30 min. The point of the second step is to raise the intraembryonic concentration of ethylene glycol to near 8.5 *M* ethylene glycol by osmotic dehydration. Survival based on hatching is some 45% at this point. When 12-h embryos in 8.5 *M* glycol containing 10% PVP are then cooled to −205°C at ~100,000°C/min and warmed at about that rate, an average of about 12% survive (hatch), although in about half the runs 15–29% survive. Survivals in the absence of PVP are usually poorer but have been as high as 40%. Currently, 5% of the surviving larvae develop to adult flies (Steponkus *et al.* reported 18% hatching and 3% development to adult). Embryos that develop but do not hatch show readily detectable abnormalities in mouth parts and dorsal closure. Very high warming rates are much more critical to survival than are very high cooling rates; for example, none survive when warming is 2000°C/min. The deleterious effect of slow warming is exerted between −80 and −40°C. The lack of reciprocity between the effects of time spent cooling and time spent warming argues against ascribing death to chilling injury. Rather, it and other data argue for ascribing death to the devitrification during warming of cytoplasm that vitrified during cooling. Various lines of evidence including calorimetry indicate that a warming rate of ~100°C/min is high enough to prevent devitrification of 8.5 *M* ethylene glycol + PVP (54 wt % glycol). The fact that survival requires much higher warming rates strongly suggests that that concentration of glycol is not being attained in the embryos as a whole or in compartments within the embryos. Superimposed on the effect of warming rate is an effect of embryo age. The survival of 14- to 15-h embryos is more than double that of 12-h embryos.

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World wide some 10,000 to 20,000 lines of mutant *Drosophila* are maintained by frequent repeated transfer of breeding stocks. Maintenance of stocks in this manner is time consuming and costly, and it can result in genetic drift or the loss of stocks as a result of poor reproductive capacity, accident, mixup, or contamination. The ability to cryobiologically preserve such stocks indefinitely would therefore be of substantial value.

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The classical approach to successful cryobiological preservation involves slow or equilibrium freezing. Cells tend to supercool even in the presence of extracellular ice, and the supercooling establishes a chemical potential or osmotic pressure difference between cell water and that in the exterior. In response to this differential, water flows out of the cell and freezes externally. If the cell is cooled slowly enough, the progressive osmotic efflux of water keeps the cell near its equilibrium water content and minimally supercooled. If the cell is not cooled slowly enough, it becomes extensively supercooled and eventually undergoes intracellular freezing when cooled to its nucleation temperature. Since such intracellular ice formation is usually lethal, a requirement for successful cryobiological preservation by this classical approach is that a cell or cell aggregate be cooled slowly enough so that its water content is reduced to near the value required to abolish supercooling before it has cooled to its nucleation temperature. The quantitative value of "slowly" enough can be computed from the physical–chemical differential equations that determine the driving force for efflux and the differential equation that determines the rate of water loss in response to the driving force (9, 12).

Although cell dehydration is necessary to avoid intracellular freezing and consequent death, it is rarely sufficient for high survival. Cryoprotective solutes must be present to protect against slow freezing injury, the genesis of which appears to be the decrease in the size of the unfrozen channels in which the cells lie as water becomes progressively converted to ice (10), the resultant increased solute concentration in those channels, or the osmotic shrinkage of cells in response to the increased solute concentration (19, 25). The protection conferred by solutes like glycerol, ethylene glycol, and dimethyl sulfoxide is related to their colligative effects on one or more of these events. To protect, the solute must usually be in the cells as well as around them.

Thus, to be successfully preserved by the slow freezing approach, the cell or cell aggregate must be permeable to both water and the cryoprotective solute. Unfortunately, the intact *Drosophila* embryo is permeable to neither because of waxes in the vitelline membrane that surround the embryo proper. Fortunately, first Lynch *et al.* (8) and then we (11) developed methods for permeabilizing high percentages of 12- to 14-h embryos with high survival, in our case by exposing them for precise times to the alkane heptane containing low and precise amounts of alcohol. The treated embryos are permeable to water and to the cryoprotectant ethylene glycol.

This then in theory should permit them to be preserved by the equilibrium freezing approach just described. Myers *et al.* (18) have calculated that the avoidance of intracellular freezing would require a cooling rate of <1°C/min. This point raises the second formidable barrier to the cryopreservation of *Drosophila;* namely, intact (and presumably permeabilized) embryos are highly chill sensitive in the absence of any external or internal ice, and they become increasingly sensitive as the temperature falls toward -25°C (14, 15, 18). The sensitivity becomes so high that intact 12- to 14-h embryos are all killed by -25°C when cooled at the low rates needed to prevent intracellular freezing (15). Younger embryos are even more sensitive. Similarly, when permeabilized 13-h embryos are equilibrated with 1.5 or 2 *M* ethylene glycol and cooled at these rates, none survive cooling to -35°C (6a).

Calculations using the experimentally determined activation energies between 0 and -25°C indicate that the temperature at which killing from chilling occurs can be suppressed by cooling and warming at very high rates. For example, the median lethal

temperature will be reduced to −65°C if the cooling and warming rates are 20,000°C/min (15). Unfortunately, in the presence of the usual concentrations of cryoprotectants (~1 to 1.5 *M*), lethal intracellular freezing will be induced in *Drosophila* at some 10,000-fold lower cooling rates. The only way to avoid ice at such high cooling rates is to introduce very high concentrations (i.e., >50 wt%) of glass-inducing solutes in and around the embryos. The solutes must not only be permeating, but because of the high concentrations required they must also have low toxicity. Steponkus *et al.* (26) reported that 8.5 *M* ethylene glycol partially meets the requirements when added in two steps: (i) 2.1 *M* glycol is allowed to "fully" permeate the embryos at room temperature, and (ii) the intracellular concentration of ethylene glycol is then raised to high levels by the osmotic removal of water, which is achieved by exposing the embryos to 8.5 *M* glycol for 5–8 min at 0°C. The embryos are then cooled at ~25,000°C/min in nitrogen slush at −205°C and warmed at similar rates. Using this approach, they were the first to report survivals of embryos cooled to such temperatures. Under their best conditions, a mean of 18% hatched. However, only 3% of the resulting larvae were able to develop to adults.

In this paper we report data that confirm their findings and further elucidate the mechanisms of injury. They concluded that damage could be a consequence of chilling injury, ice formation during cooling, or ice formation (devitrification) during warming. We conclude that the chief cause of injury is the third of these possibilities; namely, that critical portions of vitrified embryo cytoplasm devitrify during warming because of insufficient intraembryonic ethylene glycol. The chief experimental basis for that conclusion is that survival is much more dependent on very high warming rates than it is on very high cooling rates. This demand for high warming rates is consistent with the existence of a deficiency in the concentration of intraembryonic glycol.

METHODS

Rearing of Flies and Obtaining Eggs

Flies of the Oregon R-P2 strain of *Drosophila melanogaster* were maintained by the method of Travaglini and Tartof (29) at 24–25°C with minor modifications. To obtain eggs for experimental use, trays of 2% agar smeared with a paste of rehydrated active dry yeast were placed in a fly cage for 3 h in earlier experiments and 1 h in later experiments.

At the end of the collection period, the eggs were washed off the agar trays with room-temperature distilled water and passed through appropriately sized USA Standard Testing Sieves to reduce contamination by adult body parts and yeast. The collected eggs were spread on moistened filter paper in a 100 × 15-mm polystyrene petri dish (Falcon 1001). Approximately 1000 eggs were then transferred by camel's hair or sable brush in a monolayer to each of some 8–10 gridded Nuclepore or Poretics polycarbonate (PC) membrane filters (25 mm diameter, 10 or 12 μm pore size). Just prior to permeabilization, the weight of eggs was measured and eggs were removed or added so that the egg mass on the filter was between 6 and 8 mg [650–850 eggs (13)]. Mazur *et al.* (11) showed that permeabilization was impaired when the number of eggs exceeded 1000. The PC filters were then placed on moistened filter paper in a Falcon 1001 petri dish.

Obtaining Embryos at the Desired Developmental Stage

To obtain 12-h embryos [stage 14 (32)], the dish containing the eggs was held for combinations of time at 24, 22, and 17.5 ± 0.5°C calculated to produce the desired developmental stage at about 0830 h the following morning, considering time 0 to be

the midpoint of the 1- or 3-h egg-laying period. In making these calculations the respective development rates at the three temperatures were taken from data in Ashburner and Thompson (1) to be 0.9, 0.75, and 0.5 × the rate at 26°C. Staging was confirmed by morphological appearance of the embryos after permeabilization (32). Most were early stage 14. Fewer than 10% were stage 15. At the conclusion of the overnight incubation at 17.5°C, the dish containing the embryos was placed in a 4°C refrigerator until the initiation of permeabilization. Storage of 12- to 15-h embryos at 0°C produces no decrease in viability for up to 16 h (15). During the 6–7 h at 4°C, slow development occurred as evidenced by an increase in the percentage of stage 15 embryos to about 25% by late afternoon.

Permeabilization Procedure

The optimal procedure from Mazur *et al.* (11, Fig. 11) was used. Embryos sandwiched between two Nuclepore or Poretics filters are placed in a Millipore Swinnex holder (SX00-02500). They are dechorionated by 2.6% sodium hypochlorite and rinsed thoroughly with 200 ml of water, and the water is removed by isopropanol. Traces of isopropanol were removed by 2 min air drying (a critical step), and the permeabilization was effected by 90 or 110 s exposure to a mixture of *n*-heptane and 0.3% or 0.4% 1-butanol (time and concentration of alcohol are both critical). Pure *n*-heptane (15 ml) was then passed through the filter sandwich, and the filters were separated and quickly transferred to successive washes in D-20 *Drosophila* tissue culture medium (3) as modified by Zalokar and Santamaria (33) during which time it was demonstrated the residual alkane evaporated. The permeabilized sample was held at 23–24°C in a lidded 35 × 10-mm polystyrene culture dish (Falcon 3001) for 45 min (30 min in a few instances), in most cases on the bench top, but in later experiments in a sealed box with a relative humidity near 100%. This incubation produces higher survivals of permeabilized eggs.

Assessment of the Degree of Permeabilization

An aliquot of 100–150 eggs was removed from the filter by camel's hair brush, initially prior to the 45-min incubation, but later after the incubation, and transferred to a second filter. This second filter was floated with the eggs face up on a solution of 0.1% rhodamine B in D-20 for 5 min, during which time additional rhodamine solution was gently dropped over the eggs. The filter and adherent eggs were then washed free of rhodamine and the eggs were scored at 20× magnification under a microscope to determine whether they were ruby red, dark pink, light pink, or unstained. Mazur *et al.* (11) showed that this color sequence correlates well with the rate at which the embryos undergo osmotic dehydration in 0.75 *M* sucrose in D-20. This dehydration in sucrose, which takes place in 1–6 min in red-stained eggs, demonstrates that they have become permeable to water. Analogous experiments in ethylene glycol showed that they have also become permeable to ethylene glycol and to glycerol, although permeation is substantially slower in the latter. They appear impermeable to sucrose.

The parallel unstained filters at this point were often cut in half. One-half filter from the four to five permeabilization runs of that day was incubated without further treatment to determine percentage hatching and, in some later experiments, the percentage of larvae that developed to adults.

Exposure to Ethylene Glycol and Other Nonelectrolytes

Prior to cooling to cryogenic temperatures, the standard treatment was to hold embryos in 2 *M* ethylene glycol for 30 min at room temperature followed by an exposure for 5 min (4.5–5.5 min) on ice (sample temperature, 5°C) to 8.5 *M* ethylene glycol,

often containing 10% (w/v) of the polymer polyvinylpyrrolidone (PVP) or occasionally 6 or 12% (w/v) bovine serum albumin (BSA). Details are as follows: The bottom surface of the PC filter with its adherent eggs was wiped on the rim of the Falcon 3001 dish as it was removed from the D-20, in later experiments placed on absorbent paper for 2–3 s, and then floated with the eggs face up on 2 *M* ethylene glycol in D-20 in a second 3001 Falcon dish. About 0.1 to 0.2 ml of additional 2 *M* ethylene glycol was dropped several times onto the eggs, and the eggs were allowed to remain in contact with that solution at 23–24°C, usually for 30 min, on the bench top or later in the sealed high-humidity box.

Toward the end of the exposure to 2 *M* glycol, 1 ml of concentrated vitrification solution (usually 8.5 *M* ethylene glycol and 10% (w/v) PVP) that had been prechilled to 0°C in ice was transferred with a chilled pipette to a 3001 Falcon dish resting in a 140-mm glass petri dish containing ice and water and in turn resting on ice. The PC filter and adherent eggs were removed from the dish containing 2 *M* ethylene glycol, wiped on the rim, blotted ~2 s on absorbent paper, and floated on the cold 8.5 *M* ethylene glycol. About 0.1 to 0.2 ml portions of that cold glycol were dropped on top of the eggs several times. The measured temperature of the solution after these manipulations was 5°C. The wipings and blottings between transfers were to minimize dilution of the target solution by carryover. The same procedures were used for concentrated glycerol and sucrose solutions.

The composition of the chief concentrated solutions is given in Table 1 in various units. Molar solutions were prepared by adding D-20 solution to the required weight of ethylene glycol, glycerol, sucrose, PVP, or BSA to make a given volume of solution. Molal solutions were prepared by adding a kilogram (or appropriately smaller mass) of D-20 to the required number of moles or grams of the above sol-

TABLE 1
Principal Solutions Used in the Study[a]

Solute	Polymer	Molarity (mol/liter) (solution) (*M*)	Molality (mol/kg) (H_2O) (m)	wt solute(g)/ 100 g solute + H_2O (w/w)%
Ethylene Glycol	—	2	2.25	—
	—	6.5	10.06	38.4
	—	8.5	15.85	49.6
	PVP[b]	8.5	18.68	53.7
	—	9.0	17.69	52.3
	—	9.5	19.75	55.1
Glycerol	—	5	7.78	41.7
	—	6.5	12.16	52.8
	—	8.5	21.84	66.8
Sucrose	—	1.0	1.27	30.3
	—	1.5	2.21	43.1
	—	2.0	3.50	54.6
	—	2.5	5.43	65.0
	—	3.0	8.58	74.6

[a] The solvent added to these solutes was D-20 (0.26 osmolal). For purposes of calculating molalities and weight percents, a kilogram of D-20 was considered equivalent to a kilogram of water.

[b] The concentration of PVP in this solution is 10 g PVP/100 ml solution = 22.0 g PVP/100 g D-20.

ute (The D-20 was considered equivalent to water). Interconversions among molar, molal, and weight percent of ethylene glycol, glycerol, and sucrose in water were based on polynomial fits to density data in the 63rd edition of the Handbook of Chemistry and Physics. Interconversions for solutions containing PVP were based on measured densities. Osmolalities of glycerol and sucrose were computed using the osmotic coefficients determined by Scatchard *et al.* (23). Osmolalities of ethylene glycol solutions are based on the freezing point depressions given in the Handbook for various weight percents of solute in water. The PVP was Plasdone C-30 from GAF. It has a viscosity average mol wt of 38,000. The other chemicals were HPLC or ACS certified grades, from Fisher and Sigma.

Exposure to Low Subzero Temperatures

In what we shall refer to as the standard procedure, the Nuclepore filter with its adherent embryos was removed from the concentrated ethylene glycol vitrification solution, wiped on the rim of the Falcon dish, blotted once or twice for several seconds on absorbent filter paper, and then abruptly plunged into a mixture of solid and liquid nitrogen (N_2-slush) at about -205°C using fine watchmakers forceps as a handle. The N_2-slush was prepared a few minutes before a run by putting boiling liquid nitrogen (LN_2) into a strip-silvered Dewar and applying laboratory line vacuum (~ 15 mmHg) to the Dewar. Evaporative cooling resulted in the formation of a substantial amount of solid nitrogen. Variations in the cooling procedure included omissions of the blotting, plunging into boiling liquid nitrogen, and abrupt application of the filter to the smooth surface of a bronze or copper cylinder that had been prechilled to -196°C.

Some experiments involved cooling at much lower rates. In those runs, the PC filter and its adherent embryos were transferred from the concentrated ethylene glycol vitrification solution to a 1-mm circular depression in a circular bronze plate. The plate, which was on ice, was 89 mm in diameter and 8 mm thick. A matching bronze lid (89 mm diameter and 6 mm thick) was applied, and the lidded plate was then placed on an 89 × 45-mm bronze cylinder immersed to near its top in LN_2. Both the plate and its lid had 36-gauge copper-constantan thermocouples inserted into small holes drilled in the rim.

Continuing with the standard procedure, after 20–60 s in N_2-slush (time at that temperature is irrelevant) the Nuclepore filter with its adherent eggs was as abruptly as possible plunged into about 40 ml of a solution of 0.75 *M* sucrose in D-20 at 24°C. After ~10 s, it was then transferred to a Falcon dish containing 1–2 ml of that same solution where it remained for a total of 2 min. Variations in this procedure included transfer from N_2-slush to LN_2 before rapid warming in D-20/sucrose, and transfer from N_2-slush or LN_2 to the surface of a rectangular copper plate (50 × 120 × 12 mm) resting on the bench top at room temperature.

Some experiments involved warming at much lower rates. In one variant, the lidded bronze plate described above was transferred from the bronze cylinder at -196°C to a bronze cylinder of the same dimensions partly immersed in room-temperature water. The resulting warming rate was 50°C/min between -120 and -20°C. Another variant used the same approach except that the lidded bronze plate was much smaller (57 mm diameter, 5 mm thick). The warming rate with it was ~200°C/min. The third variant was to remove the Nuclepore or Poretics filter from N_2-slush or LN_2 and hold it for 10 s in room-temperature air. The resulting warming rate between -150 and -20°C was 1900°C/min. When warming was completed in all these variants, the Nuclepore or Poretics PC filter was transferred to a Falcon dish with 0.75 *M* sucrose in D-20 for a total of 2 min.

Measurement of Cooling and Warming Rates

Rates of 300°C/min and below were measured during experiments by 36-gauge copper-constantan thermocouples attached to a Leeds and Northrup potentiometric recorder. The measurements of higher cooling and warming rates were obtained using a Nuclepore filter through which a 40-gauge copper constantan thermocouple had been threaded and fixed in position with 3 μl cellulose acetate (clear nail polish). A pair of No. 5 watchmaker forceps held closed with a small O-ring served as the handle. The filter was subjected to the same exposure to concentrated ethylene glycol + PVP and the same wiping and blotting procedures as used in the standard experimental procedures. It did not contain eggs as the mass of the nail polish was similar to the mass of adherent eggs. The reference junction of the thermocouple was placed in an ice bath, and the copper leads were attached to a Data Translation DT-707T interface panel which in turn was connected to a DT-2805 analog and digital I/O board in a Tandy 2025 AT computer with a 286 microprocessor. Data were acquired at a sampling rate of 2000 Hz and a gain of 500. The acquisition control software was written in FORTRAN 5.0 (Microsoft Corp) using the Data Translation PC-LAB 3.02 subroutine library. The acquired voltages were median-filtered prior to being converted to temperatures on the basis of polynomial fits to the emf–temperature relation for copper-constantan thermocouples given by Powell *et al.* (20). Subsequent data processing was performed with FORTRAN programs developed for that purpose. The sampling rate during data acquisition was sufficiently high that the system could determine the cooling rate of a bare 40-gauge thermocouple plunged into N_2-slush; namely, 10^6 °C/min.

The measured cooling rates between −20 and −150°C for a filter plunged into N_2-slush and LN_2 were 110,000 ± 13,000 (N = 14) and 55,000 ± 6000°C/min (N = 7), respectively. The measured warming rates between −150 and −20°C for an ethylene glycol-treated filter subjected to the standard wipe and blot and transferred from LN_2 into D-20/sucrose was 120,000 ± 15,000°C/min (N = 7). We emphasize that these rates apply to the junction of the thermocouple. The rates experienced by individual embryos could well be a factor of 2 or more different.

Assessment of Development and Hatching

Embryos that were exposed to 2 *M* ethylene glycol solutions for times sufficient for it to permeate were, regardless of subsequent experimental treatment, finally placed in a solution of 0.75 *M* sucrose in D-20 for 2 min to permit the efflux of a portion of the intraembryonic glycol. The PC and adherent embryos were then transferred to three successive No. 3001 Falcon dishes containing D-20 alone, and finally to a third dish containing 0.2 or (later) 0.4 ml of D-20 containing 20 μg/ml of gentamicin (Sigma) to prevent contamination by what was identified as *Pseudomonas fluorescens*. Tests showed that two- to threefold higher concentrations of the antibiotic were not injurious. This last dish was capped and placed on filter paper moistened with 0.14 *M* NaCl in a 100-mm Falcon 1001 petri dish. The larger dish was in turn covered with its lid and placed in a sealed 30 × 30 × 30-cm Lucite box, the door of which was clamped against gaskets on the frame. The box contained a sheet of blotting paper on the bottom side of a perforated shelf. One end of the blotting paper was immersed in a reservoir of water and sand to keep it saturated. The box was kept in an incubator held at 24 ± 1°C.

The embryos were incubated in this system for 20–30 h and then scored to determine the number that remained unhatched and a qualitative estimate of the number of larvae. The number of observable larvae was not used to calculate percentage sur-

vivals, since some could not be recovered. We also scored the number of nonhatching embryos that showed development as evidenced chiefly by the appearance of air-filled tracheal tubes or movement within the vitelline membrane. The term "percentage development" refers to the number developing but not hatching plus the number hatching, all divided by the total number of embryos present.

At the time of counting, a sample of the D-20 incubation medium was removed and its osmolality determined with a Wescor 5500 vapor pressure osmometer to ensure that there had been neither substantial concentration by evaporation nor dilution by condensation of water in the D-20 during the 20–30 h of incubation. In most cases the measured osmolality was between 258 and 290 mosmolal. The osmolality of D-20 is 260 mosmolal.

It should be noted that the permeabilized embryos were not incubated under mineral oil as others have done because Mazur *et al.* (11) determined that the percentage hatching of permeabilized embryos was actually higher in the absence of mineral oil provided that the above precautions were taken to keep the air surrounding the embryos at very high humidity.

Development of Larvae to Adult Flies

In late morning of the day following an experiment, larvae that had emerged were transferred by camel's hair brush from the PC filter or surrounding D-20 medium to moistened *Drosophila* growth medium in 95 × 15-mm glass vials (~6 ml Formula 4-24 Carolina Biological Supply medium wet with 6 ml glass-distilled water). (The incubation of unhatched embryos on the filter was continued until the afternoon at which time final counts of percent hatching were made.) Some 10 μl of a dilute suspension of yeast (3 mg/ml) was added to the medium and the vials were incubated at 24°C, ~65% relative humidity. The next day, 10 μl of a suspension containing 30 mg/ml yeast was added to each vial. The number of adults was determined 11–12 days later.

Assessment of the State of the Cuticle of Treated Embryos after Incubation

The larvae were counted ~24 h after the experimental procedure and then transferred to an area of the PC filter separate from the unhatched eggs. Both areas were placed in small shell-vials containing Hoyer's solution (4:1 glacial acetic acid:glycerol) and capped. The samples were examined by A. P. Mahowald for developmental abnormalities in cleared cuticle preparations processed according to the method of van der Meer (31). After the embryos had been cleared, and mounted in Permount on slides, they were examined by either dark-field or phase contrast at 250× and 400× magnification.

Controls

An aliquot of each day's collection of eggs was used to determine the percentage hatching of embryos not subjected to dechorionation or permeabilization. About 100 eggs were placed on each of six 13-mm Nuclepore filters which were floated on a small quantity of water in plastic wells. Three of the filters were incubated at 24°C as described above for permeabilized eggs, except incubation was 40–48 h. The other three filters were held some 18 h at 17.5°C and the incubation was then completed at 24°C as above. Both methods yielded similar values for percentage hatching, which ranged from 80 to 95%.

Statistical variation for N replicate samples is reported as standard errors.

RESULTS

Effect of Permeation by Ethylene Glycol

Our strategy, like that of Steponkus *et al.* (26), has been first to equilibrate the embryos with a moderate concentration of ethylene glycol at room temperature and then to increase the intracellular concentration to high levels primarily by osmotic dehy-

TABLE 2
Survival of Permeabilized[a] 12-h *Drosophila* Embryos as a Function of Concentration of Ethylene Glycol at 23 and 0°C

Concentration ethylene glycol (*M*)	Temperature (°C)	Exposure times (min)	Approx. time for 90% to return to isotonic vol. (min)	% Hatching		*N*
				Absolute	Normalized[b]	
2.0	23	30	16	70 ± 3	84 ± 3	6
2.5	23	30	24	68 ± 8	77 ± 5	2
3.0	23	30	26	59 ± 4	68 ± 8	2
3.5	23	30	25	48 ± 8	54 ± 7	2
2.0	0	60	59	74 ± 6	85 ± 2	2
2.5	0	60	59	80 ± 2	92 ± 8	2
3.0	0	110	106	52 ± 4	60 ± 1	2
3.5	0	110	100	52 ± 1	60 ± 4	2

[a] Embryos were permeabilized by exposure to 0.3% butanol in heptane for 110 s followed by 45 min incubation in D-20 at 24°C before treatment with ethylene glycol. The percentages of parallel samples that stained ruby red, dark pink, and light pink with rhodamine were 80 ± 1, 10 ± 1, and 9 ± 1, respectively ($N = 8$).

[b] Normalized to the hatching survival of unpermeabilized controls.

dration. Table 2 shows the survivals after the first step. Exposure times of 30 min at room temperature were sufficient to allow the embryos to return to apparent normal volume after the initial osmotic shrinkage. A return to normal, isotonic, volume indicates that the intracellular concentration of ethylene glycol has approached the external concentration. With 2 and 2.5 *M* ethylene glycol, survivals normalized to untreated controls were some 80%. With 3 and 3.5 *M* concentrations, they fell to about 60%.

Reducing the exposure temperature to 0°C might be expected to reduce chemical toxicity, but as can be seen from column 4 that it also reduces the rate of permeation, i.e., the time for the embryos to return to normal volume increased two- to fourfold. Apparently, the increase in required times cancels out any reduction in the rate of toxicity injury since survivals after exposure at 0°C are similar to those at 24°C.

We selected 30-min exposure at room temperature to 2 *M* ethylene glycol as our standard treatment. Table 3 shows the effects of other variables. Our previous study (11) concluded that mixtures of 0.3 or 0.4% 1-butanol in *n*-heptane produced the best combination of a high-percentage permeabilization and high survivals. Table 3 shows that when permeabilization was followed by exposure to 2 *M* ethylene glycol, the 0.3% butanol mixture yielded slightly higher survivals and equally good permeabilization, and so it was selected as the standard. In later experiments we reduced the exposure time to 0.3% butanol–heptane from 110 to 90 s since our previous work had shown that an exposure of 110 s represented the boundary beyond which there was a substantial increase in injury from permeabilization per se.

If permeabilization induced some injury, it might exacerbate injury associated with subsequent ethylene glycol exposure. Consequently, it seemed possible that the insertion of a recovery period between the two steps would be beneficial. That proved to be the case, for when a 45-min incubation period was inserted between permeabilization and ethylene glycol exposure, survivals were significantly higher than when such incubation was omitted. One concern was that the benefit might be illusory in that the 45-min incubation might be allowing time for the 12-hr embryos to partially restore their permeability barriers and thus

TABLE 3
Survival of Permeabilized 12-h *Drosophila* Embryos after Exposure to 2 *M* ethylene Glycol in D-20 for 30 min at Room Temperature (23°C)

Variable	% Hatching		N
	Absolute[a]	Normalized[b]	
Permeabilized with heptane			
+ 0.3% 1-butanol, 110 s	63 ± 3	76 ± 3	16
+ 0.4% 1-butanol, 85 s	57 ± 5	69 ± 6	12
Incubation at 24°C after permeabilization			
None	54 ± 4	64 ± 4	12
45 min	65 ± 3	80 ± 3	16
Transferred from 2 *M* ethylene glycol to 0.75 *M* sucrose in D-20 for			
0 min	58 ± 6	70 ± 7	8
2 min	65 ± 3	80 ± 3	13
5 min	54 ± 5	65 ± 5	7
10 min[c]	31 ± 5	37 ± 6	4

Note. The 12-h embryos were permeabilized with 0.3% 1-butanol or 0.4% 1-butanol in heptane. They were then exposed to 2 *M* ethylene glycol in D-20 for 30 min at 23°C either immediately after permeabilization or after 45 min incubation at 24°C. After the ethylene glycol exposure, the embryos were either returned directly to D-20 or were first exposed to 0.75 *M* sucrose in D-20 for 2 or 5 min.

[a] The rhodamine staining percentages on parallel samples were

	Red	Dark pink	Light pink	Unstained	N
0.3% Butanol, 110 s	84 ± 3	7 ± 1	8 ± 2	1 ± 0.5	10
0.4% Butanol, 85 s	80 ± 3	10 ± 1	9 ± 1	1 ± 0.4	9

[b] Normalized to the hatching survival of unpermeabilized controls.

[c] These values are not included in calculating the mean survivals in the first four rows.

undergo less dehydration or less glycol permeation during subsequent exposure to 8.5 *M* ethylene glycol. Mazur *et al.* (11) had observed that the permeabilization efficacy of butanol–alkane mixtures dropped abruptly when embryos became older than 14 h. To test this possibility, we compared the rhodamine staining of permeabilized embryos subjected and not subjected to 45 min incubation. As shown in Table 4, there was no difference. Nevertheless we decided that in future experiments we would

TABLE 4
Rhodamine Staining of Permeabilized 12-h Embryos before and after 45-min Incubation at 24°C

Percentage 1-butanol in heptane (exposure time)	Time of staining[a]	Red	Dark pink	Light pink	Unstained	N
0.3% (110 s)	Preincubation	76 ± 5	7 ± 1	12 ± 3	5 ± 2	9
	Postincubation	80 ± 2	10 ± 1	8 ± 1	1.4 ± 0.5	18
0.4% (85 s)	Preincubation	79 ± 3	10 ± 1	10 ± 2	1 ± 0.5	6
	Postincubation	82 ± 0.3	12 ± 2	5 ± 1	1 ± 1	3

[a] "Preincubation" means staining with rhodamine was carried out immediately after the completion of permeabilization. "Postincubation" means that embryos on polycarbonate filters were incubated for 45 min at 24°C prior to staining with rhodamine.

carry out the rhodamine staining after the incubation rather than before.

At the end of subsequent treatments, the ethylene glycol has to be removed from the embryos by returning them to D-20 medium lacking ethylene glycol. In many cells, mouse embryos for instance, the abrupt transfer of cryoprotectant-loaded cells to cryoprotectant-free media may cause abrupt osmotic swelling and damage or death. An effective way to prevent this is to first transfer the cryoprotectant-loaded cells into a medium that lacks cryoprotectant but contains a nonpermeating solute like sucrose at about half the osmolality of the cryoprotectant in the cells (6). The external nonpermeating solute puts a controllable limit on the maximum osmotic swelling that the cell can undergo as the intracellular cryoprotectant diffuses out. After sufficient time to allow the efflux of most of the cryoprotectant (a time that depends on the permeability of the cell to the cryoprotectant), the cell is then transferred to a sucrose-free medium.

The bottom portion of Table 3 compares the effect of introducing such a sucrose treatment with its omission. A 2-min exposure to 0.75 *M* sucrose prior to the return of the embryos to D-20 yielded significantly higher survivals. Interestingly, though, longer exposures to the sucrose solutions were decidedly detrimental. As we shall see later, this time-dependent damage is probably a consequence of the osmotic shrinkage that embryos undergo in the sucrose solutions as the intraembryonic ethylene glycol diffuses out.

Consequences of Short Exposure to High-Concentrations of Ethylene Glycol

The second step in the vitrification strategy is to raise the concentration of both extra- and intraembryonic ethylene glycol to levels likely to prevent ice crystal formation during cooling or warming, i.e., concentrations sufficient to induce vitrification during cooling and to prevent devitrification during warming. Generally to do so, the concentrations of glass-forming solutes have to approach or exceed 50 wt% (5). One could in theory simply hold the embryos in the highly concentrated solutions sufficiently long to allow the intracellular and extracellular concentrations to approach equality. But in *Drosophila* embryos this would require an hour or more at room temperature and would almost certainly be lethal. The alternative strategy, based on that first proposed by Rall and Fahy (22), is to use the initial osmotic shrinkage that occurs when cells are placed in hyperosmotic solutions of permeating solutes to bring about the required rise in the intracellular concentration of glass-forming solute. In practice, this means exposing the embryos to concentrated ethylene glycol for short periods at reduced temperatures that will allow substantial water efflux but little ethylene glycol influx.

Table 5 shows the survivals that resulted when 12-h embryos equilibrated with 2 *M* ethylene glycol were exposed for 3 to 7 min at 5°C to 5, 6.5, 8.5, 9, or 9.5 *M* ethylene glycol. As shown in Fig. 1, survivals decreased with increasing ethylene glycol concentration. The effect of exposure time is less clear, but the indications are that a 7-min exposure was more damaging than a 3- or 5-min exposure. Although 5 and 6.5 *M* ethylene glycol were somewhat less damaging than 8.5 *M*, we shall see that the 6.5 *M* concentration, unfortunately, does not give any survival after cooling to −200°C. Consequently, most of our subsequent studies have been carried out using 5 min exposure to 8.5 *M* ethylene glycol, which in the absence of further treatments yields about 50% survival. In most subsequent experiments, the ethylene glycol has been supplemented with 10% (w/v) PVP. The survivals after 5 min exposure at 5°C to that mixture are about the same as those with 8.5 *M* ethylene glycol alone.

Effect of Glycerol

We carried out a few experiments in which glycerol was substituted for ethylene

TABLE 5
Survival (Hatching) of Permeabilized[a] 12-h *Drosophila* Embryos after Exposure to 2 *M* Ethylene Glycol for 30 min at 23°C Followed by Exposure to 5.0 to 9.5 *M* Ethylene Glycol for 3, 5, or 7 min at 5°C

Concentration ethylene glycol (*M*)	Exposure time (min)	% Hatching		
		Absolute	Normalized[b]	*N*
5.0	5	69 ± 9	78 ± 10	3
6.5	3	48 ± 3	64 ± 3	4
	5	59 ± 4	73 ± 4	14
	7	38 ± 8	50 ± 9	4
8.5	3	49 ± 5	61 ± 5	6
	5	47 ± 3	59 ± 3	27
	7	33 ± 8	42 ± 9	5
8.5 + 10% PVP	5	46 ± 3	54 ± 3	19
9.0	5	34 ± 7	41 ± 8	4
9.5	5	40 ± 4	42 ± 5	4

[a] Permeabilized with 0.3% 1-butanol in *n*-heptane for 90 or 110 s or with 0.4% of 1-butanol in heptane for 80 or 85 s.
[b] Normalized to the percentage hatching of untreated unpermeabilized controls in that experiment.

glycol in the final vitrification solution (the embryos were preloaded with 2 *M* ethylene glycol as before). As can be seen from the bottom curve of Fig. 1, 5 min exposure to glycerol at 5°C was far more damaging.

Survival of Embryos in Ethylene Glycol After Rapid Cooling to −150°C and Below and Rapid Warming

Table 6 summarizes the effects of a variety of rapid cooling and warming procedures on subsequent development and/or hatching of 12-h embryos. The column "development" refers to embryos that developed to the point of manifesting air-filled trachea or movement but did not hatch plus those that did hatch. The variables included the concentration of ethylene glycol (6.5 or 8.5 *M*), whether or not a macromolecule was added to the glycol, the method of cooling and the method of warming, details of which are given under Methods. Several points emerge clearly. First, survivals were far poorer with 6.5 *M* ethylene glycol than with 8.5 *M*. Indeed with the 6.5 *M*, none hatched. Second, even with 8.5 *M* glycol, survivals using isopentane at −150°C as the coolant gave essentially 0% hatching and poor development (21%). Third, when the 8.5 *M* ethylene glycol contained PVP or bovine serum albumin, both hatching and development were higher than when the macromolecules were omitted. Fourth, there was little difference in survival whether the coolant was boiling liquid nitrogen (LN_2) or liquid–solid nitrogen slush (N_2-slush). We emphasize this result because the former yields a lower cooling rate than the latter, a point to which we return later.

The greatest number of experiments [52] have been carried out using 8.5 *M* ethylene glycol containing 10% (w/v) PVP (Plasdone C-30). This procedure yielded 12 ± 1% hatching and 54 ± 2% development. That was about as high as those produced by any of the other combinations in the bottom half of the table. It is difficult to discern any significant differences among these combinations, i.e., PVP vs BSA, concentration of the macromolecule, or cooling in LN_2 vs cooling in N_2-slush. We also found no influence of varying the average molecular weight of the PVP from 3000 to 360,000.

The diagonally marked bars in Fig. 2 show the frequency distribution of hatching survivals obtained in the 52 runs using 8.5 *M* ethylene glycol with 10% PVP, rapid cooling in N_2-slush, and rapid warming in

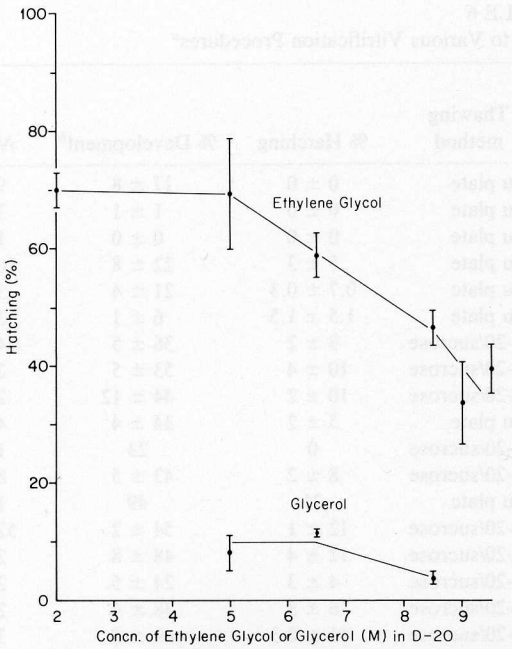

FIG. 1. Effect on the survival of permeabilized *Drosophila* embryos of a 5-min exposure at 5°C to various concentrations of ethylene glycol or glycerol. The embryos were preloaded with 2 *M* ethylene glycol by a 30-min exposure at 23°C to that concentration. Survival after that first step was 70% (left-most point on the ethylene glycol curve).

0.75 *M* sucrose in D-20. The distribution is bimodal. In about half the runs survivals ranged from 15 to 29% with a modal value of 20–24% and a normal distribution. In the other half, survivals were less than 14% with a mean value between 5 and 9% and a modal value of 0 to 4%. Figure 2 also shows that in the absence of polymer (cross-hatched bars), the bimodal aspect was absent and the average survival shifted to lower values. Paradoxically, however, this latter group yielded the three highest survivals we have obtained for 12-h eggs; namely, 30, 35, and 40%.

Possible Sources of Variability

The sources of variability could be biological, they could be physical aspects of the procedure, or they could be both. Biological sources might include small run-to-run differences in the developmental stage of treated embryos. The samples in a given experiment were aliquots of a single egg collection, all of which were held at 17.5°C for ~18 h in a single dish to allow development to the proper stage. After that time, the samples were held at 4°C for use during the four to five runs in a day. Slow development occurred during the ~6-h at 4°C as evidenced by a progressive increase in the percentage of stage 15 embryos in successive runs, the values being 7 ± 1% (N = 17), 11 ± 2% (N = 12), 14 ± 2% (N = 7), and 27 ± 2% (N = 5) in runs 1, 2, 3, and 4, respectively. The average times at 4°C before each run were 0.8, 3.3, 5.0, and 5.4 h. There was no significant correlation between the run number and the survival of either 8.5 *M* ethylene glycol controls (Fig. 1) or of experimental samples subjected to ultrarapid cooling to −205°C and ultrarapid warming. In the latter case, however, there was a statistically nonsignificant (P = 0.20) trend toward higher survivals in the later runs, a point to which we return later. We have carried out a limited number of experiments with 10-h embryos using 8.5 *M* ethylene glycol and N_2-slush cooling and obtained poorer results than with 12-h embryos.

We turn now to physical or chemical sources of variability, in order of the sequence in which they would be applicable:

Degree of permeabilization. It is possible that sublethal damage resulting from too great a degree of permeabilization might become lethal during subsequent cryogenic exposure, or it is possible that inadequate permeabilization might lead to poor survivals because of inadequate permeation of 2 *M* ethylene glycol or inadequate osmotic dehydration in 8.5 *M* ethylene glycol. However, we find no significant correlation between the percentage of embryos that stained ruby red with rhodamine and the low-temperature survivals of parallel samples from that run (P = 0.20). This is in part

TABLE 6
Survival of 12-h Embryos Subjected to Various Vitrification Procedures[a]

Concentration ethylene glycol (*M*)	Polymer concentration (w/v)	Freezing method	Thawing method	% Hatching	% Development[b]	*N*
6.5	—	Cu block	Cu plate	0 ± 0	17 ± 8	9
		Isopentane	Cu plate	0 ± 0	1 ± 1	3
		N_2-slush	Cu plate	0 ± 0	0 ± 0	1
8.5	—	Cu block	Cu plate	5 ± 3	22 ± 8	10
		Isopentane	Cu plate	0.7 ± 0.3	21 ± 4	3
		N_2-slush	Cu plate	1.5 ± 1.5	6 ± 1	2
		N_2-slush	D-20/sucrose	9 ± 2	36 ± 5	14
8.5	20% PVP	LN_2	D-20/sucrose	10 ± 4	53 ± 5	2
	10% PVP	LN_2	D-20/sucrose	10 ± 2	44 ± 12	2
	10%/20% PVP	LN_2	Cu plate	5 ± 2	34 ± 4	4
	5% PVP	LN_2	D-20/sucrose	0	23	1
	20% PVP	N_2-slush	D-20/sucrose	8 ± 2	43 ± 5	8
			Cu plate	21	49	1
	10% PVP	N_2-slush	D-20/sucrose	12 ± 1	54 ± 2	52
	5% PVP	N_2-slush	D-20/sucrose	12 ± 4	48 ± 8	2
	12% BSA	LN_2	D-20/sucrose	4 ± 3	24 ± 5	2
	6% BSA	LN_2	D-20/sucrose	6 ± 3	38 ± 4	2
	12% BSA	N_2-slush	D-20/sucrose	13 ± 0.3	49 ± 5	3
	6% BSA	N_2-slush	D-20/sucrose	5 ± 2	33 ± 8	3

Note. In the series without polymer, the percentages of embryos in parallel samples that stained ruby red, dark pink, or light pink with rhodamine B were 79 ± 2, 10 ± 1, and 8 ± 1%, respectively. The percentage unstained was 1.8 ± 0.6%. In the series with polymers, the corresponding staining percentages were 80 ± 1, 10 ± 1, and 8 ± 1%. In the series without polymers, the hatching percentages of unhandled controls, and those exposed to 6.5 or 8.5 *M* ethylene glycol but not cooled below 0°C were 79 ± 3, 60 ± 6, and 47 ± 5%, respectively. In the series with polymers, the hatching percentages of intact embryos, permeabilized embryos before ethylene glycol exposure, and those after ethylene glycol/polymer exposure were 82 ± 2, 68 ± 3, and 41 ± 4, respectively.

[a] Embryos were permeabilized by 90- or 110-s exposure to 0.3% 1-butanol in heptane and then, after 45 min incubation in D-20, were exposed to 2 *M* ethylene glycol in D-20 at room temperature for 30 min. Exposure time to the concentrated solutions in columns 1 and 2 was 4.5 to 5.5 min at 5°C.

[b] Percentage development is the percentage hatching plus the percentage showing tracheal air tubes but not hatching.

because the degree of permeabilization is quite reproducible between runs. We have also performed a limited number of experiments ($N = 4$) in which exposure to 0.3% butanol in heptane was varied from 30 to 90 s. There was no clear effect on survival after low-temperature exposure. Hatching after exposures to butanol–heptane of 30, 50, 70, and 90 s was 4, 3, 6, and 5% respectively, and development was 28, 18, 34, and 27%, respectively. We carried out a detailed comparison between exposures of 90 and 110 s. The hatching survivals were 10.6 ± 2.2% ($N = 22$) and 9.2 ± 2.2% ($N = 9$), respectively, and development was 47.4 ± 4.7% and 46.2 ± 4.0%.

Survival after ethylene–glycol treatment. There was no correlation between survival after low-temperature exposure and the survival of parallel controls subjected only to 2 *M* plus 8.5 *M* (with or without PVP) ethylene glycol. Depending on the tests and transformations used, the correlation coefficients ranged between 0.45 and 0.30, and the corresponding *P* values between 0.30 and 0.17.

Effects of cooling and warming rate. All the samples to this point had been sub-

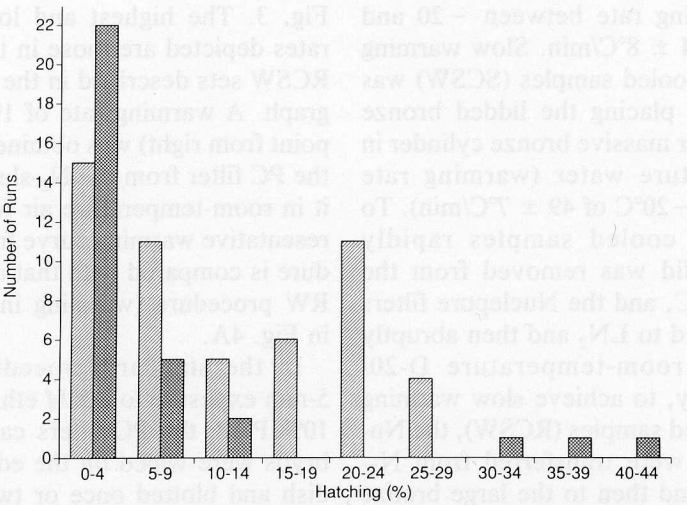

FIG. 2. Frequency distribution of the survival of permeabilized 12-h *Drosophila* embryos in replicate runs in which samples were cooled rapidly to −205°C and warmed rapidly. Prior to subzero cooling the embryos were preloaded with 2 *M* ethylene glycol and then exposed for 5 min to 8.5 *M* ethylene glycol with 10% PVP (diagonal bars) or without PVP (cross-hatched bars) at 5°C. Cooling and warming were by abrupt immersion in N_2-slush at −205°C and room-temperature 0.75 *M* sucrose in D-20, respectively.

jected to very rapid cooling and very rapid warming. Table 7 summarizes the results of an important series of experiments in which the effects of cooling and/or warming at much lower rates were assessed. Four combinations of rates were examined: rapid cool–rapid warm (RCRW) in which samples were cooled in N_2-slush and warmed in room-temperature D-20/sucrose (the standard to this point), rapid cool–slow warm (RCSW), slow cool–rapid warm (SCRW), and slow cool–slow warm (SCSW). Slow cooling (SC) was effected by placing the Nuclepore filters bearing the embryos in the large lidded bronze plate described under Methods and placing the bronze plate on a massive bronze cylinder which had been thermally equilibrated in liquid nitro-

TABLE 7
Comparative Effects of Cooling and Warming Rates on the Survival of 12-h Embryos Exposed to −200°C[a]

Sequence	Cooling rate	Warming rate (°C/min)	% Development[b]	% Hatching	*N*
RCRW	110,000	120,000	46 ± 6	10 ± 3	9
RCSW	110,000	49	0.0	0.0	6
SCRW	74	120,000	39 ± 6	6 ± 3	8
SCSW	74	49	0.5 ± 0.5	0.0	6

[a] Embryos were permeabilized by exposure to 0.3% 1-butanol in heptane for 90 s. They were then incubated 45 min at room temperature, exposed to 2 *M* ethylene glycol in D-20 for 30 min at room temperature and then to 8.5 *M* ethylene glocyl + 10% (w/v) PVP for 4.5 to 5.5 min at 5°C. They were then cooled to −196 to −200°C and warmed thereafter at the rates indicated. The percentages of parallel samples that stained ruby red, dark pink, or light pink with rhodamine B were 74 ± 3, 15 ± 2, and 9 ± 2, respectively. The percentage of unhandled intact embryos that hatched was 81 ± 3%. The percentage of permeabilized eggs that hatched before and after exposure to ethylene glycol was 82 ± 4 and 59 ± 8%.

[b] To tracheal stage or hatching.

gen. The cooling rate between −20 and −120°C was 74 ± 8°C/min. Slow warming of the slowly cooled samples (SCSW) was carried out by placing the lidded bronze plate on another massive bronze cylinder in room-temperature water (warming rate from −120 to −20°C of 49 ± 7°C/min). To warm slowly cooled samples rapidly (SCRW), the lid was removed from the plate at −196°C, and the Nuclepore filters were transferred to LN_2 and then abruptly immersed in room-temperature D-20/sucrose. Finally, to achieve slow warming of rapidly cooled samples (RCSW), the Nuclepore filters were transferred from N_2-slush to LN_2 and then to the large bronze plate at −196°C. The bronze plate was then lidded and allowed to warm as with SCSW. The lid on the bronze plate served two important functions. One is that it substantially reduced vertical temperature gradients and thus ensured that the temperatures and the rate of temperature changes experienced by the embryos were close to those of the bronze plate. Second, the lid eliminated the formation of frost on the embryos.

The results in Table 7 are clear: (i) Any combination that involved slow warming was lethal; (ii) when warming was rapid, there was relatively little difference between the effects of slow and rapid cooling, although the former was somewhat more detrimental; and (iii) the reciprocal combinations (RCSW and SCRW) yielded very different survivals (i.e., 0 and 39% development) even though both produced about the same total exposure time during cooling from −20 to −120°C and warming from −120 to −20°C; namely, 1.4 to 2.0 min. If chilling injury were the main contributor to injury one would expect that time spent during cooling would have essentially the same effect as an equivalent time spent during warming.

The effect of warming rate on the survival of embryos previously cooled rapidly in N_2-slush is examined in more detail in Fig. 3. The highest and lowest warming rates depicted are those in the RCRW and RCSW sets described in the previous paragraph. A warming rate of 1900°C/min (3rd point from right) was obtained by removing the PC filter from the N_2-slush and holding it in room-temperature air for 10 s. A representative warming curve from that procedure is compared with that achieved in the RW procedure (warming in D-20/sucrose) in Fig. 4A.

In the standard procedure, after the 5-min exposure to 8.5 *M* ethylene glycol + 10% PVP, the PC filters carrying the embryos were wiped on the edge of the petri dish and blotted once or twice to remove excess ethylene glycol. If the wiping and blotting were omitted, the mass of adhering ethylene glycol/PVP increased from ~5 to

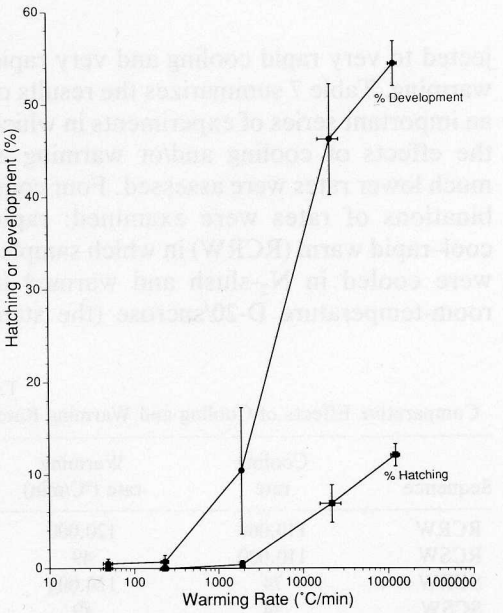

FIG. 3. Effect of warming rate on the subsequent development and hatching of embryos that had been previously cooled by abrupt immersion in N_2-slush. Prior to subzero exposure, the permeabilized embryos were exposed to the standard sequence of 2 *M* ethylene glycol followed by 5 min exposure at 5°C to 8.5 *M* glycol containing 10% PVP. The procedures for achieving the various warming rates are given in the text.

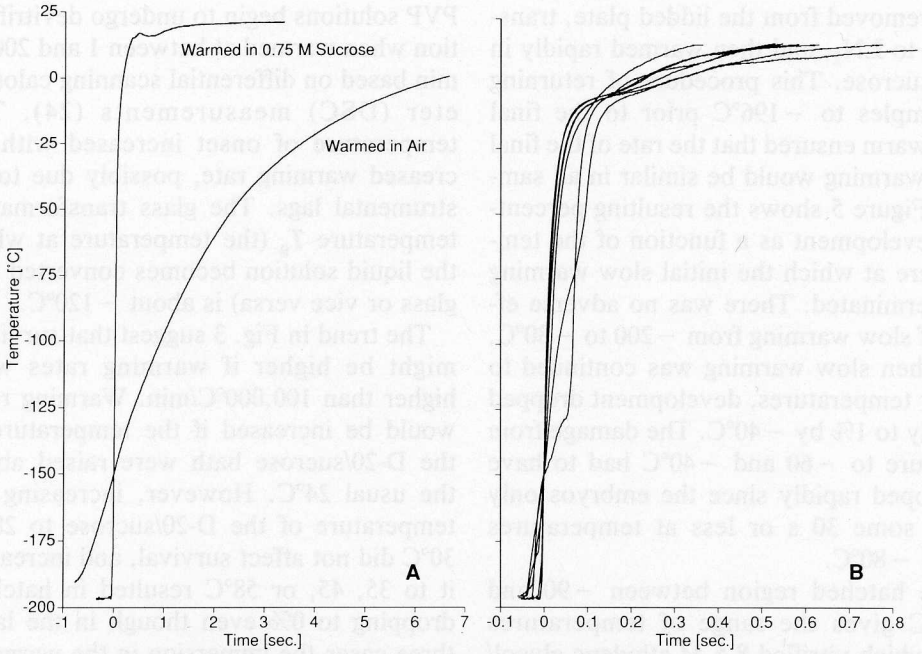

FIG. 4. (A) Examples of warming curves when PC filters previously exposed to 8.5 *M* ethylene glycol plus 10% PVP in the standard manner were rapidly cooled to −196 or −205°C in LN_2 or N_2-slush and then warmed either by abrupt immersion in room-temperature 0.75 *M* sucrose in D-20 or by holding in room-temperature air. The curves are normalized to 0 time at −150°C. (B) Replicates of ultrarapid warming runs.

~50 mg, and the measured warming rate obtained when the filter was transferred from LN_2 to the D-20/sucrose dropped from 124,000 to 22,000°C/min. That is the second point from the right in Fig. 3. The greater mass of the ethylene glycol vitrification solution also lowered the cooling rate by a comparable amount, but as seen in Table 7, survival is much less sensitive to slower cooling.

The effect of warming rate shown in Fig. 3 is dramatic. Lowering the rate from 100,000°C/min (−150 to −20°C in 0.1 s) to 2000°C/min (−150 to −20°C in 4 s) totally abolished survival and greatly reduced the percentage undergoing any development.

To gain information on the temperature range at which slow warming became lethal, we performed the following experiment. Embryos on Nuclepore PC filters were rapidly cooled in N_2-slush and then transferred to the smaller version of the bronze plate referred to above that had been precooled to −196°C in LN_2. The small plate was then lidded and transferred to a large bronze cylinder immersed either in a dry-ice alcohol bath at ~ −75°C or in an ice bath, depending on the upper temperature limit desired for warming. In the former case, when the temperature of the lidded plate rose to −120 or −100°C, the plate was transferred from the cylinder in the dry-ice bath back into LN_2. In the latter case, it was transferred from the cylinder in the ice bath back into LN_2 after it had slowly warmed to −80, −60, or −40°C. The warming rates to these temperatures were 140 to 250°C/min, some three to four times that in the experiments of Table 7. After being returned to ~ −196°C, all Nucleopore filters and their adhering embryos, including one that underwent no warming,

were removed from the lidded plate, transferred to LN_2, and then warmed rapidly in D-20/sucrose. This procedure of returning all samples to −196°C prior to the final rapid warm ensured that the rate of the final rapid warming would be similar in all samples. Figure 5 shows the resulting percentage development as a function of the temperature at which the initial slow warming was terminated. There was no adverse effect of slow warming from −200 to −80°C, but when slow warming was continued to higher temperatures, development dropped sharply to 1% by −40°C. The damage from exposure to −60 and −40°C had to have developed rapidly since the embryos only spent some 30 s or less at temperatures above −80°C.

The hatched region between −90 and −65°C gives the range of temperatures over which vitrified 8.5 *M* ethylene glycol/

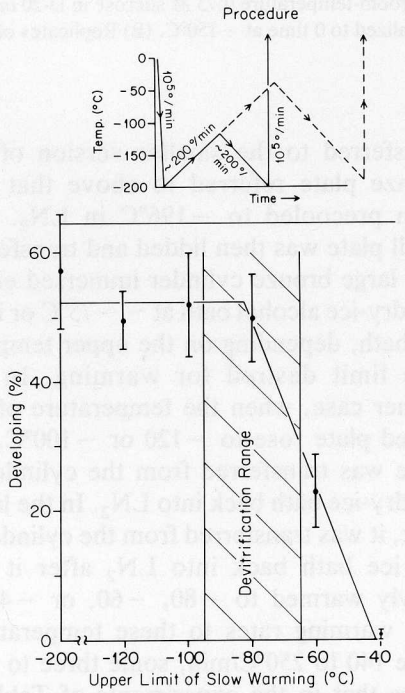

FIG. 5. Temperatures over which the slow warming of rapidly cooled embryos becomes injurious. The inset illustrates the procedure, details of which are given in the text.

PVP solutions begin to undergo devitrification when warmed at between 1 and 200°C/min based on differential scanning calorimeter (DSC) measurements (24). The temperature of onset increased with increased warming rate, possibly due to instrumental lags. The glass transformation temperature T_g (the temperature at which the liquid solution becomes converted to a glass or vice versa) is about −120°C.

The trend in Fig. 3 suggest that survivals might be higher if warming rates were higher than 100,000°C/min. Warming rates would be increased if the temperature of the D-20/sucrose bath were raised above the usual 24°C. However, increasing the temperature of the D-20/sucrose to 28 or 30°C did not affect survival, and increasing it to 35, 45, or 58°C resulted in hatching dropping to 0% even though in the latter three cases the immersion in the warm solutions was held to ~4 s. Increasing the D-20/sucrose temperature by 5–10°C should not actually increase the warming rate substantially at subzero temperatures since it represents only a small increase in the early temperature differential between filter and bath. Steponkus *et al.* (26) used small copper electron microscope grids as the support for cooling and warming some 25 embryos at a reported 25,000°C/min in N_2-slush. They did not report the warming rate, but it was presumably similar. However, when we substituted these grids for the PC filters, we obtained 1% hatching and 32% development after cooling and warming.

Dehydration, Toxicity, and Vitrification

The presumed effect of the brief 5 min exposure to 8.5 *M* ethylene glycol at 5°C is to raise the internal concentration of glycol from 2 to ~8.5 *M* by dehydrating the embryo. As shown in Fig. 1, over 40% of the embryos survive this treatment. Yet when 8.5 *M* glycerol is substituted for 8.5 *M* ethylene glycol, only 5% survive. The increased damage could be related to toxicity

of the glycerol or it could be a consequence of the lower permeability of embryos to glycerol (8, 11). The lower permeability would result in greater embryo shrinkage during the 5-min exposure time at 5°C.

The cells of *Drosophila* embryos, like most cells, are essentially impermeable to sucrose (8, 11). Sucrose, then, permits one to study the consequences of osmotic dehydration uncomplicated by permeation of the external osmoticum. The first series of experiments examined the effects of 5 min exposure at 5°C to concentrations of sucrose in D-20 ranging from 0.75 to 3 *M* on embryos that had *not* been preloaded with 2 *M* ethylene glycol. As shown in Fig. 6A, the response is a sigmoidal drop in survival with increasing molar concentration. Osmotic response, however, is related to solution osmolality more than to molarity. In Fig. 6B we plot survival as a function of the computed residual water content of the embryos after exposure to the several sucrose solutions. First molarities were converted to molalities (Table 1) and then to osmolalities using osmotic coefficients published by Scatchard *et al.* (23). If the embryos behave like ideal osmometers (and Lin *et al.*, (7) report that they do), then the water content as a fraction of the isotonic water content is 0.260/(osmolality of sucrose plus D-20). The total osmolalities of 0.75, 1, 1.5, 2, 2.5, and 3 molar sucrose in D-20 were 1.22, 1.68, 2.93, 4.91, 8.28, and 13, respectively (the last being an extrapolated value). Remarkably, 40% survived the removal of a calculated 95% of their intracellular water by 2 *M* sucrose. The extreme dehydration was qualitatively confirmed under the microscope.

Since a 20-fold dehydration will produce a large increase in the osmolality of internal endogenous solutes, we wondered whether that by itself would be sufficient to induce vitrification during rapid cooling and prevent devitrification during rapid warming. It was not sufficient; no embryos suspended in the 2 *M* sucrose solution (±10%

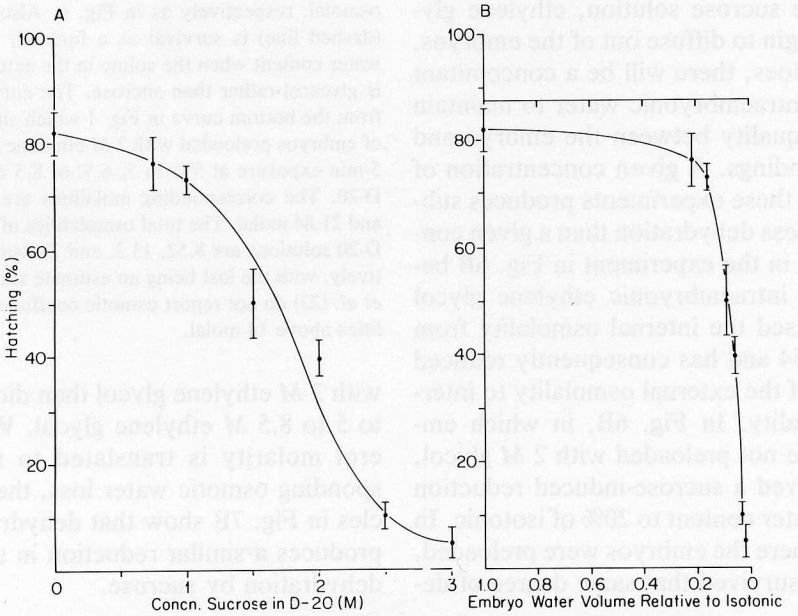

FIG. 6. (A) Effect of a 5-min exposure to various concentrations of sucrose in D-20 at 5°C on the survival of 12-h permeabilized embryos. The embryos were not exposed to any ethylene glycol. (B) Survival as a function of the extent of dehydration produced by sucrose D-20 calculated under the assumption that the embryos behave as ideal osmometers (see text).

PVP) hatched, or even developed, after rapid cooling in N_2-slush and rapid warming in D-20/sucrose. The conclusion is clear: Ethylene glycol (or its equivalent) must be present within the embryos to obtain survival with vitrification procedures.

Consequently, we examined the effect of 5 min exposure at 5°C to 0.75, 1.5, 2, 2.5, and 3 *M* sucrose in D-20, but this time on embryos that had been preequilibrated with 2 *M* ethylene glycol. Figure 7A shows hatching survivals as a function of sucrose molarity and Fig. 7B shows survival as a function of the calculated volume of intraembryonic water assuming that osmotic reequilibration has occurred solely by water loss and not by efflux of intraembryonic ethylene glycol. Although this is a reasonable approximation since the permeability of permeabilized embryos to water is higher than that to ethylene glycol (8, 11), it likely underestimates the extent of embryo dehydration during the 5-min exposure to sucrose. As soon as the embryos are transferred from the 2 *M* ethylene glycol to the glycol-free sucrose solution, ethylene glycol will begin to diffuse out of the embryos, and as it does, there will be a concomitant efflux of intraembryonic water to maintain osmotic equality between the embryo and its surroundings. A given concentration of sucrose in these experiments produces substantially less dehydration than a given concentration in the experiment in Fig. 6B because the intraembryonic ethylene glycol has increased the internal osmolality from 0.26 to 2.54 and has consequently reduced the ratio of the external osmolality to internal osmolality. In Fig. 6B, in which embryos were not preloaded with 2 *M* glycol, 76% survived a sucrose-induced reduction of their water content to 20% of isotonic. In Fig. 7B where the embryos were preloaded, only 13% survived that same degree of dehydration.

In Fig. 1 we noted that 5 min exposure to 5 to 8.5 *M* glycerol at 5°C produced far lower survivals of embryos preequilibrated

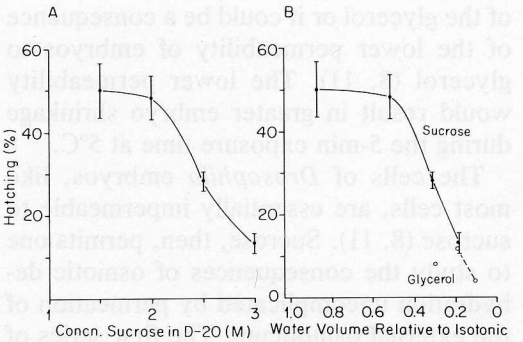

FIG. 7. (A) Effect of a 5-min exposure to various concentrations of sucrose in D-20 at 5°C on the survival of 12-h permeabilized embryos. Here, unlike Fig. 6, the embryos were preloaded with 2 *M* ethylene glycol prior to the exposure to sucrose. (B) Survival as a function of the intraembryonic water volume produced by the exposure to sucrose assuming ideal osmotic response and assuming no efflux of intraembryonic glycol; i.e., the relative embryo water volume is calculated as the total intraembryonic osmolality prior to sucrose exposure divided by the osmolality of the external sucrose/D-20 solution. The intraembryonic osmolality is taken as equal to the osmolality of the 2 *M* ethylene glycol/D-20 solution with which had previously been equilibrated; namely, 2.54 osmolal. The total osmolality of the external 1.5, 2, 2.5, and 3 *M* sucrose solutions in D-20 was 2.93, 4.91, 8.28, and 13 osmolal, respectively as in Fig. 6. Also shown in B (dashed line) is survival as a function of computed water content when the solute in the external medium is glycerol rather than sucrose. The curve is derived from the bottom curve in Fig. 1 which shows survival of embryos preloaded with 2 *M* ethylene glycol after a 5-min exposure at 5°C to 5, 6.5, or 8.5 *M* glycerol in D-20. The corresponding molalities are 7.78, 12.16, and 21.84 molal. The total osmolalities of the glycerol/D-20 solutions are 8.52, 13.3, and 24 osmolal, respectively, with the last being an estimate since Scatchard *et al.* (23) do not report osmotic coefficients for molalities above 14 molal.

with 2 *M* ethylene glycol than did exposure to 5 to 8.5 *M* ethylene glycol. When glycerol molarity is translated to the corresponding osmotic water loss, the open circles in Fig. 7B show that dehydration by it produces a similar reduction in survival as dehydration by sucrose.

Development to Adult Flies

Toward the latter portion of this investigation we began determining the fraction of

emerged larvae that are capable of developing into adult flies. To date, in 34 runs in which 12-h embryos in 8.5 *M* ethylene glycol + 10% PVP were subjected to rapid cooling to −205°C and rapid warming, and in which 16.8% hatched, 35/721 or 4.8% of the resulting larvae developed to adult flies for an overall efficiency of 0.8%. So far there is no correlation between the percentage of embryos that hatched in a given run and the fraction of larvae developing to adults. In runs in which hatching was 10% or less, 5/99 or 5.1% developed to adults. In runs where hatching was 20% or greater, 22/416 or 5.3% developed to adults. We have not yet determined the survival to adulthood of embryos older than 12 h. Nor have we yet determined whether the adults are fertile since most of the individual runs produced only a single survivor or the survivors were the same sex. As of now, 72% of the adults were males.

We also have preliminary data for the effects of treatments prior to low-temperature exposure on the percentage of larvae from 12-h embryos capable of developing to adult flies. About 60% of larvae from intact untreated embryos develop to adults. Permeabilization in heptane containing 0.3% butanol drops that percentage to 35%. Exposing the permeabilized embryos to 2 *M* ethylene glycol for 30 min at room temperature with or without subsequent exposure to concentrated ethylene glycol vitrification solutions for 5 min at 5°C drops the percentage to around 20%, but the results are too variable and the number of replicates is too small to draw definitive conclusions about the significance of differences among the various glycol treatments. The sex ratio was close to 50:50 and the adults were fertile.

Histological Manifestations of Injury

Head involution and dorsal closure are major developmental events occurring during the 12-h stage we are studying (predominantly stage 14). We have used the Hoyer method to examine the state of the cuticle in two samples of 175 embryos that failed to hatch after our standard procedure (rapid cool to −205°C/rapid warm in 8.5 *M* ethylene glycol + 10% PVP). Forty-seven percent of these embryos underwent development to air-filled trachea. Of the 175 unhatched embryos, 13% appeared normal, 64% had defective mouth parts, 61% manifested defective dorsal closure, and 15% showed other severe defects or no development. Some showed more than one defect. Twenty of the 22 larvae that had emerged and were recovered in these experiments had normal cuticle. However, we have noted that larvae emerging from embryos exposed to −205°C often manifested impaired motion.

Survival as a Function of Embryonic Stage

We pointed out earlier that slow development occurred during the 5–6 h that 12-h embryos were held at 4°C in a given day and that survival after low-temperature exposure showed a nonsignificant tendency to increase in successive runs. To investigate this trend, experiments have recently been performed in which the initial 12-h embryos were held at 17.5 or 24°C instead of 0°C until the initiation of permeabilization 1.5, 4 to 4.5, and 6.2 to 6.5 h later. We calculated the embryo age at those times by assigning a relative growth rate of 0.5 and 0.9 for the two temperatures, respectively (see Methods). Figure 8 shows the percentages of embryos that developed or hatched after the standard exposures to ethylene glycol and ultrarapid cooling and warming. There was a marked increase in hatching and development as embryo age increased from 12 to 15 h, and then a precipitous drop as it increased further to 15.5 and 17.7 h. The hatching in one sample of 14- to 15-h embryos was 49%, the highest we have observed to date. Two other samples gave 41 and 43%. The percentage of permeabilized embryos (those staining ruby red with rhodamine) showed a slow decline from 85

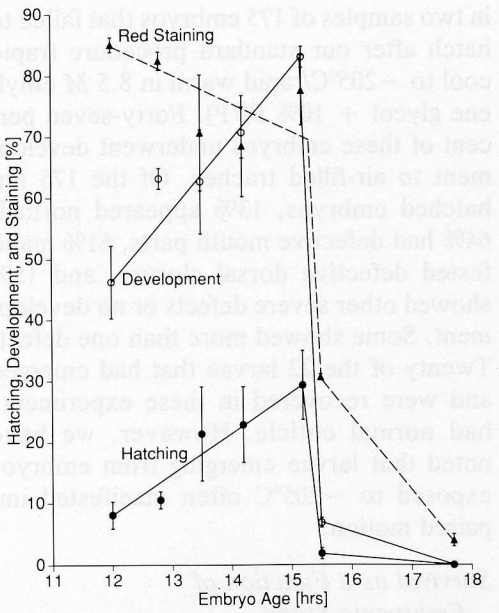

FIG. 8. Permeabilization and survival of "vitrified" *Drosophila* embryos vs developmental age. The embryos, immersed in 8.5 *M* ethylene glycol/10% PVP, were rapidly cooled to −205°C and rapidly warmed in the standard manner.

to ~75% as embryo age increased from 12 to 15 h, but then dropped precipitously in older embryos in parallel with the drop in survival after cryogenic exposure. By 17.7 h, 96% of the embryos did not stain at all. When embryos were permeabilized at the 12- to 14-h stage and then allowed to develop to the 16-h stage, there was no improvement in the percentage staining with rhodamine at 16 h. It is possible that the permeability barrier reformed during the 2- to 4-h incubation. But that seems remote because we know that it does not reform with 45 min incubation at room temperature (Table 4).

Hatching survivals of controls that were exposed to 2 *M* and then to 8.5 *M* ethylene glycol (+10% PVP) at age 15 h were 52 ± 10%, close to the values in Table 5 and Fig. 1 for 12-h embryos held at 4°C. But the survivals of the 12-h control embryos in Fig. 8, which were held at 4°C for ~10 min were substantially lower (16 ± 5%).

DISCUSSION

The extreme sensitivity of 12-h embryos to chilling injury (15) precludes their being cryobiologically preserved by standard slow-freezing procedures designed to avoid intracellular freezing. Although chill sensitivity was studied in intact embryos, there is no reason to believe that permeabilized embryos are less sensitive. Cooling rates high enough to outrun the accelerating chilling injury ordinarily result in lethal intraembryonic ice formation. The only way to avoid such a result is to introduce sufficiently high concentrations of glass-promoting solutes in the embryos to induce the vitrification of their cytoplasm. The concentrations have to approach or exceed 50 wt %, concentrations that are likely to be toxic.

Rall and Fahy (22) and Rall (21) solved the problem of achieving these high concentrations in mouse eight-cell embryos without lethal toxicity by introducing the glass-promoting solute in two steps. First, the embryos were placed in solutions of the solute at concentrations about one-fourth of that ultimately required for vitrification for periods of time long enough to allow the solute to fully permeate. This exposure was not damaging. Then the embryos were placed for short times at 0°C in the full-strength solution, the result of which was to osmotically dehydrate the mouse embryos and thereby increase the concentration of intracellular glass inducer to close to the value in the exterior. The dehydration also increases the concentration of endogenous macromolecules, which Rall (21) suggests assists in inducing vitrification. High percentages of the embryos survive subsequent cooling to −196°C and warming if cooling is some 10°C/min or faster and if warming is some 100°C/min or faster. These cooling rates are presumably high enough to induce vitrification of the mouse embryo cytoplasm and the warming rates are high enough to prevent devitrification, although there is not direct evidence to that effect.

Steponkus *et al.* (26) opted for a similar two step procedure to vitrify 13-h *Drosophila* embryos using ethylene glycol plus 6% bovine serum albumin as the glass-inducing solute. Ethylene glycol was presumably chosen because studies by Lynch *et al.* (8), later confirmed by us (11), show permeabilized embryos to be substantially more permeable to glycol than, for example, to glycerol. They exposed permeabilized embryos to 2.125 *M* ethylene glycol for 20 min and then exposed them to 8.5 *M* ethylene glycol plus 6% BSA for 5–8 min at 0°C, before placing them on electron microscope grids and cooling them at a reported 25,000°C/min in N_2-slush. The warming rates presumably were equally high. They report that these procedures yielded a mean hatching of 18%, and that 3% of the resulting larvae developed to adult flies.

We have essentially confirmed their results. The percentages of 12-h embryos found by us to survive the previtrification steps of permeabilization (we used heptane; they used hexane), exposure to 2 *M* ethylene glycol for 30 min at room temperature, and exposure to 8.5 *M* ethylene glycol with or without polymer are closely similar to the values reported by them. The mean percentage hatching we obtain for 12-h embryos after cryogenic exposure under our current optimal conditions is 12% vs their value of 18% for 13-h embryos. Both we and they obtained instances of higher survival (30–41% in our case). In both our experiments and theirs a similar percentage of larvae develop to adult flies (5 and 3%, respectively).

Causes of Damage from Cooling to −200°C

The two most likely causes of damage and death are chilling injury and intraembryonic ice formation. Mazur *et al.* (15) have shown that the rate of chilling injury accelerates as the temperature falls so that by −25°C, nearly all embryos are killed in about 20 min in the absence of any detectable ice formation. If chilling injury continues to accelerate at lower temperatures with the same very high activation energies, computations indicate that the embryos would have to be cooled and warmed at some 20,000°C/min for half of them to survive cooling to −65°C.

The extent of chilling injury was shown to be a function of time at given temperatures and not of cooling rate per se; consequently, it ought to be independent of whether that time is expended during cooling or warming; that is, the effects of cooling rate and warming rate ought to be symmetrical. But the results in Table 7 show that the effects are highly *asymmetrical:* Slow warming at 50°C/min when preceded by very rapid cooling is far more damaging than slow cooling at 75°C/min when followed by very rapid warming. Indeed, as shown in Fig. 3, warming at even 1900°C/min is far more damaging than cooling at 75°C/min even though in the latter case the embryos spend some 80 s between −20 and −120°C whereas in the former case they spend only ~3 s.

Although such marked asymmetry between the effects on survival of cooling and warming rate is not easily explicable on the basis of chilling injury, it is consistent with the formation of ice in vitrified systems. It is well known (4) that the cooling rates required to induce vitrification in aqueous solutions, including cytoplasm, are substantially lower than the warming rates required to prevent the devitrification (freezing) of the glassy solution. Our hypothesis, then, is that the prime factor responsible for the injury or death of rapidly cooled–rapidly warmed embryos is the formation of intraembryonic ice in a critical portion of the embryo by devitrification during warming.

We noted in connection with Table 6 that when the concentration of ethylene glycol in the second step was lowered from 8.5 to 6.5 *M,* there was no survival after cooling to −150°C or below and little or no develop-

ment. There is no reason to believe that the kinetics of chilling injury would be influenced by such a change in the concentration of ethylene glycol, but there is every reason to believe that in the 6.5 *M* solution, the probability of vitrification during cooling would be substantially reduced, and that even if vitrification were induced, the probability of devitrification during warming would approach unity (see below). [A concentration of 6.5 *M* is, in fact, sufficient to induce the vitrification of glycerol solutions (21), but the ability of a solute to induce vitrification and prevent devitrification depends more on the weight percent of solute present than on its molarity, and 6.5 *M* glycerol is about the same weight percent as 8.5 *M* ethylene glycol (Table 1).]

We also noted in connection with Table 6 that the percentage of embryos undergoing hatching and development was about the same when samples were cooled in liquid nitrogen as when they were cooled in nitrogen-slush, even though the cooling rate in the former was about half that in the latter (50,000 vs 110,000°C/min). This finding is consistent with the relative insensitivity to cooling rate noted several paragraphs above.

Why does survival require warming rates of 100,000°C/min? Work with mouse embryos exposed to solutions of glycerol or other solutes with weight percents comparable to those used here have shown that the warming rates required to obtain high survival (and presumably prevent devitrification) are a thousandfold lower than the ~100,000°C/min required in the present study (21). Furthermore DSC measurements by Schreuders *et al.* (24) show that 8.5 *M* ethylene glycol + 10% PVP vitrifies during cooling at ≤200°C/min and does not devitrify during warming at 200°C/min.

Our assumption has been that at the conclusion of the two step exposure to the first 2 *M* ethylene glycol at 23°C and then 8.5 *M* ethylene glycol at 5°C, the intraembryonic and intracellular concentration of ethylene glycol is close to 8.5 *M*. At the end of the 30-min exposure to the first 2 *M* step, the embryos appear to have returned to normal volume after an initial shrinkage. That ought to mean that the intracellular ethylene glycol concentration is close to 2 *M*. Relatively little ethylene glycol should permeate the embryo during the second step involving exposure to 8.5 *M* ethylene glycol both because the exposure time is short (5 min) and the temperature is low (5°C). But osmotic water efflux in response to the strongly hypertonic external solution should, and in fact does, occur—the embryos become highly flattened. The presumption is that this dehydration raises the intracellular ethylene glycol concentration to 8.5 *M*.

But these assumptions may not be quantitatively accurate. We cannot at 30× magnification, for example, detect the shrinkage of embryos in D-20 solution that is 115% of isotonic. Consequently, since water content is the reciprocal of external osmolality, this is tantamount to saying that embryos that appear to have returned to normal volume might in fact contain only 85% of their normal water content. Furthermore, physiological processes like volume regulation might intervene. Volume regulation refers to the tendency of many anisotonic cells to return to isotonic volume by pumping ions in or out as required (6). If that occurs in *Drosophila* then the attainment of isotonic volume would not represent equality of intra- and extraglycol concentrations. Finally, the embryo may have compartments that are poorly penetrated by ethylene glycol.

Suppose, then, prior to being plunged in N_2-slush, the intraembryonic concentration of ethylene glycol is not uniformly 8.5 *M*. What would be the consequences with respect to devitrification? As shown in Fig. 9 the warming rate required to prevent devitrification in solutions is very sensitive to

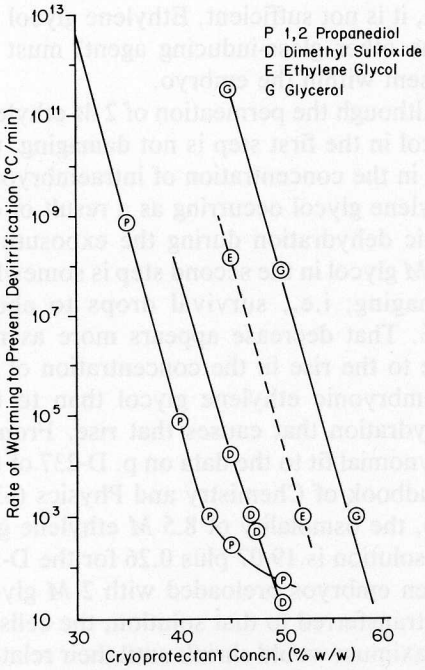

FIG. 9. Warming rate required to prevent the devitrification of vitrified solutions of various concentrations of ethylene glycol and other solutes (modified from Fahy, 4).

the weight percent concentration of glass-inducing solute present. A 5% decrease in the weight percent concentration increases the warming rate requirement about 5000-fold. With respect to water, the weight percent of ethylene glycol plus PVP in the 8.5 *M* glycol/10% PVP solutions is 54 wt % (Table 1). According to curve E of Fig. 9, the warming rate needed to prevent devitrification of such a solution is about 50°C/min, a value consistent with Schreuders *et al.*'s (24) DSC findings. But if the intraembryonic solution were 5 wt % lower with respect to ethylene glycol and if it behaves like curve E, the warming rate required to prevent devitrification would jump about 5000-fold to about 200,000°C/min. This would be consistent with our finding that warming rates have to be on the order of 100,000°C/min to obtain significant survivals.

The Role of PVP and Other Polymers

We have noted in connection with Table 6 and Fig. 2 that average survivals of 12-h embryos after very rapid cooling to −205°C and warming are higher when the 8.5 *M* ethylene glycol contains 10% (w/v) PVP. Perhaps more important than the average survival is that in the presence of PVP, the modal survival was 20–24%. In the absence of PVP, the modal value was less than 5%. The superior results with 10% PVP appear not to be unique to that polymer or that concentration. Thus, survivals with 8.5 *M* ethylene glycol containing 5% PVP or 12% BSA were similar to those with 10% PVP. A small number of runs with higher and lower molecular weight PVPs yielded similar results. Steponkus *et al.* (26) obtained their optimal 18% hatching with 6% BSA. It seems safe to assume that neither PVP nor BSA can penetrate the permeabilized embryo, especially in only 5 min at 5°C. Although it is possible that the two macromolecules confer protection by, for example, reducing the rigidity of the extraembryonic glass and thereby reducing thermal stresses, a simpler explanation is that they confer a degree of protection by increasing the osmolality of the external medium, which in turn leads to greater osmotic dehydration of the embryos during the 5-min exposure at 5°C than would occur in 8.5 *M* ethylene glycol alone. The greater dehydration would cause the concentration of glycol to rise from 2 *M* to a higher value than would be the case in the absence of the PVP. We see from Table 1 that in an 8.5 *M* ethylene glycol solution without PVP, the molality of ethylene glycol is 15.8 and the weight of ethylene glycol per 100 g of ethylene glycol plus water is 49.6%. In an 8.5 *M* ethylene glycol that contains 10% PVP, the molality rises to 18.7 and the weight of ethylene glycol per 100 g ethylene glycol plus water rises 4% to 53.7%. The higher molality in the latter case means a higher os-

molality and osmotic pressure. (The osmotic contribution of 10% PVP is small because of its high average molecular weight.) Even though the embryo will not contain PVP, it does contain nonaqueous components amounting to 25% of the normal wet weight (13). A substantial portion of those components will be proteins and other macromolecules, and they too will be concentrated several fold by the dehydration occurring during the 5-min exposure to the concentrated ethylene glycol.

It is a common observation that mixtures of inorganic compounds vitrify more readily and devitrify less readily than do the compounds individually (30). The same is true of aqueous solutions of organic solutes. Thus, Sutton (27, 28) has found that the critical cooling rate required to induce vitrification of a given concentration of butane-2,3-diol is markedly reduced by the addition of PVP, and especially by 400- to 8000-dalton polyethylene glycol (PEG). Put differently, the weight percent of butanediol needed to prevent crystallization at a given cooling rate is reduced by more than 10% in the presence of PEG and other solutes. Although not measured by Sutton, the mixed solutes probably exert similar effects on the warming rate required to prevent devitrification.

Relative Importance of Dehydration vs Intraembryonic Ethylene Glycol to the Induction of Vitrification and the Avoidance of Devitrification: Toxicity of Intraembryonic Ethylene Glycol

We pointed out in connection with Fig. 6 that even though 40% of embryos survived the osmotic removal of 95% of their water that is calculated to occur in 2 *M* sucrose, none of these embryos hatched after subsequent ultrarapid cooling and warming, or even showed development. Clearly, although dehydration may be an important component to obtaining survival by inducing vitrification and avoiding devitrification, it is not sufficient. Ethylene glycol (or some other glass-inducing agent) must be present within the embryo.

Although the permeation of 2 *M* ethylene glycol in the first step is not damaging, the rise in the concentration of intraembryonic ethylene glycol occurring as a result of osmotic dehydration during the exposure to 8.5 *M* glycol in the second step is somewhat damaging; i.e., survival drops to about 45%. That decrease appears more ascribable to the rise in the concentration of intraembryonic ethylene glycol than to the dehydration that causes that rise. From a polynomial fit to the data on p. D-237 of the Handbook of Chemistry and Physics (63rd ed.), the osmolality of 8.5 *M* ethylene glycol solution is 19.07 plus 0.26 for the D-20. When embryos preloaded with 2 *M* glycol are transferred to that solution, the cells as a maximum would shrink until their relative water volume was 2.54/19.3 or 13% of isotonic. When embryos that do not contain glycol are shrunken to that extent in sucrose (Fig. 6) some 75% survive.

Pertinent to this point is that when embryos preloaded with 2 *M* ethylene glycol are dehydrated by exposure to high concentrations of glycerol or sucrose for 5 min at 5°C, the survivals are far lower than when they are dehydrated by comparable molalities of ethylene glycol (Figs. 1 and 7). This may well be related to the fact that the embryos are more permeable to ethylene glycol than to glycerol or sucrose. A higher permeability means a lower reflection coefficient and as the reflection coefficient drops, the maximum initial cell shrinkage is reduced, as is the time the cells remain in that maximally shrunken state. A priori, if the shrinkage is less, the intraembryonic ethylene glycol will become less concentrated. But because of the cross couplings between water and ethylene glycol fluxes, an analysis by irreversible thermodynamics would be required to determine whether this is in fact so.

Role of Developmental Stage

Because we had determined that alkane/alcohol permeabilization became substantially less effective in embryos older than 14 h (11) and because permeabilization was essential, we used 12-h embryos until late in the study. But it now appears that superimposed on the critical role of warming rate is an important contribution of developmental stage. Survival after ultrarapid cooling and warming is substantially higher in 14- to 15-h embryos. We suggest two explanations. One possibility is that less devitrification occurs in the older embryos because all regions are more effectively permeated by ethylene glycol. The other is that older embryos may be more tolerant of small amounts of intraembryonic ice. The two are not mutually exclusive. With respect to the second, we note that the critical embryological steps of dorsal closure and head involution are essentially complete by 15 h, and that it is these events that were pathological in a high proportion of treated 12-h embryos.

Although the older experimental embryos did not receive prior exposure to 4°C as did the 12-h embryos in our other experiments, that does not explain their superior survivals after subsequent exposure to −205°C. The 12-h embryos in Fig. 8 were held at 4°C for only 10–20 min, and yet their survival, if anything, was poorer than that of embryos in the other experiments that were maintained at 4°C for 1 to 6 h (Fig. 2) prior to permeabilization and cryogenic exposure. Interestingly, in the case of 12-h controls subjected to 8.5 *M* ethylene glycol 10% PVP but not to −205°C, the omission of ≥1 h at 4°C prior to permeabilization and exposure to glycol was decidedly deleterious; the survivals (16%) were only a third of those in other experiments that received longer exposures at 4°C (Fig. 1). However, this heightened sensitivity vanished in 15-h embryos. It may be that ≥1-h exposure to 4°C protects 12-h embryos from ethylene glycol and associated osmotic dehydration by virtue of inducing the synthesis of stress proteins. Exposure at 0°C is known to induce such synthesis in *Drosophila* larvae (2). Myers and Steponkus (18) and Mazur *et al.* (15) have noted that exposure to 0°C confers a small degree of protection against chilling injury below −15°C. But that protection does not seem relevant here because it disappears in a few minutes when embryos are returned to room temperature as occurs during permeabilization and the subsequent 45-min incubation. The basis of the higher hatching survivals of 14- to 15-h embryos exposed to −205°C is being pursued.

Although overall survivals to adulthood are currently less than 1% (No. adults/No. embryos cryopreserved) it should be emphasized that the *Drosophila* embryo is among the more complex systems that have survived exposure to cryogenic temperatures to any degree. The 12- to 15-h stages being frozen contain some 50,000 cells that have differentiated into highly organized tissues and organ systems.

ACKNOWLEDGMENTS

We thank J. W. Hall for determining survivals of unfrozen controls and S. Scott for maintaining the flies and harvesting eggs.

Note added in proof. Since the submission of this paper, P. Steponkus and S. Caldwell have confirmed that hatching survivals after cryogenic exposure improve as embryos age from 12 to 15 h; namely, from 18 to 49–55%. [Annual meeting, Society for Cryobiology, June 14–19, 1992 (*Cryobiology* **29**, 763, 1992, abstract)]. They also report that with minor changes in permeabilization procedure, 11% of the resulting larvae developed to adults. More recently, P. Mazur, K. Cole, P. Schreuders, J. Hall, and A. Mahowald report (*Science* **258**, 1932–1935, 1992) that with a developmentally more precise way of staging the embryos and with modifications in post-treatment culture techniques, 60–75% of treated Oregon R embryos survive, and a mean of 40% of the resulting larvae develop to fertile adults.

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