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Characterization of Intraembryonic Freezing in *Anopheles gambiae* Embryos[1,2]

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Intraembryonic freezing (IEF) in *Anopheles* mosquito embryos has been evaluated by differential scanning calorimetry with respect to embryo age, temperature, rate and duration of cooling, and absence or presence of extraembryonic ice. The initial temperatures for intraembryonic ice nucleation were -30.1 ± 0.3, -28.4 ± 0.4, and -29.1 ± 0.2°C for embryos incubated for 15 h at 17°C, 15 h at 26°C, and 24 h at 26°C, respectively, after oviposition. The first value is slightly but significantly lower than the latter two. These values were obtained on embryos in which the surface water was removed by brief drying; however, the values were nearly identical when external water and ice were present. Not only were the embryos of all three ages able to supercool at least transiently to −26°C, but they could remain supercooled for up to 4 h at −20°C after being cooled to −20°C at 10°C/min or (in the case of embryos incubated for 15 h at 26°C) at 100°C/min. The amount of freezable water in single embryos has been calculated from the differential scanning calorimetry measurements to be 3.45 ± 0.08, 3.46 ± 0.08, and 3.53 ± 0.06 μg for embryos incubated for 15 h at 17°C, 15 h at 26°C, and 24 h at 26°C, respectively. The differences are not significant. The corresponding values for the total water contents for embryos of the three ages were 4.04 ± 0.20, 3.72 ± 0.16, and 3.98 ± 0.10 μg, values that also did not differ significantly. Water thus makes up ~74% of the total weight of the embryo (~5.3 μg) and about 91% of that water is freezable. Total water contents were determined gravimetrically after extensive air and vacuum drying. The kinetics of dehydration were determined during the air drying. They differed substantially among the three ages. The embryos incubated for 15 h at 17°C lost water at about four times the rate of those incubated for 15 h at 26°C and 10 times the rate of the embryos incubated for 24 h at 26°C.

Malaria, a human disease caused by the *Plasmodium* parasite and transmitted by the mosquito *Anopheles*, kills somewhere between 1 and 2 million people annually. Because of the evolution of both insecticide resistance and drug resistance, much current malaria research focuses on the genetics of *Anopheles* involved in the transmittance of the disease in an effort to create transgenic strains of *Anopheles gambiae* that block the *Plasmodium*. Significant problems encountered in genetically engineering the mosquitoes include the difficulty in maintaining adult mosquito stocks and the threat of genetic drift. These problems have motivated our efforts to develop a method for the cryopreservation of mosquito embryos (28).

The initial effort was based on existing knowledge of the problems associated with the cryopreservation of *Drosophila melano-*

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gaster embryos. The problems in *Drosophila* were formidable. Briefly, they were the extreme impermeability of the eggs to both water and cryoprotectants and the consequent need to develop permeabilization procedures, the high chill sensitivity at subzero temperatures in the absence of ice, and the strong age dependence of both chill sensitivity and the effectiveness of permeabilization procedures. Permeabilization was mandatory because cryopreservation requires that cells be permeable to both water and cryoprotective solutes. Even when that was achieved, chill sensitivity accelerated so rapidly at subzero temperatures that classical slow-freezing techniques could not be used. Alternatively, Steponkus *et al.* (23) and Mazur *et al.* (9) were forced to develop vitrification procedures involving extremely high cooling and warming rates. Developmental age of the embryos was critical in part because young embryos were prohibitively chill sensitive (12, 15, 16) and embryos approaching hatching became refractory to permeabilization (9, 10).

Some of the problems with *A. gambiae* embryos are similar to those in *Drosophila.* But there are important differences. Valencia *et al.* (25, 26) have been studying *Anopheles* embryo permeability and permeabilization at the Laboratory of Parasitic Diseases at the National Institutes of Health (NIH). Like *Drosophila,* the intact embryos are impermeable to the cryoprotectant ethylene glycol. Unlike intact *Drosophila* embryos, which are highly impermeable to water at all developmental stages, *Anopheles* embryos are slightly permeable to water when young but become highly impermeable when older. A consequence of the modest water permeability of the young embryos is that, although the full *Drosophila* permeabilization procedure is effective, it is lethal. Valencia *et al.* (26), however, have developed modifications that are relatively innocuous. Older embryos constitute a different problem: They cannot be permeabilized even by the full *Drosophila* procedure.

We in Oak Ridge have been studying the chill sensitivity of intact *Anopheles* embryos; chill sensitivity is the term assigned to embryo death at low temperatures in the absence of ice formation. Preliminary results indicate first that chilling injury appears at +8°C and that the rate of injury increases as the temperature is lowered to 0°C, and, second, that the death rate at 0°C is much higher in young embryos than in older ones (11). The terms *young* and *old* are defined specifically in terms of the duration and temperature of incubation between egg laying and experiment. By the former, we mean eggs incubated for 15 h at 17°C. By the latter we mean eggs incubated for time/temperature combinations such as 15 h at 26°C and 24 h at 17 or 26°C.

An important factor in the cryopreservation strategy is the kinetics of chill injury at subzero temperatures, a question that is tantamount to asking about chill injury in unfrozen, supercooled, embryos. To determine the limits of embryo supercooling, we have used differential scanning calorimetry to measure the temperature at which intraembryonic water freezes in both the absence and the presence of extraembryonic ice and the time over which deep supercooling can be maintained. When intraembryonic freezing (IEF) does occur, the next question is what fraction of the total embryo water freezes. Differential scanning calorimetry gives the quantity of frozen water. Measuring total water content required complete air drying of the embryos. That also permitted us to assess the kinetics of dehydration in air and the age dependence of those kinetics.

MATERIALS AND METHODS

A differential scanning calorimeter (DSC-7, Perkin Elmer, Norwalk, CT) was used to characterize IEF in *A. gambiae* mosquito embryos. The DSC-7 measures the amount of heat absorbed or released by a sample as the temperature is changed. The instrument does this by determining the amount of electrical energy required to keep the temperature of the test sample equal to the temperature of the reference sample that contains no water. Ice

formation in the embryo and its environs will produce a thermogram that shows an exothermic peak. Conversely, the melting of that ice produces an endothermic peak. The first value of concern was the onset temperature of the endotherm or exotherm and the temperature at which they reached the maximum rate of energy release or absorption (peak temperature). The second value was the area under the peak. It represented the heat energy that was absorbed or released during the phase change. From this area, the amount of intra- or extraembryonic water that underwent the phase change was calculated. The analyses were performed using the Perkin Elmer Thermal Analysis software (Version 2.2).

Collection and Staging of Anopheles Embryos

Blood-fed female *Anopheles* mosquitoes were obtained weekly from the Laboratory of Parasitic Diseases, NIH, Bethesda, Maryland. The mosquitoes were maintained in a controlled environment room at 80% humidity and 26°C. A piece of cotton saturated with 15% Karo syrup in double-distilled water was provided as a food source. Embryos were collected from the adult mosquitoes 4–6 days after the blood meal. Approximately 40–50 mosquitoes were aspirated from their container and anesthetized with carbon dioxide gas. Mosquitoes were then transferred to an inverted 9.0-cm Petri dish (Falcon No. 1001) containing 9.0-cm Whatman No. 1 filter paper. After allowing 20–30 minutes for the mosquitoes to recover from the carbon dioxide, the filter paper was moistened with 2 ml of double-distilled water. The mosquitoes were then placed in the darkened, controlled environment room for approximately 45 min to induce oviposition. The midpoint of this collection period was defined as time zero. The adults were then removed from the Petri dish and embryos were incubated either in a water bath at 17°C or in a 12 × 12 × 12-in. box in an incubator at 26°C and at nearly 100% humidity.

Defining Developmental Age

The embryos' developmental stages are specified in terms of the number of hours elapsed since the zero time of the embryo collection and the temperature of the incubation, rather than in terms of embryological stage. One reason for this is that there is little information on the morphological changes in *Anopheles* embryos with time. Second, even if the staging were known, it cannot be readily determined because of the opacity of the embryos. The mosquito embryos were examined at three different ages: 15h@17°C, 15h@26°C, and 24h@26°C.

The time from egg laying to hatching is about tripled at the lower temperature; i.e., it increases from 50 to 153 h (25). If one assumes that all the intervening development is correspondingly slowed, the developmental ages are roughly in the ratio of 1:3:5 in the three groups studied (15h@17°C, 15h@26°C, 24h@26°C); e.g., the 15h@17°C embryos are roughly equivalent to 5h@26°C embryos. There is some experimental support for this equivalence with respect to permeability properties and sensitivity to chilling.

In the ensuing discussion we make some comparisons with *Drosophila* embryos. In connection with such comparisons, it should be noted that *Drosophila* embryos develop about twice as rapidly as *Anopheles* embryos, based on the time to the embryological stage of germ band extension and on time to hatching (26). Thus, if one assumes this ratio holds throughout development, a 12h@26°C *Drosophila* embryo would be roughly equivalent to a 24h@26°C *Anopheles* embryo.

Postincubation Storage and Handling

Following the incubation period, embryos were washed into a sheet of 9-cm Whatman No. 1 filter paper formed into a funnel and placed into a plastic cup of water. Those that floated were then transferred to dry 13-mm polycarbonate filter membranes (12-μm pore size, Costar Scientific Corp., Cambridge, MA) and immediately placed into a 9.0-cm Petri

containing a disk of moistened Whatman No. 1 filter paper. Embryos that did not float were considered damaged or infertile and were not used in the experiments.

Between experimental runs on a given day, the embryos were stored in an 8°C water bath, since this temperature minimized subsequent embryonic development without impacting the embryo's survival (11).

Calibration and Sensitivity Determination of the Differential Scanning Calorimeter

The DSC-7 was calibrated using standard procedures (17) to an accuracy of ±0.25°C and ±0.15 J/g at a cooling/warming rate of 10°C/min. The calibration was performed using the melting of indium (melting point, 156.60°C; transition energy, 28.45 J/g) and the crystalline transition of cyclohexane (−87.06°C). The calibration was checked using the melting of double-distilled water.

The instrument's sensitivity was then examined by assessing the freezing and thawing of various numbers of *D. melanogaster* embryos, since these embryos are essentially impermeable to water and contain known quantities of water. The DSC-7 was capable of quantifying the melting and freezing of a single *Drosophila* embryo. It contains 5.4 μg of freezable water (14). Later experiments showed that the nucleation/melting of the ice in a single *Anopheles* embryo was also well within the analyzable sensitivity of the instrument. Furthermore, the DSC-7 was capable of detecting, though not accurately analyzing, exotherms and endotherms with areas as small as 0.0267 mJ, a value that corresponds to the freezing of the water in approximately 0.024 *Anopheles* embryo.

Determination of the Nucleation Temperature of Intraembryonic Water

In the absence of extraembryonic water. This series of experiments was designed to measure the nucleation temperature and amount of freezable water in *Anopheles* embryos at the three different stages of development in the absence of external water. The DSC-7 was first allowed to reach thermal equilibrium at 25°C (approximately 20 min). During this time, 30–50 *Anopheles* embryos were transferred by a small sable brush to a moist $\frac{1}{8}$-in. polycarbonate membrane filter (punched from 25 mm, 12.0-μm pore size, Nuclepore Corp., Pleasanton, CA, or Costar Scientific Corp., Cambridge, MA), arranged in a monolayer, and counted. The membrane filter and embryos were then floated on water to prevent dehydration prior to the next steps of the experiment. Two microliters of mineral oil (Chemline light mineral oil, American Drug Industries, Chicago, IL) was added to the bottom of an aluminum volatile-sample pan (Perkin Elmer). The filter membrane with embryos was placed on a 9.0-cm paper filter on top of a fritted glass filter inserted into a flask attached to house vacuum. Vacuum was applied for a total time of approximately 1 min to remove surface water around the embryos. After the completion of this step, all visible extraembryonic water had been removed from the embryos/filter. After about 30 s of that total, 4 μl of mineral oil was dropped on the embryos and pulled through by the vacuum. The mineral oil was added to minimize dehydration of the embryos and to displace residual extraembryonic water. The filter membrane was then loaded into the DSC-7 sample pan with the embryos facing down, the pan lid was put in place and sealed using a crimper, and the pan was loaded into the DSC-7. A second crimped reference pan containing 2 μl mineral oil was placed in the DSC-7 to serve as a reference.

The experimental procedure was as follows: (1) cool from 25 to −50°C at 10°C/min, (2) hold at −50°C for 1 min (to allow the sample to reach thermal equilibrium), and (3) warm from −50 to 25°C at 10°C/min.

In the presence of extraembryonic water. This set of experiments was designed to determine if the freezing of extraembryonic water affects the intraembryonic nucleation temperature of *Anopheles* embryos of different ages. The same experimental protocol was used for these experiments as for those conducted in

the absence of external water, except that 2 μl water was placed in the bottom of the aluminum sample pan instead of the mineral oil. This double-distilled water contained Snomax (10 mg/liter), a freeze-dried preparation of the bacterium *Pseudomonas syringae* that initiates the nucleation of water above −10°C (24). The filter membrane containing the embryos was not dried by vacuum, but instead was blotted on a Kimwipe to remove excess water. Once the filter membrane and embryos were transferred to the water in the sample pan, 15 μl mineral oil was added on top of the membrane to minimize both the evaporation of the water and the possibility of small droplets of water condensing on the interior walls of the pan. The reference pan was empty. The subsequent cooling and warming procedures were the same as in the previous experiments in which extraembryonic water was absent.

Effects of long-term holding at −20°C. These experiments were performed to determine if *Anopheles* embryos could be held at subzero temperatures for extended periods without IEF occurring. The embryo samples were prepared as in the nucleation temperature runs in the absence of external water (using mineral oil to coat the embryos), but the cooling/warming procedures were different. In one set of experiments, the samples were (1) cooled from 25 to −20°C at 10°C/min, (2) held at −20°C for 240 min, and (3) warmed from −20 to 25°C at 10°C/min. The other experiments were the same except that the cooling rate was 100°C/min.

Total Water Content and Drying Kinetics of Anopheles Embryos

This set of experiments was performed to determine both the total amount of water in *Anopheles* embryos of three developmental ages and their rate of dehydration in room air. Between 100 and 255 embryos were placed in a monolayer in groups of five on a preweighed 13-mm polycarbonate filter membrane and counted. Surface water was removed from the embryos by placing the membrane filter on top of a sintered glass filter-and-flask assembly and applying vacuum for approximately 1 min. The membrane filter with embryos was weighed on an analytical balance with a resolution of ±10 μg (AG245, Mettler-Toledo, Switzerland). This weight (recorded approximately 75 s after removal from the vacuum), minus the filter weight, was taken as the time zero weight for fully hydrated *Anopheles* embryos. The membrane filter was then weighed periodically for the next several hours. To determine the absolute dry weight of the embryos, the filter membrane with embryos was then placed in a vacuum box (less than 25 mm Hg absolute pressure) for several days and weighed.

Statistical Analysis

Means, standard errors and standard deviations, and *t* tests (two-tailed assuming unequal variances) were calculated using Excel (Version 4.0 for Windows and Version 5.0a for the Macintosh, Microsoft Corp., Redmond, WA). These *t* tests were performed when comparing embryos that were the same age, but were subjected to different treatments. One-way analyses of variance (ANOVAs) and *t* tests corrected using the Bonferroni method were performed when comparing embryos that were different ages, but were otherwise subjected to the same treatments. These calculations were performed using InStat (Version 1.15 for DOS, GraphPad Software, San Diego, CA). Mean values are reported as plus or minus the standard error. Values of P less than 0.05 were considered significant.

RESULTS

Nucleation Temperature of Embryonic Water

The IEF of *A. gambiae* mosquito embryos was evaluated with respect to the developmental stage, temperature, rate and duration of cooling, and absence or presence of extraembryonic ice formation.

Nucleation in the absence of extraembryonic water. As illustrated in Fig. 1, intraem-

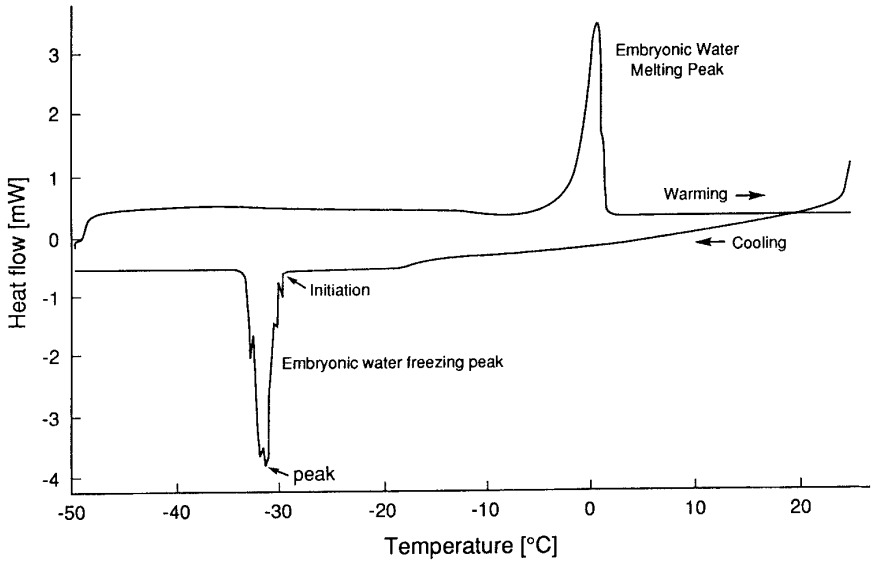

FIG. 1. Thermogram from a typical nucleation temperature experiment. This experiment was performed using 35 of the 15h@26°C embryos. Both the cooling and warming traces are shown. The intraembryonic freezing peak was initiated at −29.5°C, with the embryonic peak maximum occurring at −31.3°C.

bryonic freezing in mosquito embryos was typically manifested in the DSC-7 as a single, somewhat jagged peak or exotherm.

The average exotherm began at −30.1, −28.4, and −29.1°C for 15h@17°C, 15h@26°C, and 24h@26°C embryos, respectively (Table 1). The corresponding maximum peak temperatures were reached at −32.9, −31.3, and −31.2°C. The differences, with respect to developmental age, were small, although the values for the youngest embryos (15h@17°C) were significantly lower than those for the 15h@26°C embryos. The evidence that the exotherm arises from the freezing of water comes from the warming curve, which showed only a single smooth endotherm that occurred at 0°C. The conclusion is that the embryos remain supercooled to below

TABLE 1
Nucleation Temperature of *Anopheles* Mosquito Embryos in the Absence[a] and Presence[b] of Extraembryonic Water

Developmental stage	Major embryonic peak initiation temperature		Major embryonic peak maximum temperature	
	Water absent	Water present	Water absent	Water Present
15 h @ 17°C	−30.1 ± 0.3°C	−30.6 ± 0.3°C	−32.9 ± 0.2°C	−32.6 ± 0.3°C
15 h @ 26°C	−28.4 ± 0.4°C	−29.8 ± 0.2°C	−31.3 ± 0.1°C	−31.9 ± 0.2°C
24 h @ 26°C	−29.1 ± 0.2°C	−29.3 ± 0.2°C	−31.2 ± 0.2°C	−31.2 ± 0.1°C

[a] Values are averages ± standard errors for 9, 7, and 8 experiments using 15 h @ 17°C, 15 h @ 26°C, and 24 h @ 26°C embryos, respectively.

[b] Values are averages ± standard errors for 4, 6, and 5 experiments using 15 h @ 17°C, 15 h @ 26°C, and 24 h @ 26°C embryos, respectively.

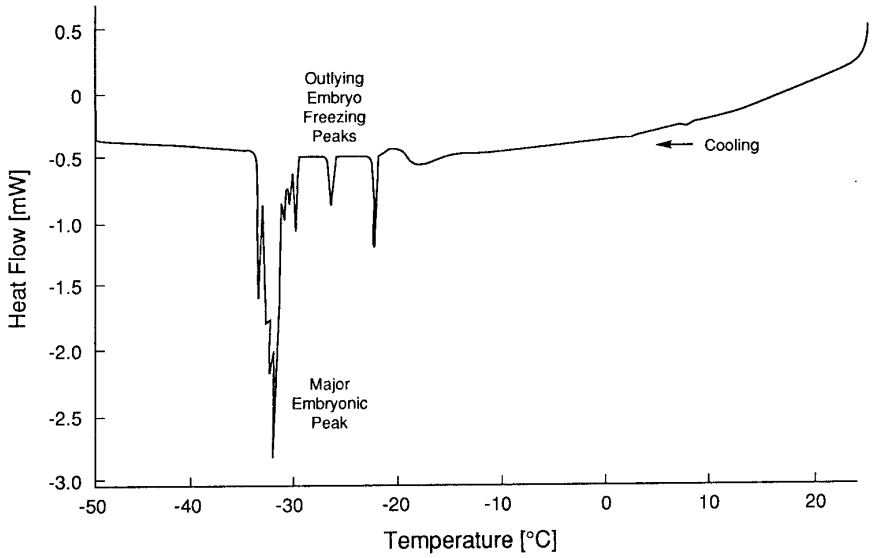

FIG. 2. Thermogram showing a nucleation experiment involving 26 of the 15h@17°C embryos. Only the cooling trace is shown. The two rightmost peaks are outliers, with the rightmost having an area equal to the freezing of two embryos and the middle peak having an area equal to the freezing of a single embryo.

−26 to −32°C and only then undergo nucleation and freezing.

The jaggedness of the exotherm during cooling indicates, first, that not all embryos supercooled to the same extent before nucleating and, second, that the nucleation of one embryo, or group of embryos, did not induce immediate nucleation in the remaining embryos. Essentially, the embryos are isolated compartments of water that nucleate in a stochastic fashion. Although the embryos nucleate stochastically during cooling, they melt simultaneously during warming, as evidenced by the smoothness of the single endotherm.

In some of the nucleation experiments, small outlying exotherms occurred during cooling before the major exothermic embryonic peak. A small number of these peaks were well below the size of a single embryo. These peaks were attributed to the freezing of small droplets of residual extraembryonic water and were omitted from the analysis. Others, such as those shown in Fig. 2, were attributed to the freezing of individual embryos. These outlying peaks occurred between −22.1 and −28.6°C and usually had an energy release equivalent to the freezing of a single embryo (though instances occurred with an energy release equivalent to two or three embryos). The mean initiation temperature of the outlying embryonic peaks were −24.3 and −25.1°C for 15h@17°C and 24h@26°C embryos, respectively. There was a single instance of an outlying peak (at −26.6°C) in the 15h@26°C embryos.

The thermograms showed a small transition at about −20°C (Figs. 1–3). This transition is believed to reflect a thermal event in the mineral oil, for it occurred in samples containing mineral oil without embryos. It will not be discussed further.

Nucleation temperatures in the presence of extraembryonic water. The peak initiation and peak maximum temperatures were also measured when the embryos were in the presence of extraembryonic water (ice). Figure 3 shows the clear differentiation between the exotherms from the freezing of intra- and extracellular water. The latter were above −10°C. The peak initiation temperatures of the former

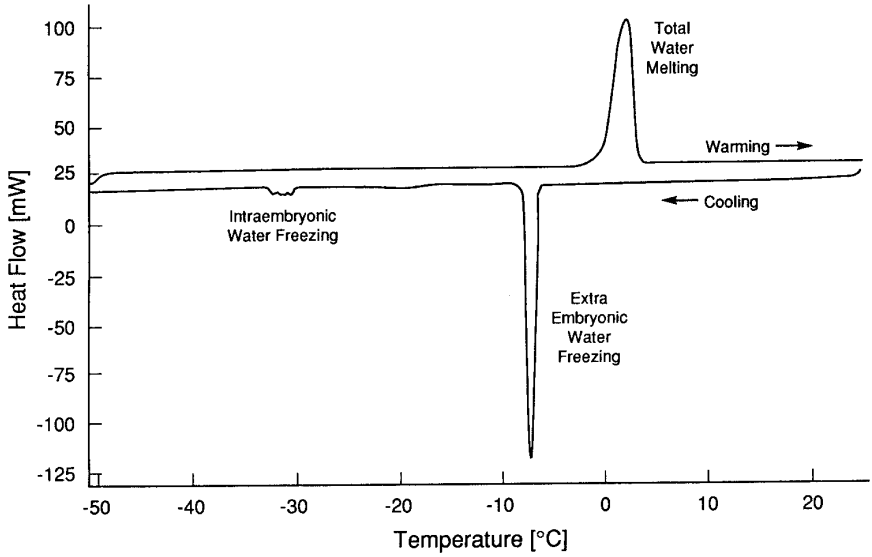

FIG. 3. Thermogram showing both the cooling and warming traces of a nucleation experiment involving 44 of the 24h@26°C embryos with 2 μl of extraembryonic water containing Snomax. The freezing of the extraembryonic water occurred at −6.5°C, a temperature similar to that observed for water/Snomax in the absence of embryos. In contrast, the intraembryonic water did not freeze until −29.7°C. As expected, the melting occurred at close to 0°C for both the intra- and extraembryonic water. Note that the scale of the ordinate of this thermogram has been expanded to show the freezing/thawing of the extraembryonic water, thus compressing vertical height of the intraembryonic water peak.

were −30.6, −29.8, and −29.3°C for 15h@17°C, 24h@26°C, and 15h@26°C embryos, respectively. The corresponding peak maxima occurred 2° lower at about −32°C (Table 1).

The lower peak initiation and peak maximum temperatures were essentially the same as the corresponding values for embryos in the absence of external water, although the values for 15h@26°C embryos in the presence of external water are significantly lower than in the absence.

Nucleation due to long-term exposure subzero temperatures. Although IEF rarely occurred above −27°C when the embryos were cooled at 10°C/min to −50°C and immediately rewarmed, it is possible that IEF might occur above −27°C if the embryos were held for extended periods. To test this possibility, embryos at the three developmental stages were cooled to −20°C at 10°C/min, held at that temperature for 4 h, and then warmed at 10°C/min. No IEF occurred. The experiments were repeated on 15h@26°C embryos using a cooling rate of 100°C/min. Again, no IEF occurred during the 4-h hold at −20°C.

Intraembryonic Water Content

Amount of freezable water. The amount of heat absorbed during melting is proportional to the mass of intraembryonic ice. Unlike pure ice, which melts at 0°C, the ice in frozen solutions (such as that in embryos) melts progressively over a range of temperatures. As seen in Fig. 1, absorbed heat is first discernible (as a rise above the baseline) at about −9°C. The total amount of heat absorbed during melting was calculated from the area under the endotherm from that point to the time (temperature) at which the peak returned to the baseline. However, it is a reasonable assumption that the contents of an embryo respond similarly to that of a (isotonic) 0.15 *m* NaCl solution. Phase diagram data (Fig. 4) show that a

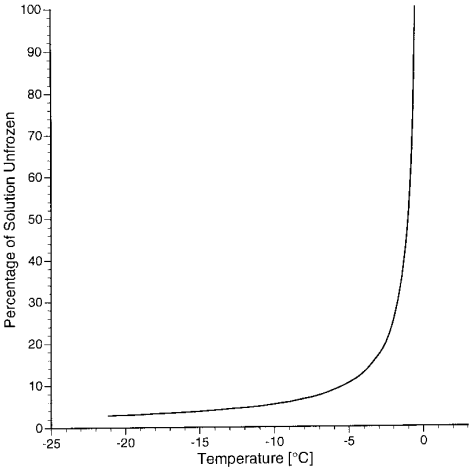

FIG. 4. Percentage of water in a 0.15 *m* NaCl/water solution that is unfrozen at subzero temperatures. Computed from phase diagram data in Seidell (22) and the International Critical Tables (27).

frozen 0.15 *m* aqueous sodium chloride solution begins to melt at −21°C and that, when it has warmed to −9°C, 5.9% of the total ice will have melted. Consequently, although we could not calorimetrically detect heat absorption below −9°C, we think it appropriate to add 5.9% to the absorbed heat that we detected in the visible endotherm.

The translation from heat absorbed to the mass of ice that has melted requires a value for L_f, the heat of fusion. We use the value of L_f at 0°C (79.7 cal/g, 333.5 J/g) even though some melting is occurring progressively over lower temperatures, and at lower temperatures L_f is reduced. The reason for using the 0°C value of L_f is as follows. It takes the same amount of heat to melt 1 g of ice at −10°C (which could be done, for example, under hydrostatic pressure) and warm the resulting liquid water to 0°C as it does to first warm 1 g of ice to 0°C and melt it at 0°C. The latent heat of fusion is reduced in the first case by close to 5 cal (the 0.5 cal/g/deg difference between the specific heats of ice and water × 10°C). Consequently, it takes 74.7 cal to melt the ice at −10°C, but it then takes the absorption of an additional 10 cal to warm the now liquid water to 0°C, for a total of 84.7 cal. In the second case, it takes the absorption of 5 cal to warm the 1 g of ice from −10 to 0°C, and it then takes 79.7 cal to melt it, for the identical total of 84.7 cal. The total heat absorbed during the melting of a frozen solution containing 1 g of water will also be 84.7 cal, although the partition between L_f and heat capacities for the warming and melting of frozen solutions will be intermediate between the two ice examples.

The mass of ice per embryo is given in column 2 of Table 2. It was calculated as described above using the melting peaks. The mean values ranged from 3.45 to 3.53 for the three developmental ages. These differences are not significant.

Alternatively, the mass of ice was calculated from the heat released during freezing. As noted above, the molar heat of fusion decreases with temperature. It can be calculated using a refinement of Kirchhoff's law by the equation (2, 5, 6)

$$L_f = L_f^0 + 9.080T_{°C} - 0.02649T_{°C}^2 + 0.000216T_{°C}^3,$$

where L_f^0 is the heat of fusion at 0°C (1436 cal/mol) and $T_{°C}$ is the Celsius temperature. The temperature used in this calculation was the peak maximum temperature, since this

TABLE 2
Amount of Water per *Anopheles* Embryo

Stage of embryonic development	Amount of freezable water per embryo (μg)[a]	Total amount of intraembryonic water (μg)
15 h @ 17°C	3.45 ± 0.08[b]	4.04 ± 0.20[c]
15 h @ 26°C	3.46 ± 0.08[b]	3.72 ± 0.16[b]
24 h @ 26°C	3.53 ± 0.06[b]	3.98 ± 0.10[c]

[a] Based on warming data.
[b] Values are averages ± standard errors for seven experiments.
[c] Values are averages ± standard errors for three experiments.

represented the temperature at which the greatest number of embryos underwent freezing. When the mass of ice per embryo was calculated using the corrected values of L_f, the masses were 3.59 ± 0.05 (SE), 3.64 ± 0.08, and 3.71 ± 0.07 μg for the 15h@17°C, 15h@26°C, and 24h@26°C embryos. The differences among the three ages are not significant. These masses of ice calculated from the exotherms during cooling are 4–5% greater than those calculated from the endotherms during warming, but the differences are not significant.

Amount of total water. To calculate the total amount of water in an average embryo, the fully hydrated mass and the fully dehydrated mass of known numbers of embryos were both measured gravimetrically. The fully hydrated weights of the 15h@17°C, 15h@26°C, and 24h@26°C embryos were 5.40 ± 0.11, 5.14 ± 0.26, and 5.27 ± 0.14 μg/embryo, respectively. The corresponding dry weights for the embryos were 1.36 ± 0.11, 1.41 ± 0.14, and 1.29 ± 0.07 μg/embryo. Thus, as shown in Table 2, the corresponding total amounts of water in embryos were 4.04, 3.72, and 3.98 μg/embryo. These amounts of water constitute 74.7 ± 2.3 (SE), 72.7 ± 1.5, and 75.5 ± 1.1% of the total mass of the fully hydrated embryos (~5.3 μg). The fractions of the total water that are freezable (column 2 versus column 3 in Table 2) are 85, 93, and 93% for the three ages of embryos, respectively. These differences are not significant.

Drying Rate

The rate at which embryos at the three developmental ages lose water when exposed to air at ~50% relative humidity and 22 to 23°C is shown in Fig. 5. The younger the embryo, the greater the rate of dehydration. Thus, 15h@17°C, 15h@26°C, and 24h@26°C embryos lost 30% of their initial weight (~40% of their water) in 11, 92, and 112 min, respectively.

DISCUSSION

This study has been concerned with a number of properties of intraembryonic water in embryos of the mosquito *A. gambiae* that are relevant to their cryobiological preservation, but are also of more general biological interest. These included the amount of water in embryos of three developmental ages, the fraction of that water that can freeze, and the temperature at which it freezes. The experiments also included measurements of the permeability of eggs to water vapor as a function of developmental age.

Intraembryonic Nucleation Temperature

The osmolality of isotonic media for mosquito tissues, and presumably that of the eggs, is ~0.3 osmolal (26). Although such a solution has a freezing point of about −0.5°C, our DSC-7 measurements in the absence, or near absence, of external water showed that intraembryonic freezing in the great majority of instances begins only below −28°C, and that the modal temperature of nucleation lies between −30 and −34°C (Fig. 1, Table 1). In a small number of instances, a few embryos nucleated at somewhat higher temperatures, i.e., about −24°C (Fig. 2). They could have represented damaged eggs or unfertilized eggs that had not developed normal permeability barriers.

The fact that the eggs supercool about 30° before nucleating indicates that they contain no effective heterogeneous nucleating agents. In the absence of nucleating agents, pure water freezes at about −36°C by homogeneous nucleation (20). In the presence of effective nucleating agents such as silver iodide and ice-nucleating bacteria (see below), it freezes above −10°C by heterogeneous nucleation. The most effective nucleator of supercooled water is, of course, ice itself, which nucleates at or just below 0°C.

Ice nucleation is a stochastic process (18). The stochastic nature is difficult to see in bulk samples of water or aqueous solution because the first nucleation results in the freezing of the entire sample. It can be seen, however, in the exotherms generated during the cooling of the *Anopheles* eggs. The exotherms show a number of jagged peaks as opposed to the

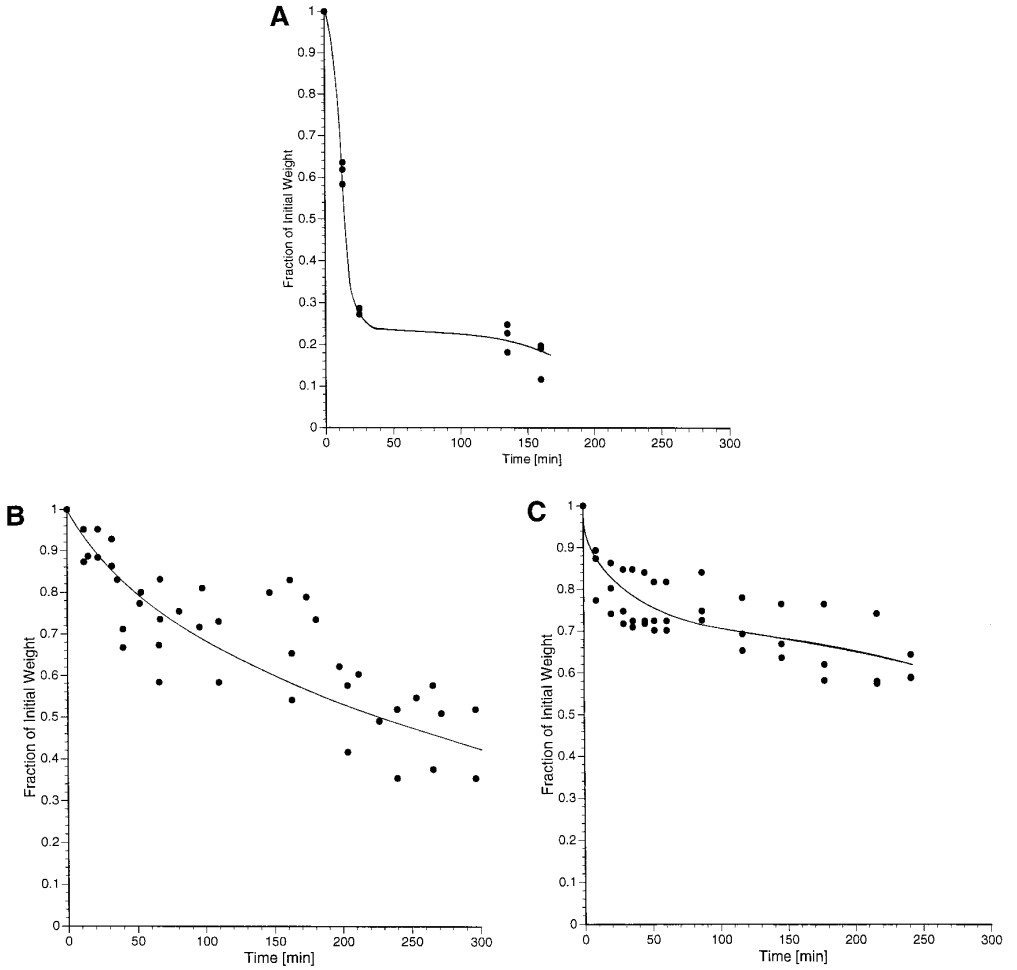

FIG. 5. Kinetics of the dehydration of embryos of the three ages exposed to room air (22–23°C and 50–60% relative humidity). The embryo ages were (A) 15h@17°C, (B) 15h@26°C, and (C) 24h@26°C.

endotherms during warming, which show only a single smooth peak. The mean temperatures for the initiation of the major exotherm have higher standard deviations and standard errors than those of the modal or peak-maximum temperatures (Table 1). When outlying peaks from the freezing of single embryos occur at higher temperatures, their nucleation does not affect the nucleation temperature of the bulk of the embryos (compare Figs. 1 and 2). These findings indicate that although most of the embryos nucleate about −30°C, the nucleation of one embryo does not affect the nucleation temperature of the other embryos in the sample. The nucleation temperature of the embryos is also essentially independent of their developmental stage, although there are statistically significant suggestions that it might be slightly lower in the youngest embryos studied (15h@17°C).

To determine the embryo's chilling sensitivity (in the absence of intraembryonic freezing) versus exposure time, the supercooled intraembryonic water has to be stable with time. That was the case, at least at −20°C, for no intraembryonic nucleation occurred over 4 h.

Such a result is consistent with homogeneous nucleation theory in that the nucleation of water 8 to 10°C above the observed nucleation temperature occurs approximately 20 orders of magnitude more slowly. Another implication of this extreme temperature sensitivity is that homogenous nucleation (and presumably intraembryonic nucleation) is far more sensitive to the temperature than to cooling rate or time at a subzero temperature (4, 7). Furthermore, our experiments show that the stability of the intraembryonic liquid water is independent of whether the embryos are cooled to −20°C at 10 or 100°C/min.

Lack of Effect of External Ice

The extent to which cells supercool is often affected by whether or not external ice is present. When it is present, it can pass through the plasma membrane below certain temperatures or otherwise induce ice nucleation of supercooled protoplasm (7, 13). Our differential scanning calorimetry studies, however, show that the presence of external ice essentially does not affect the nucleation temperature of the *Anopheles* embryos. In our preliminary experiments, added extracellular water usually supercooled to about −20°C before nucleating. This made it difficult to know whether exotherms above −28°C reflected an upward shift of the nucleation temperature of the embryos because of the presence of external ice or just reflected the freezing of the external water. To eliminate this problem, we added a small quantity of freeze-dried membranes of *Pseudomonas syringae,* an ice-nucleating bacterium. It caused the external water to nucleate above −10°C, and thus clearly separated its freezing from that of the embryos.

The fact that the embryos supercool some 20° below the temperature at which external ice forms and that they supercool as much in the presence of external ice as in its absence indicates that external ice crystals are unable to penetrate the egg to nucleate the supercooled water within. While it is possible that the barrier to ice nucleation is the plasma membranes of the embryo cells, it is more likely that it lies in the egg shells that surround the embryo proper. The shell is composed of an outer chorion and an inner vitelline membrane (1). Depending on its age, the anophelian egg possesses low to nearly undetectable permeability to liquid water. Some of the barrier to water permeation lies in the chorion, for its removal by hypochlorite results in some increase in permeability (25). But most of the barrier lies in the vitelline membrane. In 15h@17°C *Anopheles* embryos, that barrier can be removed or breached by exposure to the alkane heptane. Such treatment produces a marked increase in water permeability (26). The effectiveness of the alkane suggests that the barrier is at least partly composed of waxes. The water permeability properties of *Drosophila* eggs are similar, except that they are even more impermeable to water than are *Anopheles* eggs prior to permeabilization by alkane (10, 21, 25).

Age-Dependent Differences in the Permeability of Eggs to Ice, Liquid Water, and Water Vapor

The close similarity of all the nucleation temperatures in Table 1 indicates that external ice is unable to come in contact with the supercooled intraembryonic protoplasm regardless of the embryo age. Although ''permeability'' to ice is not affected by embryo age, the permeability to liquid water and to water vapor decidedly is. Figure 5 shows that the permeability to water vapor as measured by the rate of dehydration in air decreases dramatically with age. Moreover, the rapid dehydration of the youngest embryos (15h@17°C, Fig. 5A) is probably an underestimate. Valencia *et al.* (26) observed under the microscope that embryos of that age undergo severe morphological collapse after 2 to 3 min in air. Our gravimetric technique may underestimate their drying rate because some dehydration is likely to have already occurred prior to the first weighing, and because there is likely to be some confounding between the loss of intraembryonic water and

the evaporation of residual surface water [see Schreuders *et al.* (21) for further discussion]. Permeability to liquid water under an osmotic gradient also decreases with increasing developmental age. Thus, the time for 25% of the embryos to shrink in 0.75 *M* sucrose/isotonic saline increases from 5 min in 15h@17°C embryos to approximately 60 min in 15h@26°C embryos (25).

These differences in the penetration of ice, liquid water, and water vapor would appear to indicate that the pores for water transport in the egg shells are either nonexistent or extremely small even in the youngest eggs tested. For an ice crystal to pass through a barrier, the barrier must possess pores of sufficient diameter to allow the passage of the assemblage of molecules that constitutes the crystal. Probably, such pores would have to be some 10 Å in diameter or more (7). In contrast, liquid water and water vapor could diffuse through the barrier as single molecules.

What causes the barrier to the permeation of liquid water and water vapor to increase with embryo age? As indicated, the barrier in 15h@17°C embryos appears primarily to be waxy. Older embryos may become less permeable because there are changes in the chemical composition or structure of the wax. But there are other possibilities. One is that as *Anopheles* embryos age they are known to become increasingly melanized and sclerotized (1). These processes, which involve protein crosslinking, may augment the barrier.

Amount of Freezable Water versus Amount of Total Water

The mass of freezable water in the embryo was estimated from the area under the exotherm during cooling to −50°C and from the area under the endotherm during warming. The two estimates agree closely, namely, 3.4–3.7 μg/embryo. The value was independent of embryo age. The calculation of the mass of water from the heat released during freezing used the value of the heat of fusion, L_f, applicable to the modal temperature at which freezing occurred (about −30°C). As mentioned under Results, L_f decreases by ~0.5 cal per degree below 0°C. The calculation of the mass of intraembryonic ice from the heat absorbed during warming used the value of L_f at 0°C but added 5.9% to the mass for the undetectable gradual absorption of heat between −20 and −9°C.

The total mass of water was determined gravimetrically to be 3.7 to 4.0 μg/embryo. It, too, was independent of age. The mean quantity of intraembryonic ice (for the three embryo ages) after cooling to −50°C was thus 85–93% of the total embryo water (based on melting data). The mean total water constitutes 74.3% (w/w) of the total mass of the fully hydrated embryo; i.e., the solids content of the embryo is 25.7%.

The total mass of water in fully hydrated intact *Anopheles* embryos is about half that in *Drosophila* (7.9 μg/embryo) (21), but the fractional water contents are similar, namely, 74.3 versus 76.2% (w/w). The percentage of that water that is freezable appears to differ in the two species, however, for the differential scanning calorimetry data of Myers *et al.* for the mass of freezable water (14) yield a value of 68% versus our 91%. The difference could be real or it could be a consequence of differences in procedure. Myers *et al.* determined the amount of ice in *Drosophila* embryos cooled to −35°C; We determined it in embryos cooled to −50°C. Additional ice could have formed in the latter case, although the amount would probably be small. Mazur *et al.* (12) observed no additional freezing below −35°C in *Drosophila* embryos using differential thermal analysis, and, as illustrated in Figs. 1–3, we detected none in *Anopheles.* That conclusion is supported by the equivalence of the mass of ice in *Anopheles* calculated from the exotherm above −35°C and the mass of ice calculated from the endotherm after warming from −50°C. Although Myers *et al.* computed the mass of intraembryonic ice from the endotherm during warming, they did not appear to compensate for the undetect-

able melting of about 6% of the ice that must occur above −21°C (Figs. 1 and 4).

Cryobiological Implications

To achieve cryobiological preservation, cells must survive cooling to below −100°C, and in the process of cooling they must not freeze intracellularly. As we have seen, ice forms in intact *Anopheles* embryos at −28 to −33°C. There are two approaches to avoiding such freezing. One is to cool the embryos slowly enough to allow freezable intracellular water to flow out of the cells osmotically. The other is to use a combination of high concentrations of glass-inducing agents and high cooling rates to cause the protoplasm to vitrify during cooling rather than to freeze (3, 8, 19). In *Anopheles*, both approaches require that the eggs be rendered permeable to water and cryoprotective solutes, for the intact egg is poorly permeable to both. Valencia *et al.* (26) have developed effective procedures for the permeabilization of young (15h@17°C) eggs, but the procedures are ineffective for older eggs.

If permeabilization is achievable, the decision between a slow cool/osmotic dehydration and a rapid cool/vitrification cryopreservation strategy depends on the chill sensitivity of the embryos independent of ice formation and on whether such chill sensitivity accelerates below 0°C as in *Drosophila* (12). Since the present study shows that intraembryonic ice does not occur in *Anopheles* over a 4 h period at −20°C, it will be possible to determine the kinetics of chill injury down to that temperature. Such studies are in progress.

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