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The Kinetics of Embryo Drying in *Drosophila melanogaster* as a Function of the Steps in Permeabilization: Experimental

P. D. SCHREUDERS,* J. N. KASSIS,†‡ K. W. COLE,† U. SCHNEIDER,†§ A. P. MAHOWALD,¶ P. MAZUR*†

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The *Drosophila* embryo is surrounded by an outer chorion and an inner vitelline membrane that are an effective barrier to the movement of water. The chorion can be removed by 2.5% hypochlorite (1). If surface water is then removed by isopropanol (2), the vitelline membrane can be rendered permeable to water by heptane containing 0.3% 1-butanol (3). Here, we determined quantitatively the effects of these three steps on the permeability of the embryo to water. Permeability was assessed by exposing embryos to air at 22–23°C and approx. 56% r.h. and determining weight loss with time. We also determined the effect of the three permeabilization steps on the water and solids contents of the embryos initially, after equilibration with air, and after complete drying. The intact embryo contains 76% (w/w) water and 24% solids. The successive permeabilization steps produce small increases in the former and small decreases in the latter. Intact embryos require 5–12h to lose half their water, the longer times being applicable to older embryos. After permeabilization steps (1), (1)+(2), and (1)+(2)+(3) the times for 50% water loss decrease to 1.6, 1.9, and 0.1 h, respectively. Copyright © 1996 Published by Elsevier Science Ltd

Drosophila melanogaster Water and solids content Permeabilization protocol Membrane permeability Embryo dehydration

INTRODUCTION

The *Drosophila* embryo is surrounded by a multilayered eggshell which, amongst its functions, acts as a barrier to the movement of both water and solutes. It is this barrier that is a major obstacle to experimental techniques requiring the insertion of compounds into the embryo. Examples of these techniques are cryopreservation (Mazur *et al.*, 1992b; Steponkus *et al.*, 1990), *in vivo*, staining (Strecker *et al.*, 1994), and the effects of drugs on embryo development (Inman, 1984). One of the most demanding of these techniques is the introduction of cryopreservatives to permit the vitrification of embryos for long term storage. Successful cryopreservation by vitrification requires introduction of quantities of ethylene glycol sufficient to produce concentrations > 50% (w/w) in the embryos (Mazur *et al.*, 1992b; Schreuders and Mazur, 1993; Steponkus *et al.*, 1990). Furthermore, the permeabilization barriers must be breached without unduly injuring the embryo.

The eggshell

The eggshell can be divided into two primary layers; the outer *chorion* and the inner *vitelline membrane*. The chorion is an open structure with an *endochorion* sandwiched between the *innermost chorionic layer* and the *exochorion*. The endochorion contains cavities with a floor and a roof separated by vertical pillars. These cavities are normally believed to be air-filled (Margaritis *et al.*, 1980). However, our gravimetric evidence indicates that they are water-filled under the conditions in this study, probably because these conditions include brief exposure to 70% ethanol as part of the egg collection procedure.

*Oak Ridge Graduate School of Biomedical Sciences, University of Tennessee, Knoxville, TN, U.S.A.
†Fundamental and Applied Cryobiology Group, Biology Division, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, TN 37831-8080, U.S.A.
‡Earlham College, Richmond, IN 47374-4095, U.S.A.
§Deutsches Institut für Reproduktionsmedizin GmbH, Hannoversche Str. 24, 31848, Bad Münder, Germany
¶Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, U.S.A.

The vitelline membrane is approx. 300 nm thick. Based on its freeze-cleaving properties, its outermost surface is covered by a hydrophobic waxy coating. This coating is less than 5 nm thick and consists of multiple layers of overlapping plaques 0.5–1.0 μm in diameter (Margaritis *et al.*, 1980; Papassideri *et al.*, 1991). This layer is believed to be the primary barrier to transport into and out-of the embryo, however its composition is largely unknown.

By-passing the chorion

The chorionic barrier is circumvented through its mechanical or chemical removal. Typically, mechanical removal involves prying an embryo from the chorion using forceps or a needle (Ashburner, 1989; Limbourg and Zalokar, 1973) or rolling embryos between layers of adhesive tape (Ashburner, 1989). The disadvantage of both methods is that they involve handling the embryos individually or in small groups. Thus, while successful, these techniques are not suitable for applications requiring large numbers of embryos.

Chemical dechorionation avoids this problem by treating large numbers of embryos simultaneously. Most chemical methods are performed using a variant of the protocol presented by Hill, 1945. He recommended the use of 3% aqueous sodium hypochlorite to dissolve the chorion. However, various concentrations, ranging from 2.6 (Fausto-Sterling *et al.*, 1974) to 5% (Arking and Parente, 1980), have been used. Both we (Cole *et al.*, 1993; Mazur *et al.*, 1992a) and Steponkus' group (Lynch *et al.*, 1989; Steponkus and Caldwell, 1993b) have found that exposure to 2.6% aqueous sodium hypochlorite (50% Clorox) works well. The removal of the chorion still leaves the embryos essentially impermeable to liquid water and, as will be shown in this study, it only modestly improves their permeability to water vapor. The removal does, however, make the inner vitelline membrane, the major barrier to water permeability, accessible to the solvents used in its permeabilization.

By-passing the vitelline membrane

A variety of physical methods have been used in attempts to bypass the barrier of the vitelline membrane. One recent method involved vortex mixing of the embryos with silicon carbide whiskers, but success was limited (Raloff, 1990). Another unique method was Kuroda *et al.*'s (1989) use of a laser microbeam to penetrate the micropile of the *Drosophila melanogaster* embryo. With 15% glycerol used as a cryoprotectant, the embryos were frozen and thawed with 1–2% survival to adulthood. However, the poor survival and the high labor requirements make this technique impractical for general use. Another potential method, the abrasion of the embryos using alumina dust, has been used successfully to permeabilize the embryos of the blowfly following their dechorionation, but has not been applied to *Drosophila* (Davies, 1949).

Chemical methods for permeabilizing the embryos have been more successful and permit the permeabilization of bulk samples. The chemical permeabilization of the vitelline membrane's hydrophobic waxy layer is believed to result from the extraction or emulsification of the compounds that form this barrier. A variety of organic solvents and solutions have been used for this extraction. The majority use a linear alkane, in some cases, with an added alcohol. The solvents include hexane (Strecker *et al.*, 1994), hexane/isopropanol (Steponkus and Caldwell, 1993a, b), heptane (Limbourg and Zalokar, 1973), heptane/butanol (Cole *et al.*, 1993; Mazur *et al.*, 1992a), and octane (Arking and Parente, 1980; Limbourg and Zalokar, 1973; Strecker *et al.*, 1994). Ether/ethanol (Widmer and Gehring, 1974) solutions and the anionic detergent Triton-X100 (Sayles *et al.*, 1973) have also been used.

Two successful protocols for the permeabilization of *Drosophila* embryos have been developed using alkane/alcohol as the permeabilizing solvent. One of these was developed by Steponkus' group (Lynch *et al.*, 1989; Steponkus and Caldwell, 1993b). The other was developed by Mazur's group (Cole *et al.*, 1993; Mazur *et al.*, 1992a). We have found that the addition of 0.3% butanol to the *n*-heptane in our protocol (Cole *et al.*, 1993; Mazur *et al.*, 1992a) significantly improves the degree of permeabilization of 12–15h embryos. Similarly, Steponkus *et al.* included isopropanol in their hexane. Their protocol involves the washing of the embryos in isopropanol followed by the removal of only excess alcohol before immersion in the hexane.

There are two potential reasons for the increases in effectiveness and repeatability that result from alcohol addition. The first reason may be the presence of hydrophilic groups in the compounds extracted from the vitelline membrane. These groups would normally inhibit a compound's solvation by pure alkane. This problem is avoided by the presence of alcohol. The supposition is that the alcohol's hydroxyl group moves adjacent to the hydrophilic groups of the extract and the alcohol's hydrocarbon chain moves adjacent to the alkane. Thus, the small amounts of alcohol added would act as an interface between the hydrophilic groups of the barrier and the hydrophobic alkane.

The second role of the alcohol may be related to the fact that the alkanes are immiscible with water. Thus, the addition of an alcohol (which is soluble in both water and the alkane) may allow the solution to dissolve any small amounts of surface water present. The alkane could, then, wet the embryo and dissolve the waxy layer.

Water permeability is usually assessed in terms of the flux of water in or out of cells under an osmotic gradient in anisosmotic aqueous solutions of nonpermeating solutes. The measure of flux is generally the rate of cell shrinkage or swelling. That approach was not feasible in *Drosophila* embryos. First, as will be seen, up to the point of exposure to alkane there is little or no observable water loss in hyperosmotic solutions, i.e. the embryos exhibit little or no visible osmotic shrinkage over 4–6h.

Second, although alkane-treated embryos do shrink osmotically in a matter of minutes, the shrinkage is so anisotropic that it cannot be readily translated into numerical values of embryo volumes. Consequently, we decided to put emphasis on assessing the effects of the permeabilization steps in terms of their effects on the rate of water loss in air as measured gravimetrically. A derivative benefit of these gravimetric measurements is that they yielded data on the masses of solids and water removed from the embryos by the several steps in the permeabilization procedure.

MATERIAL AND METHODS

Embryo preparation

Egg collection and staging Flies of the Oregon R-P2 strain of *Drosophila melanogaster* were maintained at 24-26°C by the method of Travaglini and Tartof (1972) with minor modifications (Cole *et al.*, 1993). To obtain eggs for experimental use, trays of 2% agar (Bacto-Agar, Difco, Detroit, MI) smeared with a paste of rehydrated active dry baking yeast (Fleischmanns Yeast Inc., Oakland, CA) were placed in a cage for 3h or 1h. The 3h period was used in earlier drying experiments involving different age intact embryos and in experiments in which the survival was determined. The 1h collection period was used in later experiments comparing the dehydration rates of the eggs at various steps in permeabilization.

To obtain 3-, 5- to 6-, and 9-h embryos, the collected eggs were maintained at 25 ± 1°C for 3, 5–6, and 9h from the midpoint of the collection period. To obtain 12- and 15-h embryos, the embryos were held at 26°C (24°C in later experiments), room temperature (22–23°C), and 18°C for times calculated to produce 12- and 15-h embryos (as measured from the midpoint of collection) at 8–10 a.m. the following morning. In making these calculations, we used growth-rate factors of 1.0, 0.9, 0.75, and 0.5 for maintenance at 26, 24, 22, and 18°C, respectively. These factors were obtained from Ashburner and Thompson (1978). The 12- or 15- h embryos were held at 4°C to provide material of a given developmental stage through the day. However, 3- to 9- h embryos were not refrigerated, since they are substantially more chill sensitive than older embryos and are killed in a few hours (Mazur *et al.*, 1992c).

Permeabilization The steps in the permeabilization of *Drosophila* embryos are (1) dechorionation by exposure to 50% Clorox, (2) a thorough water rinse, (3) exposure to pure isopropanol to remove residual water and to provide a solvent miscible with heptane, (4) brief air-drying to remove most of the carry-over isopropanol so as not to materially affect the amount of alcohol present in the next step, (5) 90 s exposure to *n*-heptane containing 0.3% (v/v) 1-butanol, (6) a brief exposure to pure heptane to quench the permeabilization, and finally (7) evaporation of the residual heptane. As will be seen, the primary permeabilization to water and cryoprotectant occurs in step 5 (the butanol accelerates the removal), although the removal of the chorion (step 1) somewhat enhances the permeability to water.

The exposure to the various fluids is achieved by sandwiching some 600–800 embryos between two 25 mm polycarbonate (PC) filters (10–12 μm pore size, Nuclepore, Costar, Cambridge, MA or Poretics Corp., Livermore, CA). The filter sandwich is placed in a Millipore Swinnex (Bedford, MA) holder above which is attached a 30 ml glass syringe and below which is a vacuum flask. The successive fluids are drawn through the system by applying vacuum. Additional details are given below by Mazur *et al.* (1992a), and Cole *et al.* (1993). The procedure permeabilizes some 80% of the 15-h embryos used in this portion of the study, as judged by staining with the dye rhodamine B (Sigma Chemical Co., St Louis, MO), and over 90% of the embryos are viable (hatch and develop into adults).

Dechorionation

The embryos were exposed to 30 ml of 50% Clorox solution (2.6% sodium hypochlorite) (Clorox Professional Products Co., Oakland, CA) for a total of 150 s. The Clorox was removed by replacing the delivery syringe and passing 150 ml of distilled water through the PC filter sandwich. Sodium hypochlorite solutions degrade as a function of exposure to light and of temperature. Thus, refrigeration of the Clorox and the use of only fresh solutions is necessary for consistent results (Chlorine Institute, 1992). If the drying kinetics were to be determined after dechorionation, the filter sandwich was removed from the Swinnex, placed in a small petri dish (Falcon #3001, Becton Dickinson and Co., Lincoln Park, NJ) containing 1–2 ml of water, and separated. In the other cases, the embryos were subjected to the next step: treatment with pure isopropanol.

Isopropanol treatment

Because the aqueous solutions used in the previous steps are not miscible with the heptane/butanol mixture used in the final stage of the permeabilization, the residual water in the PC filter sandwich and the surface water on the embryos was removed by exposing the embryos to 20 ml of isopropanol (Fisher Chemical, Fair Lawn, NJ) for 30 s. When the drying kinetics of the embryos were to be examined following this step, the isopropanol was followed by a rinse of approx. 50 ml of water and the filters containing the embryos were floated on water in a petri dish. Otherwise, air was allowed to pass through the filters for 120 s to evaporate the residual isopropanol prior to exposure to the heptane/butanol treatment (Cole *et al.*, 1993; Mazur *et al.*, 1992a).

Heptane/butanol treatment

The embryos were then exposed to 30 ml of *n*-heptane (Fisher Chemical, Fair Lawn, NJ) containing 0.3% 1-butanol (Fisher Chemical, Fair Lawn, NJ) for 90 s. Permeabilization was quenched by washing away the heptane/butanol solution with 15 ml of pure heptane. The

filter sandwich was then removed and placed into a Petri dish containing D-20 *Drosophila* cell culture solution (Cole *et al.*, 1993; Zalokar and Santamaria, 1977). The filter sandwich was separated carefully, and the top filter was placed into a second Petri dish, also containing D-20. The transfer of the PC discs from the Swinnex to the Petri dishes and the separation of the filters was done as quickly as possible to permit rapid evaporation of the heptane and to minimize dehydration of the now permeabilized embryos.

Gravimetric determination of water loss and water and solids content

Continuous recordings of embryo weight These weights were determined using a Cahn RG electrobalance (Ventron Instruments Corp., Paramount, CA) connected to a Speedmax H Azar chart recorder (Leeds and Northrup, Philadelphia, PA), which provided a continuous trace of the weight of the embryos plus the filter. In the Cahn RG, the electrobalance proper is mounted in a glass vacuum 'bottle' equipped with two removable glass hang-down tubes 24 cm long. A thin Nichrome wire runs from each arm of the balance to stirrups at the bottom of the hangdown tubes. The two stirrups support pans and Teflon cups that hold the egg sample and counterweights, respectively.

Sample loading and measurement of surface water

Samples of 6-, 12-, and 14–15-h intact eggs in water [or 0.05% Triton (Mallinckrodt, Paris, KY), in a few early experiments] were placed on 13 mm polycarbonate (Nuclepore) filters with 12 μm pore size. The mass of eggs after the removal of surface water was between 1.7 and 13.5 mg (mostly 5–10 mg), which corresponds to 150–1200 eggs. To remove most of the surface water, the 13 mm filter was placed on a 25 mm, 12 μm pore size, Nuclepore filter which rested on the glass frit of a standard Millipore 25 mm filter funnel inserted into a vacuum flask. The removal of the surface water was initiated by applying laboratory vacuum for 30 s. Zero-time was defined as the time the vacuum was first applied. The 13 mm filter, with its adherent eggs, was then transferred to a Teflon sample cup positioned near the bottom of the hang-down tube of the Cahn RG, and recording of the weight initiated.

As illustrated in Fig. 1, the weight tracings showed an initial 2–3 min rapid drop followed by an abrupt transition to a much lower rate of water loss. We interpret the former as the removal of residual surface water and the latter as the removal of water from the embryo proper. The zero-time weight of the embryos, and, from that, the zero-time water content, was obtained by extrapolating back to zero-time as shown by the dotted line in Fig. 1. Because the inclusion of the surface water alters the scaling in plots and obscures features in the embryonic drying curve, this portion of the experimental data has been eliminated from the figures that follow.

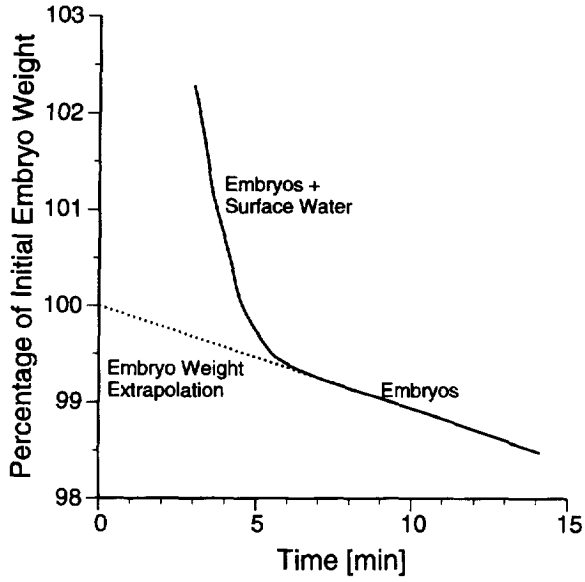

FIGURE 1. Kinetics of water loss during the earliest stages of the air drying of intact embryos. Two regions are evident: (1) An initial rapid loss in water reflecting the evaporation of water on the embryo surface followed (2) by a much slower loss of intraembryonic water. The mass of intraembryonic water at 0-time (fully hydrated) was obtained by linear extrapolation from region (2) back to 0-time (···).

Room temperature measurements

For these measurements, the glass housing of the Cahn RG was left open to air at ambient temperature (22–23°C) and ambient humidity (approx. 50–60%). Air-drying was continued until the next morning (approx. 18h). The input port of the RGs glass housing was then sealed and high vacuum applied to the RGs exit port for at least 6h, or until the curve clearly had reached a new plateau. Because the vacuum slightly affects the buoyancy of the system and hence slightly affects the apparent weight, room-air was re-admitted into the RG to obtain the value of the final dry weight. All dry eggs were then brushed off the Nuclepore filter and its weight was then determined on the RG. The weight of the eggs at time t is the recorded weight of the egg sample minus the filter weight. The weight of the eggs minus the dry weight is the weight of water in the eggs at time t.

Low temperature measurements

Measurements were also made of the rate of water loss at 0°C and below. In these experiments, the samples of eggs on the Nuclepore filter were first subjected to drying in contact with room air for about 15 min, as above. During this time, a small thermoelectric refrigerated bath (Minifreezer Model PS5, the Virtis Company, Gardiner, NY) containing room-temperature ethanol was positioned around the sample hang-down tube. Dry air or dry nitrogen was then allowed to flow through the RG for an additional approx. 15 min. With the dry air or nitrogen still flowing, the thermoelectric bath was then turned on at the highest setting, and allowed to cool to pre-selected temperatures between −6 and −20°C. The setting was then reduced to previously determined values

that would maintain the desired temperature. Since, the bath and sample are separated by the RG hang-down tube, the relation between bath temperature and sample temperature was determined in calibration measurements by positioning the junction of a 36 gauge thermocouple in the Teflon sample cup. The sample temperature was 6°C above the bath temperature when the bath was at −6 to −8°C and the differential was 10°C with a bath temperature of −18 to −19°C.

Drying, in the presence of flowing dry air or dry nitrogen, was continued at the reduced temperature for some 15–86h, depending on the temperature. Some 20% of the egg water had been lost at this point. The sample was then allowed to rewarm to room temperature. In most cases, drying was then continued, as in the room temperature experiments described above, to obtain dry weights. In a few cases it was aborted to determine embryo survival after the partial low temperature dehydration.

A single hydrated *Drosophila* egg weighs approx. 10 μg. Weighings on the RG after calibration are accurate to ±5 μg.

Determination of the drying kinetics at room temperature Most of the drying kinetics were obtained by periodic weighings of 11.5- to 14- h embryo samples on a Cahn G2 electrobalance (Ventron Instruments Corp., Paramount, CA). This balance is analogous to a standard 2-pan analytical balance but has an accuracy of ±5 μg on the 10 mg range used.

Untreated dechorionated, and isopropanol-treated embryos

The following procedure was used for intact, dechorionated, and isopropanol-treated embryos (heptane/butanol treated embryos were treated somewhat differently). The individual weights of a number of 13 mm Nuclepore polycarbonate filters (10 or 12 μm pore size) were determined. Some 50–150 intact or treated embryos of the desired age were transferred by sable brush from moistened filter paper to successive tared polycarbonate filters. In some cases, drying was initiated as soon as a filter was loaded. In other cases, the embryo-laden filters were floated on the surface of water until the initiation of drying.

The polycarbonate filters and adherent embryos were first subjected to the procedure described above for the Cahn RG experiments to remove the bulk of surface water; namely, 30 s air-drying on a Millipore glass frit. As above, the initiation of the 30 s is defined as zero-time.

The filter and embryo sample were then placed in the Cahn G2 and the initial weight obtained at 0 + 2 or 3 min. A second weighing was performed approx. 1 min later. The initial (zero-time) weight of the untreated, dechorionated, and isopropanol-treated embryos was determined by linear extrapolation backwards from these two points. The water content of the heptane/butanol treated embryos could not be determined using this method due to the rapid rate of intraembryonic water loss (see below).

The polycarbonate filters, with their adherent embryos, were then placed in empty 35 mm Falcon tissue culture dishes with the lids tilted so as to expose the embryos to room air at 22 to 23°C and approx. 56% relative humidity. Periodically, the filters and embryos were reweighed on the G2. In those experiments where survival was determined as a function of embryo water content, the dehydration of a filter sample was terminated after a given time and the percentage hatching determined. In those experiments concerned only with the physical aspects of dehydration, a given filter and its adherent embryos were weighed successively for some 5–6h. Embryo weights as a function of time were obtained by subtracting the tared weights of the filters from the measured weights.

Computations by the model to be discussed in the companion paper (Schreuders *et al.*, 1996) require measurements of the weight of the embryos after attaining a steady-state with air at ambient relative humidity. Calculations of water content vs time require values for the dry weight of the embryos. To determine the steady-state weight, the PC filters, plus embryos, were kept in room air in Falcon dishes with tilted lids for 48h or more and then weighed on the G2. To determine solids content, the filters plus embryos were held in a vacuum desiccator at room temperature and less than 25 mm Hg absolute pressure until there was no detectable further weight loss in successive weighings. To ensure that none fell off, the number of embryos on a given filter was determined both before initiating drying and after its completion. These measurements also permitted weights and water concentrations to be expressed on a per embryo basis.

Heptane/butanol treated embryos

The procedure for permeabilized eggs was somewhat different because the time required to evaporate extra-embryonic water is a significant fraction of the time for intra-embryonic dehydration. Furthermore, following the heptane/butanol treatment, the embryos have to be maintained in an isotonic salt solution to avoid dehydration or volume changes before the initiation of experimental dehydration.

Fifty embryos were loaded onto each of several pre-weighed 13 mm PC filter discs and floated on D-20 in small petri dishes. A PC filter was taken out of its petri dish, the side away from the embryos quickly blotted with 2–3 strokes on a piece of bibulous paper, and the filter then immediately transferred onto the measuring pan in the Cahn G2 electrobalance and weighed. Time zero was taken as the point where the disc was taken off the bibulous paper. It took several minutes for the first weighing because the weight was changing rapidly. The PC disc was, then, weighed once a minute and the times and weights recorded. After 40 min, by which time the weight had plateaued, the disc was placed into an empty

petri dish, the dish capped, and placed into a vacuum desiccator. The discs were taken out periodically and weighed. The dry weight of the embryos was that at which no more weight loss was observed.

The statistical analysis of these data was performed using Excel (version 4.0, Microsoft Corp., Redmond, WA).

The hatching survival of partially dehydrated intact embryos

After a drying time of 1–9h, the PC filter was placed on the surface of a 25 mm, 2% agar disk in a petri dish with the adherent embryos uppermost. The embryos were covered with 0.05 ml of water; the dish capped and placed in a 26°C incubator for 24–30h. The percentage hatching was then determined. The control consisted of embryos subjected to only 30 s drying to remove surface water. The hatching percentages after variable dehydration are normalized to the hatching of an untreated control.

Microscope observations on water loss in Drosophila embryos in air and in hyperosmotic sucrose or ethylene glycol

Approximately 100–120 untreated or dechorionated embryos were placed on Nuclepore filters. In the case of air-drying, the bottom of the filter was blotted on absorbent paper, and the filter then transferred to a dry 35 mm Falcon petri dish. Excess water on the top surface of the filter was wicked away and timing initiated when, a minute or so later, the last extra-embryonic water disappeared. The dish was placed on the desk top with the lid tilted, and the morphological appearance of the embryos observed at approx. 15–30 min intervals under 15–30× magnification. The relative humidity, as determined by a sling psychrometer, was 48–51%. In the case of exposure to hyperosmotic solutions, the PC filter bearing the embryos was transferred from D-20 to two successive dishes of 0.75 M sucrose (Bethesda Research Laboratories, Gaithersburg, MD) in D-20 or 2 M ethylene glycol (Sigma Chemical Co., St Louis, MO) in D-20, wiping the bottom of the filter on the edge of the dish between transfers. Solution was then gently pipetted on top of the embryos. The morphological appearance of the intact or dechorionated embryos was followed, at intervals, for 6h.

RESULTS

Dehydration kinetics of intact 3- to 15-h embryos in air

Dehydration at room temperature Full drying curves of intact 6- to 14-h embryos were obtained on the recording Cahn RG electrobalance. The mass of eggs varied from 1.7 to 12.8 mg. Figure 2(A) shows the weight of the embryos (normalized to the zero-time weight) as a function of time in contact with air at 23°C and ambient r.h. To obtain dry weights, drying was continued in the presence of dry air or in vacuo for up to 118h. The mean dry weight was 23.6 ± 1.1% of the original fully hydrated

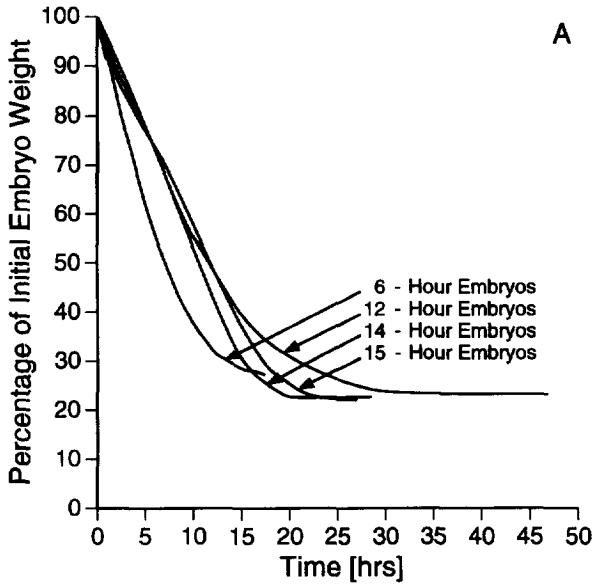

FIGURE 2. (A) Kinetics of water loss of intact 6, 12, 14, and 15- h *Drosophila* embryos during air drying at 23°C and approx. 55% r.h. (A) Percentage of 0-time embryo weight vs time.

weight. In other words, the water content of full hydrated embryos was 76.4% (w/w). These latter data were used to construct Fig. 2(B) which shows the kinetics of water loss of the embryos in the four runs.

Two of the curves exhibit long gentle undulations. The most likely causes are slight and slow changes in the r.h. of the ambient air or in its temperature. As will be discussed shortly, the drying rate is temperature sensitive.

Figure 2 shows that the rate of water loss from 6-h embryos was higher than that of 12- to 15-h embryos. That was confirmed in subsequent drying runs over a 4–9h period on a Cahn G2 balance (Fig. 3). The two lower curves in Figure 3 are the water loss kinetics for 3- and 6-h embryos. The three upper curves are the kinetics of

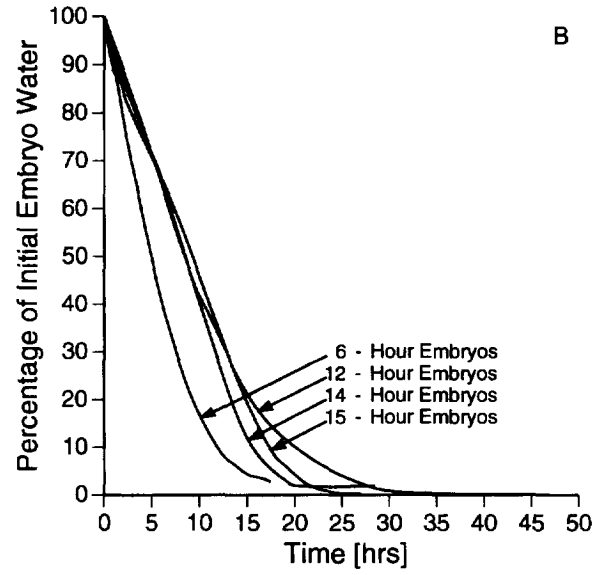

FIGURE 2. (B) Percentage of 0-time embryo water content vs time. Weights were followed continuously on a Cahn RG balance.

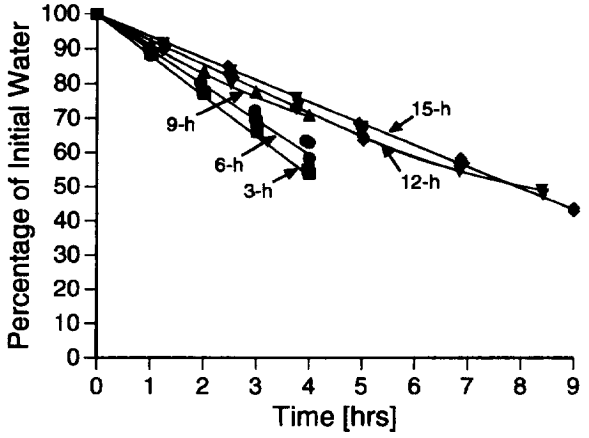

FIGURE 3. Kinetics of water loss (expressed as a percentage of the initial water content) of 3-, 6-, 9-, 12-, and 15-h intact *Drosophila* embryos during air drying at 23°C and ambient r.h. (approx. 55%). Embryo weights were measured at approximately hourly intervals on a Cahn G2 balance. The ranges for the SE were 0.05–0.65%, 0–1.25%, 0–0.50%, 0.05–0.55%, and 0.50–1.05% for the 3-, 6-, 9-, 12-, and 15-h embryos, respectively.

9-, 12-, and 15-h embryos. In one experiment the embryos at all five developmental stages came from the same batch of eggs. The 3- and 6-h embryos lose about half their water in about 4.7h; the 9-, 12-, and 15-h embryos lose half their water in about 8h. These figures are in close agreement with the values of 5.5 and 9h in Fig. 2(B).

Effect of temperature on the kinetics of dehydration of 6-h embryos The procedure for determining the effect of lowered temperature on the kinetics of water loss was as follows. Samples of embryos in the RG electrobalance were first exposed to flowing ambient air for approx. 15 min. The air was then replaced with dry air or dry nitrogen (dew point approx. 50°C) for an additional 15 min to ensure that there was no condensation on the embryo surface during subsequent cooling. The samples were slowly cooled (approx. 0.2°C/min) in a thermoelectric cooler until the bath temperature reached −6 to −8 and −15°C which corresponded to sample temperatures of −1, and −5°C (see Methods). Drying was continued at these temperatures for 15–86h. Table 1 summarizes the essential results of experiments on intact 6-h embryos. In ambient air at room temperature, the rate of water loss was 12.3%/h. The r.h. in our buildings is kept between 50 and 60%. Surprisingly, when ambient air was replaced by dry air or dry nitrogen, the rate of water loss fell to 9.3%/h. The total water loss during the approx. 30 min at 23°C was 4.9%.

Cooling was then initiated, and over that 3h period the embryos lost an additional 5.3% of their original water. Finally, the embryos were held at −1 and −5°C, in contact with flowing dry air or dry nitrogen, for 15 and 67h respectively. Over that period, the embryos lost an additional 7.1 and 8.5% of their water and the rate of water loss was 0.47 and 0.13%/h, respectively. At the initiation of low-temperature drying, the embryos had thus lost only about 10% of their water, and at the conclusion of the drying they had lost about 18%. Figs 2(B) and 3 show the rate of removal of that fraction of water was essentially constant so that the rate of water losses at room temperature and at approx. 0°C and below are directly comparable. The rates at −1 and −5°C were thus 20-fold and 72-fold lower than at 23°C. The embryos at −1 and −5°C were almost certainly not frozen. Mazur *et al.* (1992c) and Myers *et al.* (1989) have demonstrated, either by differential thermal analysis or by differential scanning calorimetry, that intraembryonic freezing of intact eggs does not occur until they are cooled to below −24°C. Furthermore, we have found that embryos held at −10°C for up to 4h in a differential scanning calorimeter show no indication of ice crystal formation (Schreuders and Heimansohn, unpubl. data).

TABLE 1. Drying rates of intact 6-h *Drosophila* eggs as a function of temperature

Step	Temperature (°C)	Step duration (h)	Water lost (%)	Drying rate (%h)
Ambient air	23	0.22	2.7	12.30
Dry air or dry nitrogen	23	0.23	2.2	9.30
Cooling	–	3.25	5.3	–
Low temperature	−1	15.5	7.1	0.47
Low temperature	−5	67.0	8.5	0.13

The natural logarithms of the drying rates from Table 1 are plotted in Fig. 4 as a function of the reciprocal of the absolute temperature. The activation energy calculated from the slope of the best fit line was 22.7 kcal/mol.

Survival of 3- to 15-h embryos as a function of water loss The water contents at each time point in the room temperature drying runs of Fig. 3 were those of separate embryo samples on polycarbonate filters. As each time point was reached, that filter was transferred to an incu-

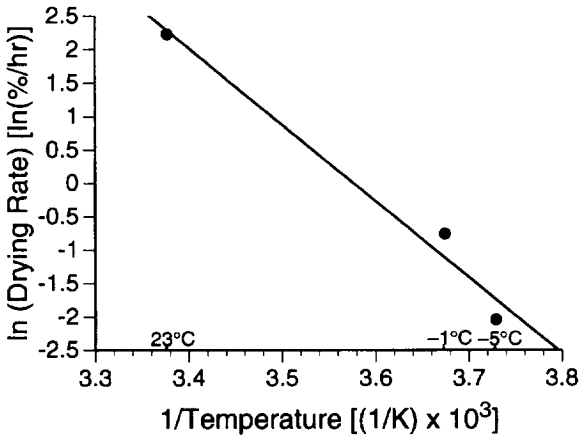

FIGURE 4. Arrhenius plot of the drying rates from Table 1. The natural logarithms of the drying rates are plotted vs reciprocal absolute temperature. The slope of the best fit line yields an activation energy of 22.7 kcal/mol.

bator to determine the hatching. Figure 5 depicts the percent survival (% hatching) as a function of the embryo water content. The results fall into two groups. The 3-, 6-, and 9-h embryos tolerated less water loss than did the 12- and 15-h embryos. In the former group, half survived the removal of 20–25% of their water, whereas in the latter group, half survived the removal of about 50% of their water.

The drying runs at −1°C in Table 1 removed only about 20% of the embryo water; nevertheless, none of these 6-h embryos survived. This was a consequence of the extreme chill sensitivity of *Drosophila* embryos of this age (Mazur *et al.*, 1992c). Because the young embryos are especially sensitive to low temperature, to dehydration, and to damage from the alkane permeabilization procedure (Mazur *et al.*, 1992a), all our subsequent studies on dehydration kinetics made use of 15-h embryos.

The effects of successive steps in the permeabilization procedure on embryo properties

The weight of the embryos was determined for each treatment (untreated, dechorionated, isopropanol treated, and permeabilized) at 100% hydration, at various times during drying, after reaching steady-state with room air (average relative humidity = approx. 56%), and following vacuum dehydration. The results for the first, third, and fourth stages are shown in Table 2. Comparisons between groups of embryo weights were performed using one of two tests, depending upon the treatment. A one-tailed *t*-test for two samples with equal variances was used to decide if the reduction in the mean embryo weights at sequential steps along the permeabilization process was significant. A paired, two-tailed Students *t*-test was used when comparing the means of groups at the same step in the permeabilization process but having differing levels of hydration. In both tests, the significance was defined as $P<0.05$.

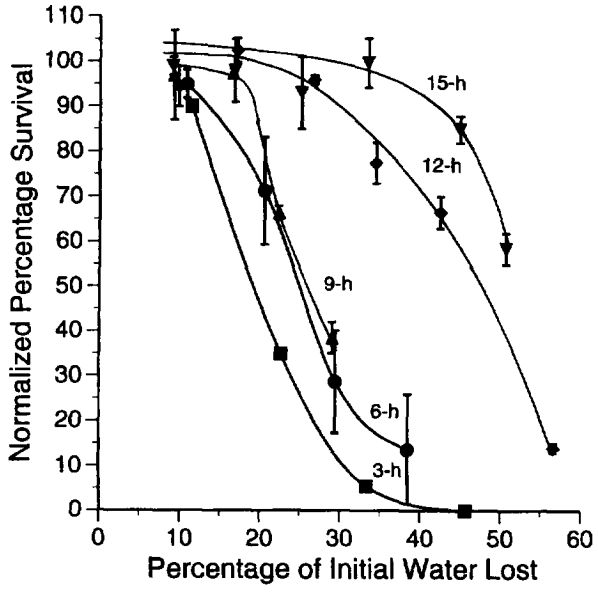

FIGURE 5. Percentage survival (hatching) of 3-, 6-, 9-, 12-, and 15-h intact embryos as a function of the extent of dehydration during air drying at 23°C and approx. 55% relative humidity. Survivals have been normalized to those of undried controls. Note that the embryo sensitivity to water loss is a strong function of age, with 12- and 15-h embryos substantially less sensitive than those at 3-, 6-, and 9-h.

Water and solids content of the embryos

100% hydration weights after various permeabilization steps

As described in Methods, the 100% hydration weights were determined by linear extrapolation back to time zero from two weighings after the surface water had evaporated. As shown in column 2 of Table 2, a fully hydrated 15-h embryo weighs 10.4 μg. (In a more limited series of measurements in connection with the data in Fig. 3 we found no differences in the hydrated weights of embryos ranging from 6 to 15h in age). Column 2 also shows that the fully hydrated weight decreases as expected, going from untreated to dechorionated, to isopropanol treated, and to permeabilized. The isopropanol and heptane/butanol treatments reduced the hydrated weights by 0.09 and 0.04 μg/embryo, respectively. However, these weight losses were too small to be significantly greater than zero statistically ($P = 0.184$ and 0.279, respectively). (Note from the footnote of Table 2, that the 100% hydration weight in permeabilized embryos was defined as the sum of the average water weight in the isopropanol treated embryos and the dry weight of the permeabilized embryos.)

Steady-state weights after various permeabilization steps

After drying to steady-state (column 3), the embryo weight with all four treatments fell to 22–26% of the fully hydrated weight. The steady-state weights also decreased with each additional treatment step. While dechorionation did not produce a significant decrease in the steady-state embryo weight ($\Delta w = 0.09$ μg/embryo; $P = 0.239$), the weight losses following the isopropanol treatment ($\Delta w = 0.34$ μg/embryo) and heptane/butanol treatment ($\Delta w = 0.08$ μg/embryo) were significantly different from zero ($P = 0.001$ and 0.017, respectively).

0% hydration weights after various permeabilization steps

The dry weights of the embryos were determined following vacuum desiccation (column 4). For embryos at all steps of the permeabilization process, the dry weights were significantly lower than those of the same embryos at steady-state with room air ($P<0.0001$). Furthermore, the 0% hydration weights decreased with each additional step in the permeabilization process. As with the steady-state weights, the primary loss in solids content occurred during the isopropanol treatment ($\Delta w = 0.33$ μg/embryo). This loss in dry weight was significant ($P = 0.002$), but the losses in dry weight from dechorionation ($\Delta w = 0.05$ μg/embryo) and from the heptane/butanol treatment ($\Delta w = 0.05$ μg/embryo) were

TABLE 2. Effect of the several steps in the permeabilization process on the gravimetrically determined water and solids contents of 15-h *Drosophila* embryos*

Treatment	Weight at 100% hydration (μg/embryo)	Weight at steady state† (μg/embryo)	Weight at 0% hydration‡ (μg/embryo)	Weight of water§ (μg/embryo)	Percentage water¶ (g H_2O/100 g embryo)	Percentage solids¶ (g solids/100 g embryo)
None	10.42±0.13	2.67±0.08	2.49±0.08	7.94±0.08	76.22±0.54	23.78±0.54
Dechorionation	9.92±0.07	2.58±0.08	2.44±0.08	7.49±0.06	75.54±0.70	24.46±0.70
Isopropanol	9.83±0.06	2.24±0.02	2.11±0.03	7.72±0.06	78.52±0.26	21.48±0.26
Heptane/butanol	9.79±0.04**	2.16±0.04	2.06±0.04	7.72	78.92±0.33**	21.08±0.33**

For each permeabilization step, embryo wieghts were measured at full hydration, after attaining steady-state wtih room air, and after vacuum drying. Weight fractions of water and solids were computed from these weights. Note that this set includes data in addition to that obtained as part of the dynamic drying experiments.

* Values are averages (±SE) for 25, 26, 18, and 12 filters of embryos following no treatment, dechorionation, isopropanol treatment, and heptane/butanol treament, respectively.

† After reaching steady-state in room air (approx. 56%, 22°C).

‡ After vacuum drying.

§ Column 2 – column 4.

¶ Percentages of water and solids are: weight water or solids/weight fully hydrated embryos ×100.

** Because of the rapid rate of dehyration of the heptane/butanol treated embryos a weight of water at 100% hydration could not be determined. Instead, the weight of water in these embryos was assumed to be 7.72 μg, the same as in the isopropanol treated embryos.

not statistically significant (P = 0.322 and 0.150, respectively).

Fractional water contents of the embryos

It was assumed that the embryo weight losses observed after drying resulted only from the loss of water from the embryo. Based upon this assumption, the weight fraction of water in a fully hydrated, untreated embryo was 0.762 (column 6). While there was no significant difference (P = 0.224) between this weight fraction of water and that found in the dechorionated embryos (0.755), dechorionation removed a significant amount of material (0.50 μg/embryo). Ninety percent of this removed material was water (0.45 μg/embryo) (column 5) and 10% was solids (0.05 μg/embryo) (column 4). It represents the chorion, water within the chorion, and, perhaps, water between the chorion and the vitelline membrane.

The weight fraction of water in the embryo was significantly altered by the isopropanol treatment (P = 0.001). Interestingly, it *increased* from 0.755 to 0.785. This change in fractional water content was the result of a 0.33 μg/embryo decrease in the solids content combined with a simultaneous increase of 0.23 μg of water/embryo. While the solids loss and water gains were significant, the sum of the two resulted in a total weight change (0.09 μg/embryo) that was not significantly different from zero (column 2).

The fraction of water in the heptane/butanol treated embryos could not be determined because of an inability to accurately estimate the mass of the embryos at zero time.

Embryo composition

Based on these results, the contents of intact *Drosophila* embryos can be broken down into the following relevant weight fractions: 76.2% of the fully hydrated, intact embryo was water, while 23.8% was solids.

The embryo water can be considered to be separated into two primary compartments, the water associated with the chorion and the water within the volume bounded by the vitelline membrane. Assuming that the intra-embryonic water is the amount of water in the dechorionated embryos, we see from column 5 of Table 2 that 0.45/7.94 or 5.7% of the water was associated with the chorion with the remaining 94.3% of the water within the embryos interior.

However, removal of the chorion reduced the embryos solids by only 2.0% (Table 2, column 4). This small decrease in solid combined with a comparatively large loss in water is consistent with the open structure of the chorion described in the Introduction. The next step in the permeabilization protocol, the isopropanol treatment, extracted 13.3% of the remaining solids. The nature of the removed material is unknown, since the visible portion of the chorion has been already removed by hypochlorite and the vitelline membrane remains intact (as indicated by embryos' impermeability). The permeabilization of the vitelline membrane with heptane/butanol resulted in the removal of only 2.0%, and possibly less, of the embryos solids. The material removed could well have included the waxy 50 Å coating, since it constitutes considerably less than 2% of embryo solids (see Discussion). Following all of these treatments, 82.9% of embryo solids remains. It is notable that dechorionation

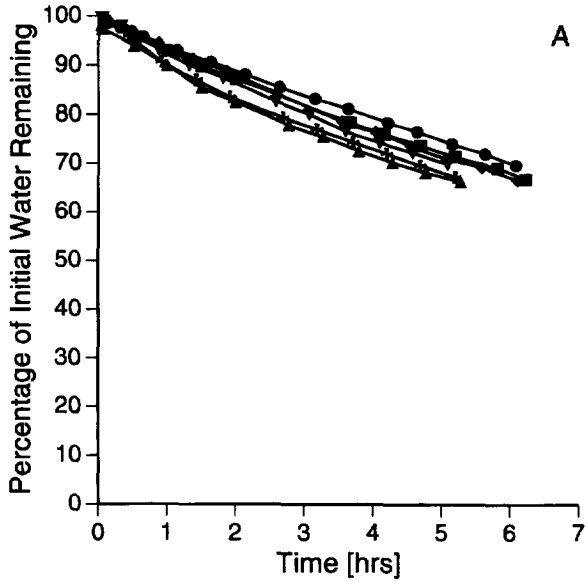

FIGURE 6. Kinetics of water loss during individual air drying runs at 23°C and approx. 55% r.h. of (A) intact 15-h *Drosophila* embryos and of embryos subjected to (B) dechorionation, (C) dechorionation and exposure to isopropanol, and (D) dechorionation and exposure to isopropanol and heptane/butanol.

and permeabilization, the treatments that resulted in the greatest alteration of the permeability, each removed only 2.0% of the embryo solids and perhaps less. The treatment that removed the most embryo solids (the isopropanol treatment, removing 13.3%) actually produced a slight decrease in the permeability, as will be seen in the next section.

Kinetics of water loss from embryos following various treatments Figure 6(A–D), respectively, show the kinetics of water loss in sets of runs on intact 15- h embryos and on embryos subjected to dechorionation, dechorionation plus exposure to isopropanol, and dechorionation plus isopropanol plus exposure to

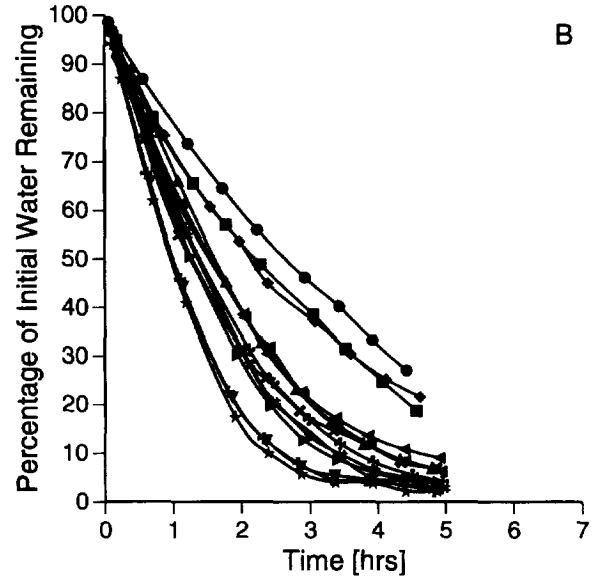

FIGURE 6. (B).

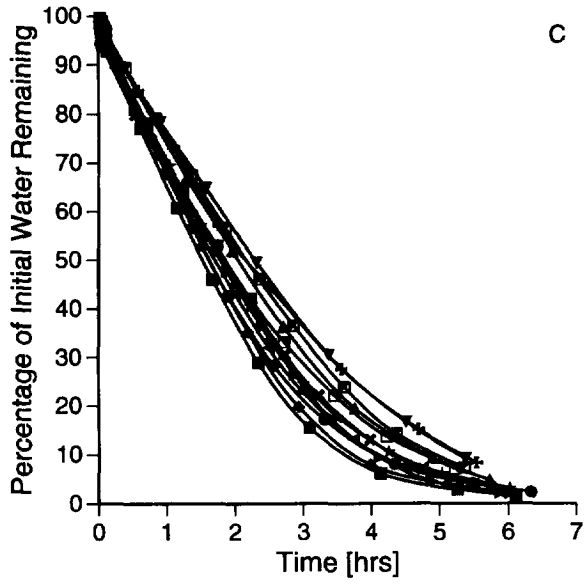

FIGURE 6. (C).

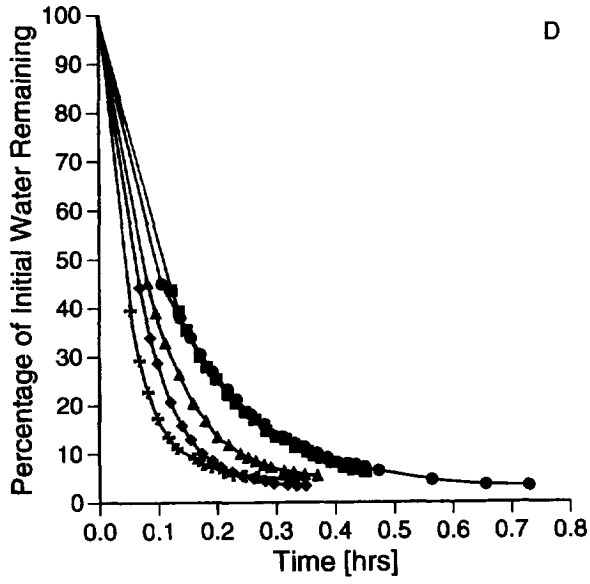

FIGURE 6. (D).

butanol/heptane. Figure 7 compares the median curves for the successive treatments. Dechorionation increased the initial rate of water loss some 3.5-fold. Exposure to 0.3% butanol in heptane increased it an additional 14.4-fold. Interestingly, the intermediate step of exposing dechorionated embryos to pure isopropanol actually resulted in a slight decrease in the rate of water loss. Another way to compare the kinetics is to compare the times required for the loss of 50% of the embryo water. These times were 9.5h for intact embryos (from Fig. 3), and were 1.6, 1.9, and 0.1h in the curves for the successive permeabilization treatments shown in Fig. 7.

The evaporation of water from the embryos can produce a drop in their temperature, and we have already noted that the drying rate of intact embryos has a sizable temperature coefficient. To test whether evaporative cooling is significant, we inserted a 40-gauge thermocouple

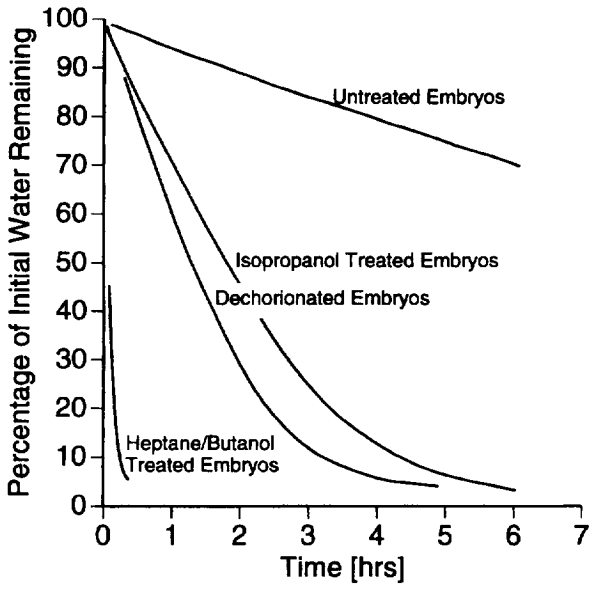

FIGURE 7. Comparative effects of the steps in permeabilization on the kinetics of water loss during the air drying of 15- h *Drosophila* embryos at 23°C. The curves are the median curves from Fig. 6(A and D).

into a mass of dechorionated eggs on a polycarbonate filter and measured the temperature during the initial stages of drying. The evaporation of surface water lowered the temperature 3–4°C for the first 3–5 min of drying, but the temperature then returned to and remained at the predrying value during the loss of *intra*embryonic water. Since the drying rates of intact and isopropanol-treated embryos are less than that of dechorionated embryos, we conclude that significant evaporative cooling does also not occur during their dehydration. Some cooling could occur in fully permeabilized embryos, since their dehydration occurs much more rapidly, but it probably was no greater than the 3–4°C cooling produced by the evaporation of the surface water.

Run-to-run variability Figure 6(A–D) show a degree of run-to-run variability in the kinetics of dehydration of intact embryos and of those subjected to a given treatment. One source of variability could have been small run-to-run differences in the ambient temperature and relative humidity or in convection patterns in the dishes during drying. We noted in Table 1 that the drying rate is temperature sensitive. However, the small run-to-run variation in the dehydration kinetics of the intact embryos [Fig. 6(A)] and of embryos subjected to dechorionation plus isopropanol treatment [Fig. 6(C)] suggests that these factors were not major contributors to variation. A second potential source of variation was run-to-run differences in the efficacy with which a given treatment removed the material that contributed to the permeability barrier. A third source could have been variation in the water content at what we defined as fully hydrated embryos, i.e. embryos at zero time. To some extent these various sources can be distinguished by whether the variation was in the zero-time weights, in the rates of dehydration, or in the final vacuum dried weight. In this analysis we consider only runs in which all of these data were collected. In intact embryos, isopropanol treated embryos and heptane/butanol treated embryos, the variation in dry masses was small (SD of 0.09, 0.04, and 0.08 μg/embryo). So too was the variation in the zero-time masses of intact and isopropanol-treated embryos. The respective SD were 0.16 and 0.24 μg/embryo.

As has been mentioned, it was not possible to directly determine the initial water contents of heptane/butanol treated embryos because of the rapidity of water loss and because of overlap between the removal of surface water and intraembryonic water. The variation among the curves in Fig. 6(D), then, could have been, in part, an artifactual consequence of substantial differences between the true zero time weight and the assumed zero time weight, or it could have been due to run-to-run differences in the efficacy with which heptane/butanol removed the permeability barrier. Or it could have been both.

The greatest run-to-run variation occurred in the dechorionated embryos [Fig. 6(B)]. This was true with respect to the fully hydrated weights (SD = 0.86 μg/embryo), the rates of dehydration, and the steady-state weights (SD = 0.59 μg/embryo). The major contributor to these higher SD were the runs depicted by the upper three curves. Excluding those three runs has no effect on the SD of the fully hydrated weight (SD = 0.87 g/embryo), but it decreases the SD of the dry weight (which is sensitive to the presence of chorion fragments) to 0.33 μg/embryo. It also substantially reduces variation in the slopes of the kinetic curves. We believe that the upper three curves are somewhat anomalous. The Clorox treatment, with rare exceptions, always removed the chorionic structures resolvable at dissecting microscope magnifications. Consequently, the run-to-run scatter combined with the fact that the three runs in question were performed on the same date suggests that the Clorox treatment used, or the age and specific lot of Clorox, may have variably affected other, unresolved, structures that contributed to the permeability barrier.

Relation between morphological manifestations of dehydration in air and gravimetric water loss

To provide a link between gravimetric measures of embryo dehydration in air and morphological measures of dehydration in hyperosmotic aqueous solution (next section), we made observations on the morphologies of embryos during the course of air drying. The first general morphological manifestation of shrinkage in air is a unilateral indentation. With further dehydration, one or both sides of the embryo collapse until, finally, the two lateral sides come into close apposition. In certain orientations this appears as a distorted flattened embryo. When intact 15- h embryos were air-dried, some 50% showed the lateral indentation in 3h. The gravimetric runs in Fig. 6(A) show that in 3h, intact embryos have lost 15–23% of their water. In the median curve in Fig. 7, 18% of the

water was lost in 3h. As drying was continued, about 50% of the embryos underwent major collapse or flattening in approx. 5h. Figure 6(A) shows that 25–33% of intraembryonic water was lost in that time. The median water loss was 29% (Fig. 7).

Morphological manifestations of dehydration occur considerably sooner in air-dried dechorionated embryos. Some 50% show indentations in 25 min. The runs in Fig. 6(B) (excluding the upper three curves) show that, in that time interval, the dechorionated embryos lose 13–21% of their water, and the water loss in the median curve is 15%. Thus, in both intact and dechorionated embryos, indentation occurred when the embryos had lost approx. 15–20% of their water. However, that morphological endpoint was reached in about 1/6th the time in the latter (0.5 vs 3h).

With further drying about 50% of the dechorionated embryos underwent collapse or flattening in approx. 1h, or about 5 times as rapidly as intact embryos. From Fig. 6(B), we see that in 1h dechorionated embryos lose 30–48% of their water (again excluding the upper three runs), and the water loss in the median curve (Fig. 7) in 1h is 37%.

Comparison between morphological manifestations of osmotic dehydration in hyperosmotic solutions and dehydration in air

The morphological manifestations of dehydration in embryos exposed to hyperosmotic solutions are somewhat different from those in air. In solutions, the first manifestation of shrinkage is the development of transverse creases. With further dehydration (which we have only observed in heptane permeabilized embryos), the embryos flatten in the microscope Z-axis to take on the elliptical shape illustrated in Fig. 13 of Mazur *et al.* (1992a). In a 5-h period, only 2–3% of intact and dechorionated embryos developed creases (9% in one case) in either 0.75 M sucrose/D-20 or 2 M ethylene glycol/D-20, and only 1–2% progressed beyond that stage.

The small amount of dehydration occurring during these 5h was insufficient to interfere with embryonic development as, after 6h (i.e. 21h since oviposition), 75–88% had hatched. We know from previous work (Mazur *et al.*, 1992a, b) that by approx. 17 h after oviposition the embryos become refractory to permeabilization by heptane/butanol, presumably as a result of the embryo (pharate larva) developing another permeability barrier–chitinized cuticle. To determine whether the appearance of that barrier during dehydration was slowing osmotic dehydration, we performed analogous experiments on embryos allowed to develop for 15 h at 17.5°C. Including time at 26°C and room temperature, such embryos were at the 8–9-h developmental stage at the start of exposure to hyperosmotic sucrose and ethylene glycol. Furthermore, at the end of 6h of exposure to the hyperosmotic solutions, they were 14–15-h developmentally; i.e. too young to have developed an impermeable cuticle. Nevertheless, they dehydrated no more rapidly than the older embryos. None of the intact embryos even showed creasing in that period in either 0.75 M sucrose or 2 M ethylene glycol/D-20. Only 1% of dechorionated embryos showed creasing in 0.75 M sucrose and only 10% in 2 M ethylene glycol. None progressed beyond the creased stage.

DISCUSSION

Effectiveness of the barrier to the transfer of water vapor

The shells of the *Drosophila* egg constitute a remarkably efficient barrier to the efflux of water (studied here) and presumably to influx as well. The initial rate of water loss in air is at least fifty times faster in alkane permeabilized embryos than in untreated embryos, and there is evidence, discussed below, that that is a substantial underestimate of the difference. Some of the barrier lies in the chorion, but most lies in the vitelline membrane. Removal of the former by hypochlorite increases the initial rate of water loss in air about 3.5-fold. Permeabilization of the latter by heptane/butanol mixtures increases it at least 15-fold, and probably considerably more. The impedance to water flow by the chorion is probably structural. Its honeycombed structure virtually eliminates convective mass transfer and it increases the distance over which the diffusion of water has to occur before it leaves the embryo.

The barrier in the vitelline membrane, in contrast, appears to be compositional. It is probably a thin layer of wax on the outside of the vitelline membrane. As reviewed by Papassideri *et al.* (1991), the evidence is, firstly, that permeability is increased by heating eggs to 46°C and by exposure to alkanes and, secondly, that frozen embryos cleave in a manner consistent with the existence of a smooth hydrophobic layer. From transverse sections, these same authors estimated the thickness of the wax layer to be only 50 Å, a value that emphasizes the remarkable effectiveness of its barrier properties. A 50 Å layer surrounding the prolate spheroidal embryo would have a volume of 2200 μm^3 and a mass of 0.0022 μg/embryo if we assume a density of one. Our gravimetric measurements (Table 1) indicated that treatment with heptane/butanol removed 0.04–0.05 μg solids/embryo but, as we pointed out, that is not statistically different from 0.0022.

Rates of dehydration in air vs dehydration under an osmotic gradient

As mentioned in the Introduction, water permeability is usually assessed by measuring the rate at which cells shrink or swell osmotically in anisoosmotic aqueous solutions. Accordingly, we thought it desirable to obtain approximate, semi-quantitative, comparisons between the effects of the permeabilization steps on water permeability in air and the effects on water permeability under an osmotic gradient. The comparisons are approximate because osmotic water permeabilities are so low prior to exposure to heptane/butanol and because the

comparison had to be based on embryo morphologies during dehydration. On the other hand, the differences in the rate of water flow in air and in solution are great.

We noted that the morphological manifestations of dehydration in air differ somewhat from those in hyperosmotic solutions. In air, the first manifestation of drying is a lateral indentation. In hyperosmotic solution, it is the appearance of transverse creases. In air, subsequent dehydration is manifested by lateral collapse of the embryo to form a distorted flattened entity; in hyperosmotic solution, subsequent dehydration (which we have rarely observed in other than heptane/butanol permeabilized eggs) converts the embryos into flattened ellipses. We noted that indentation in air corresponds to approx. 15–20% water loss. In the following discussion, we make the assumption that indentation in air and crease formation in hyperosmotic solution correspond to about the same water loss. We make a similar assumption for an equivalence between lateral collapse in air and flattening in hyperosmotic solution — that they correspond to a loss of 30–40% of embryo water.

In air, approx. 20% of dechorionated embryos exhibited the initial lateral indentation in 15 min. In hyperosmotic solutions, fewer than 10% exhibited creasing in 5h indicating that the rate of dehydration in air is at least 20 times faster than that in hyperosmotic solutions. That difference is almost certainly a consequence of the much higher driving force for water loss in air. To a first approximation, the driving force is proportional to the difference in chemical potential between intraembryonic water and the external water, which is equivalent to the logarithm of the ratio of the internal and external water activities. In solutions, $\ln a_w = -\bar{V}_w m$, where $\bar{V}_w$ is the partial molar volume of water (0.018 l/mol) and m is osmolality (Lewis and Randall, 1961). The osmolalities of 0.75 M sucrose in D-20, 2 M ethylene glycol in D-20, and D-20 itself are 1.22, 2.54, and 0.26, respectively and the corresponding water activities are 0.9782, 0.9553, and 0.9953 (computed from osmolality data in Scatchard *et al.* (1938) and density, molality, and osmolality data in the Handbook of Chemistry and Physics (Werst, 1982)). If we assume that the osmolality of an isotonic embryo is the same as D-20, then the initial driving force for water efflux of embryos in 0.75 M sucrose/D-20 and 2 M ethylene glycol/D-20 will be ln(0.9782/0.9953) and ln(0.9553/0.9953) or 0.0173 and 0.0410, respectively.

The water activity in air is numerically equal to the relative humidity/100; i.e. a_w^e was 0.5. Hence in air a_w^i/a_w^e had the value of ln (0.9953/0.5) or 0.6884. Consequently, the driving force for water efflux in air was 0.6884/0.0173 or 40× that in 0.75 M sucrose, and it was 0.6884/0.0410 or 17× that in 2 M ethylene glycol. These 17× and 40× ratios for the driving forces correspond rather well to our finding that embryos in air dehydrate at least 20× as rapidly as do embryos in hyperosmotic sucrose or ethylene glycol. Note, however, that the actual flow rate of water depends both on the driving force and on the numerical value of the proportionality constant, i.e. the hydraulic conductivity, L_p, in the case of osmotic flow and the diffusion constant and diffusional distance in the case of water loss in air. Lin *et al.* (1989) have estimated the L_p of permeabilized embryos to be 0.72 μm/(min•atm), but there are no values for L_p in intact to dechorionated embryos and the diffusion coefficients are unknown.

Kinetics of water loss in heptane/butanol permeabilized embryos

[Mazur *et al.* (1992a) Table 6] reported that 50% of permeabilized 12- h embryos (defined as those staining red or dark pink with rhodamine B after treatment with 0.3% butanol in heptane) manifested creasing after 1–3 min in 0.75 M sucrose/D-20, and 50% became flattened in 4–8 min. We noted above that in the absence of exposure to alcohol/alkane, only 1% of the embryos underwent creasing in 5h. In other words, the butanol/heptane treatment increased water permeability 100–300 fold.

If alkane permeabilized embryos dry 20 times faster in air than they dehydrate in hyperosmotic sucrose (the ratio estimated in the preceding section for dechorionated embryos), half of them should have undergone lateral collapse in air in 10–20 s (1/20th of 4–8 min). We estimated in Results that lateral collapse corresponds to the loss of 30–50% of intraembryonic water. From Fig. 6(D), however, we see that the loss of 40% of water from heptane/butanol permeabilized embryos in air took about 4 min, or 12–25 times as long as expected. The discrepancy we believe arises because the water loss curves in Fig. 6(D) may be more a reflection of approx. 5 min required to remove extra embryonic water (Fig. 1) than of the loss of intraembryonic water. In other words, we believe that Fig. 6(D) substantially underestimates the rapidity with which the alkane permeabilized embryos dehydrate in air, and therefore underestimates the contribution of the waxes in the vitelline membrane to the water permeability barrier.

There are two other possible contributors to the discrepancy between observed and expected rates of water loss of permeabilized embryos in air. One is that the during air drying, the heat of vaporization withdrawn from the embryos as liquid embryo water is converted to water vapor in air is sufficient to cool the embryos and thus slow the water loss. We noted in Results that the temperature coefficient for air drying is high. We also noted that the evaporation of water from the surface of dechorionated embryos produces a 3–4°C drop in temperature over the first 3–5 min of drying, but that the subsequent loss of intraembryonic water produces no cooling. Although the loss of intra-embryonic water from fully permeabilized embryos is not as rapid as the evaporation of surface water, it is substantially greater than that of dechorionated embryos, and it may be sufficient to produce some evaporative cooling after the surface water has disappeared. But that cooling would not be greater than the 3–4°C produced by the vaporization of surface water.

Another hypothesis is that air drying alters and increases the water barrier properties of components of the vitelline membrane left after the heptane/butanol extraction. There are several lines of evidence that the permeability of the egg shell components to water is reduced by exposure to anhydrous fluids. First, we noted in connection with Table 1 that the initial rate of dehydration of untreated embryos decreased from 12%/h in approx. 50% r.h. air to 9% in dry air or dry nitrogen. This decrease occurred in spite of the fact that the driving force for water efflux in the very low humidity air or nitrogen was far higher than in 50% air. Here, the rate of water loss (1 μg water/embryo/h) was too low to produce evaporative cooling. Consequently, it would appear then that the dry gases modified the barriers to reduce their diffusion coefficient. The second line of evidence is that the exposure of dechorionated embryos to pure isopropanol significantly reduced the rate of water loss [Fig. 6(C) vs 6(B)]. One presumed action of the isopropanol is to remove residual extra-embryonic water. Third, Cicero and Steponkus (1993) have reported that if, after the isopropanol exposure, the embryos are exposed for 2 min to air that is dried by molecular sieve, they become less readily permeabilized by subsequent exposure to hexane/butanol than we have found after 2 min exposure to air at ambient relative humidity (approx. 50%).

One puzzling aspect about the exposure to isopropanol is that although it significantly decreased permeability to water, it removed the largest amount of solids of any of the permeabilization steps (Table 2). One explanation would be that it is removing proteinaceous material unrelated to the permeability barrier. Perhaps it is removing fragments of endochorion that are not visible at dissecting microscope resolution. Perhaps it can access and remove proteins in the vitelline membrane without materially breaching the wax layer. It is interesting, and possibly relevant, that embryos of *Anopheles gambiae* are actually as effectively permeabilized by isopropanol alone as by heptane/butanol mixtures (Valencia *et al.*, 1996).

Effect of embryo age on dehydration rates

Figures 2 and 3 compared the effect of developmental age on the kinetics of water loss in air. Three and 6- h intact embryos dehydrated at a somewhat higher rate than 9- to 15-h embryos. These times refer to the embryo age at the start of drying. We noted in connection with the morphological observations in hyperosmotic solutions that normal embryonic development is not impaired by slight dehydration to the point of the appearance of transverse creases. However, it ceases with further dehydration. From Fig. 5 we saw that a water loss of approx. 20% was lethal to 50% of 3–9-h embryos and a loss of approx. 40% was lethal to 50% of 12–15-h embryos. Cuticle begins to be laid down in embryos at about the 12-h stage. At about the 17-h stage, the embryos can no longer be permeabilized by exposure to alkane, presumably because the embryonic cuticle has been fully formed and has been rendered resistant to alkane permeabilization (Mazur *et al.*, 1992a, b). If, during drying, embryonic development continues to the point where cuticle is produced and becomes impermeable, our evidence indicates that these events do not materially affect the kinetics of water loss. The water loss curves in Figs 2 and 3 are close to linear over many hours of drying. More significant, we shall see in the companion paper that a single kinetic constant suffices to quantitatively describe the observed kinetics over many hours of dehydration. These facts would indicate that either drying is preventing cuticle formation or its chitinization, or else the rate limiting barrier is the wax layer in the vitelline membrane. Another way to state the latter is that the impedance of the wax layer to water flow is much higher than the impedance of the cuticle in series with it.

Effect of water in embryos vs osmotically available water and freezable water

Our gravimetric measurements (Table 2) show that a dechorionated embryo contains 7.5 μg of water and that that water constitutes 75.5% of the mass of the embryo. [The mass of water in the intact embryo is slightly higher (7.9 μg) but the fractional water content is nearly the same (76.2%). These values lead to the conclusion that most of the mass of the chorion is water.] According to differential scanning calorimetric measurements by Myers *et al.* (1987), 5.4 μg or 72% of the water in dechorionated embryos freezes by −35°C. Probably more water would have frozen at still lower temperatures. By that measurement, then, some three-quarters or more of the embryonic water is free (available for freezing) and not bound (unavailable for freezing).

Estimates of osmotically available water in cells can be obtained from Boyle vant Hoff plots. These are plots of cell volume as a function of the reciprocal of the osmolality of a non-permeating solute. Extrapolation of these plots to infinite osmolality yields a value for the volume fraction (V_b) of the cells that is osmotically unavailable. Lin *et al.* (1989) made such measurements in permeabilized *Drosophila* eggs and reported a V_b value of 0.54. That translates to 46% (v/v) of the embryo volume being occupied by osmotically responsive water. By microscope measurement, Lin *et al.* (1989) have estimated the volume of the isotonic embryo to be 8.5×10^6 μm^3. From our measurements of mass and assuming a density of 1.09 g/cc for the fully hydrated embryo (computed using the embryo solids density found in the companion paper (Schreuders *et al.*, 1996) and the mass fractions data from Table 2), we calculate the volume of the dechorionated embryo to be 9.55×10^6 μm^3. Using the average of these values (9×10^6 μm^3), the 46% osmotically available water corresponds to 4.1×10^6 μm^3 or 4.1 μg. Thus, of the 7.5 μg water/embryo determined gravimetrically, 3.4 μg is osmotically unavailable, and 2.1 μg does not freeze by −35°C.

Physiologists refer to the ratio of osmotically responding water to total intracellular water as Ponders

R. In dechorionated *Drosophila*, *R* has a value of 4.1/7.5 or 0.55. That is an unusually low value. Dick (1966) reports only one other value that low in 17 cell types. The usual values of *R* are between 0.8 and 1. Also, there is some discrepancy between the fraction of water that is non osmotic (45%) and the fraction that does not freeze by −35°C (28%). The abnormally high value of V_b, and the consequent unusually low value of *R* may be artifactual. Lin *et al.* (1989) calculated the volumes of the eggs in hyperosmotic solutions assuming them to be prolate spheroids that shrink isotropically. We do not find that to be the case. We find that permeabilized embryos in hyperosmotic sucrose preferentially flatten in the Z-axis of the microscope. Consequently, calculations of volume based on the optical XY area and the assumption that they are prolate spheroids will progressively become overestimates as the embryo increasingly flatten in the Z-axis in increasingly hyperosmotic solutions.

As indicated, the drying of embryos is a physical process driven by thermodynamic potentials and modulated by the resistance of the system to water flow. The companion paper (Schreuders *et al.*, 1996) is concerned with the development of a physical model of the kinetics of the process and with a comparison between the predictions of the model and the experimental observations reported here.

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