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

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Previously, we gravimetrically examined the *Drosophila* embryos' rate of air drying after each step in their permeabilization; namely, (1) dechorionation, (2) isopropanol exposure, and (3) heptane/butanol exposure. Here we describe a physical model characterizing the dehydration and compare the theory with the experimental kinetics. The model is based on those of Eyring. It treats the net flux (J_{net}) through the membrane as the sum of two opposing fluxes (air → embryo {a} and embryo → air {i}). Each flux is the product of a kinetic constant (k), the number of embryos in the sample (n), and the water concentration (C). Thus, $J_{net}=k_a n C_a - k_i n C_i$. The relationship between k_i and k_a, for embryos in steady state with room humidity, is $k_i/k_a=C_a/C_i$. Determining the k values requires the mass and density of the embryo solids, n, C_i as a function of drying time, and the steady-state value of C_i. Following integration of the flux equation, linear regression was used to determine k_i. k_a was computed using the previous equation. The resulting values for k_a are 3.96, 26.70, 17.40, and 258.00 mm³/embryo·h for untreated embryos and embryos treated with, (1), (1)+(2), (1)+(2)+(3), respectively. The corresponding values for k_i ($\times 10^6$) are 484, 3680, 2800, and 47,300 mm³/embryo·h. Published by Elsevier Science Ltd

Drosophila melanogaster Kinetic model Permeabilization protocol Permeability Embryo dehydration

INTRODUCTION

Drosophila membrane permeability and cryopreservation

There are, currently, more than 10,000 mutant strains of *Drosophila*. These strains are being maintained through frequent transfer of breeding stocks. Cryopreservation of these organisms can provide a reduction in the manpower, costs, and storage space required, allowing the retention of strains that would otherwise have to be abandoned or lost. In addition, cryopreservation provides a method for minimizing genetic drift.

The primary obstacle to cryopreservation was the impermeability of the *Drosophila* embryo to both water and cryoprotectants, for successful cryopreservation requires that water must be removed from the embryo and cryoprotectant introduced. This impermeability resides in the embryo's surrounding shells; an outer chorion and an inner vitelline membrane. While permeabilization protocols for *Drosophila* embryos have existed for a number of years (Limbourg and Zalokar, 1973), it was only comparatively recently that Lynch *et al.* (1989) and Mazur *et al.* (1992b) published methodologies that permeabilized the embryos sufficiently to allow the introduction of the cryoprotectant ethylene glycol while retaining adequate viability.

In our permeability procedure, the embryos are exposed successively to 50% Clorox, isopropanol, and heptane containing 0.3% butanol. The Clorox removes the chorion; the heptane/butanol removes a wax layer on the surface of the vitelline membrane; and the isopropanol serves as mutually miscible intermediary between the aqueous Clorox and the non-polar alkane.

The companion paper (Schreuders *et al.*, 1996) was concerned with determining the effects of the several permeabilization steps on the permeability of the embryo to water. The experimental measure of permeability was the

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rate at which the embryos lost water when exposed to room air. The alternative approach of determining water permeabilities in solutions under osmotic gradient was not pursued quantitatively because, prior to exposure to the heptane/butanol, no osmotic volume changes are evident after 4 h, even in strongly hyperosmotic solutions.

The air-drying experiments in the companion paper showed that dechorionation produced a substantial increase in permeability to water vapor, and exposure to heptane/butanol produced an even larger increase. Thus, while intact embryos require 9.5 h to loose half their water, dechorionated, isopropanol-treated, and heptane/butanol-treated require 1.6, 1.9, and 0.1 h, respectively.

The purpose of the present paper is to develop a physical model of the transport of water during air drying, and to determine the coefficients that describe that transport in intact embryos and in embryos subjected to the several permeabilization steps.

Diffusion modeling

The most common approaches to biological mass transfer are based upon the work of Fick. In his paper, he stated that the flow is "directly proportional to the difference in concentration, and inversely proportional to the distance of the elements from one another" (Fick, 1855). Since that time, diffusion modeling has been enhanced to include the consideration of the effects on the flux of one component by the concentration gradient of another (Onsager, 1931a, 1931b). Typically, for a system containing 'n' species, these models are expressed in the form:

$$-\mathcal{J}_p = \sum_{q=1}^{n-1} \mathcal{D}_{pq}\mathcal{A}\nabla\mathcal{C}_q \qquad (1)$$

where the total flux ($\mathcal{J}_p$) of component 'p' is the sum of the fluxes resulting from each of the independent gradients in 'q' Each of these sub-fluxes is, in turn, the product of the area through which the flux occurs ($\mathcal{A}$), a concentration gradient of component 'q' acting as the driving force ($\nabla\mathcal{C}_q$), and the phenomenological coefficient ($\mathcal{D}_{pq}$). Note that 'q' includes the species 'p.' (Fick's law is a special case of Equation 1, where $\mathcal{D}_{pq}=0$ for all $p \neq q$.)

Unfortunately, when modeling the drying of *Drosophila melanogaster* embryos, this continuum approach is not practical because the water's phase change during drying prevents the use of the concentration gradient as the driving force. An alternate solution to this problem is to employ an irreversible thermodynamic model for the diffusion. However, these models use a chemical potential gradient as the driving force, a quantity which (while well documented for water vapor in air) becomes unknown for the interior of the embryo as drying proceeds (Cussler, 1984; Katchalsky and Curran, 1967).

These difficulties can be avoided by considering the drying process in terms of kinetics. Unlike the continuum approaches of Fick (1855) and Onsager (1931a, 1931b), kinetic models to describe diffusion are based on the models proposed by Eyring (Eyring *et al.*, 1949; Zwolinski *et al.*, 1949). These models, derived from absolute reaction rate theory, describe the diffusion process in terms of point to point jumps between nodes a distance 'λ' apart. The net flux (Q) [moles/second] is the difference between the number of particles jumping in the forward (or positive) direction and those jumping in the backward (or negative) direction. The magnitude of each of these fluxes is the product of the number of particles at a node and a kinetic constant 'k' which Eyring describes as the probability of a particle making the jump per unit time. If C_i is the molar concentration of the diffusing molecule, the amount of material in a node with a unit cross-sectional area is λC_i. Then, the net flow of a species between the adjacent nodes 'i' and 'i+1' in the series becomes:

$$Q = k\lambda C_i - k\lambda C_{i+1} \qquad (2)$$

Eyring's model and its variants have been successfully applied to a variety of biological applications including transport through cell membranes of *Arbacia* embryos (Zwolinski *et al.*, 1949), erythrocytes (Levin *et al.*, 1976), and murine embryos (Schreuders, 1989), and through the interstitial tissue of the rabbit ear (Schreuders, 1989; Schreuders *et al.*, 1994).

THEORETICAL DEVELOPMENT

The modeling of drying processes usually requires the consideration of two coupled phenomena: heat and mass transfer. The evaporation of water from the system can produce a decrease in the temperature of the object undergoing drying. A decrease in temperature, in turn, would reduce the rate of mass transfer. As noted in the experimental paper (Schreuders *et al.*, 1996), the temperature coefficient of the drying rate in intact embryos is sizable. However, thermocouple measurements reported in that paper show that evaporative cooling is not sufficient to measurably lower the temperature of intact, dechorionated, or alcohol-treated embryos. In the case of heptane/butanol permeabilized embryos, the dehydration occurs too rapidly and is too confounded with the evaporation of surface water to permit a measurement of the embryo temperature during drying, but the cooling cannot be greater than that resulting from the evaporation of surface water in dechorionated embryos; namely, 3–4°C. On the basis of these results, we assume that the drying process is isothermal in the ensuing theoretical treatment and analysis.

Definition of the fluxes and concentration

The model developed in this paper is a significant modification of Eyring's. Specifically, it has been simplified by assuming that diffusion occurs between two adjacent compartments, rather than through a series of nodes. Under this model the net flux across a membrane is described as the sum of two fluxes acting in opposition.

Each of these fluxes is, in turn, described in terms of first-order kinetics such that:

$$J = kAC \tag{3}$$

Where:

J = the flux $\left[\frac{\text{mmol}}{\text{hr}}\right]$

k = the kinetic constant $\left[\frac{\text{mm}}{\text{hr}}\right]$

A = the area through which the diffusion occurs [mm^2]

C = the concentration of water $\left[\frac{\text{mmol}}{\text{mm}^3}\right]$

The concentration is the number of moles of water divided by the volume occupied by the water, the embryo solids, and an unknown volume of mixing. The volume of mixing is assumed to be zero. Thus, the concentration is written:

$$C = \frac{W_w}{M_w\left(\frac{W_w}{\rho_w} + \frac{W_w}{\rho_s}\right)} \tag{4}$$

Where: W = weight of specie [mg]

M = molecular weight of specie $\left[\frac{\text{mg}}{\text{mmol}}\right]$

ρ = density $\left[\frac{\text{mg}}{\text{mm}^3}\right]$

s = embryo solids

w = water

Experimentally, the rate of water loss was determined gravimetrically. Thus it is convenient to work in units of mass, rather than mol. This conversion is achieved by multiplying both the left and right hand sides of Equation 3 by the molecular weight of water, M_w. On the right hand side of Equation 3, the change in units occurs in the concentration term. The flux now has units of mg/h and the concentration has units of mg/mm^3.

The total area A, through which transport occurred, was broken down into the surface area of an individual embryo 'A' multiplied by the number of embryos n. In modeling the kinetics of osmotic dehydration, it makes little difference in the goodness of the resulting fit, whether one assumes that the membrane area remains constant with the change in cell volume or assumes that it varies as the 2/3 power of the volume (Mazur, 1990). We have assumed that A remains constant throughout the dehydration process. Therefore, it can be absorbed into 'k' yielding a composite 'k'. This transformation normalizes Equation 3 such that the values of the composite kinetic constants are independent of sample size and, thus, can be compared between experiments. The resulting equation is:

$$J = knC \tag{5}$$

Where:

J = the water flux across the membrane either into or out-of the embryo $\left[\frac{\text{mg}}{\text{h}}\right]$

k = the composite kinetic constant (includes embryo surface area) $\left[\frac{\text{mm}^3}{\text{embryo}\circ\text{h}}\right]$

n = the number of embryos

C = the concentration $\left[\frac{\text{mg}}{\text{mm}^3}\right]$ The constants and variables used in the equations which follow are of the form: X^z_y. The superscript refers to the time at which the variable is examined and the subscript refers to the measured quantity's location (and composition, if not explicit). Noting that the net flux is the sum of two fluxes (one into and one out-of the embryo) and choosing the sign convention such that a positive net flux results in an embryonic weight gain, the equation for the net flux can then be written:

$$J_{net} = k_a n C_a - k_i n C_i \tag{6}$$

Where: J_{net} = the net water flux across the membrane $\left[\frac{\text{mg}}{\text{h}}\right]$

a = with respect to the air

i = intraembryonic

The case of the embryo

For the case of a discrete system (such as an embryo):

$$J_{net} = \frac{dW_{wi}}{dt} \tag{7}$$

Where: t = time (h)

Substituting Equation 7 into Equation 6 yields:

$$\frac{dW_{wi}}{dt} = k_a n C_a - k_i n C_i \tag{8}$$

Integration of the kinetic Equation 8 requires that the net flux be described using the same variable as the individual fluxes (in this case C). This conversion was accomplished by noting that:

$$\frac{dC_i}{dt} = \frac{dC_i}{dW_{wi}}\frac{dW_{wi}}{dt} \tag{9}$$

$\frac{dC_i}{dW_{wi}}$ is obtained by differentiating the definition of concentration (Equation 4) (rearranged to express W_{wi} in terms of C_i) with respect to W_{wi} to yield:

$$\frac{dC_i}{dW_{wi}} = \frac{\rho_s(\rho_w - C_i)^2}{W_{si}\rho_w^2} \tag{10}$$

Substituting Equations 8 and 10 into Equation 9 yields:

$$\frac{dC_i}{dt} = \left[\frac{\rho_s(\rho_w - C_i)^2}{W_{si}\rho_w^2}\right][k_a n C_a - k_i n C_i] \tag{11}$$

As the water loss proceeds, a point is reached where the embryo's weight becomes constant. At this time, the chemical potential of intraembryonic water is reduced to that of the water in the surrounding air. The influx and efflux of water are equal and the net water flux across the egg shells becomes zero. We define the concentration of intraembryonic water at that steady state as C_i^*. Furthermore, we assume that, even if the kinetic 'constants' have deviated from their initial values, both k_i and k_a have changed by the same ratio. Then, at this steady state, Equation 6 becomes:

$$k_a^* n C_a = k_i^* n C_i^* \tag{12}$$

If we assume the relative humidity of the air to be constant in a given drying run, C_a and C_i^* will be constant. Furthermore, we assume that the ratio k_i/k_a is constant at all times. Then Equation 12 becomes:

$$\frac{k_i^*}{k_a^*} = \frac{k_i}{k_a} = \frac{C_a}{C_i^*} \tag{13}$$

Some of the implications and limitations of these assumptions are examined in the DISCUSSION. With that fact noted, Equation 13 is substituted into Equation 11, and the result is rearranged into the standard form for integration; namely,

$$\int_0^t \left[\frac{k_i n \rho_s}{W_{si}\rho_w^2}\right] dt = \int_{C_j^t}^{C_i^0} \left[\frac{1}{(\rho_w - C_i)^2 (C_i^* - C_i)}\right] dC_i \tag{14}$$

Integration and rearrangement yielded

$$k_i t = \left[\frac{W_{si}\rho_w^2}{n\rho_s(\rho_w - C_i^*)}\right]\left[\frac{C_i^0 - C_i^t}{(\rho_w - C_i^t)(\rho_w - C_i^0)}\right] + \left[\frac{W_{si}\rho_w^2}{n\rho_s(\rho_w - C_i^*)^2}\right] ln\left[\frac{(C_i^0 - C_i^*)(\rho_w - C_i^t)}{(C_i^t - C_i^*)(\rho_w - C_i^0)}\right] \tag{15}$$

This equation is of a form such that a plot of the right hand side of the equation ($f(C_i^t)$) vs time yields a line of slope k_i. The first aim of the analysis was to compute the kinetic constant k_i for embryos subjected to the several steps involved in permeabilization, allowing comparison of the rates of water loss.

METHODS

Embryo collection and the permeabilization protocol

Computation of the water concentrations used in Equation 15 requires five experimental values. Four of these are static values; namely, the embryo weight at 100% hydration, the embryo weight at steady state with room air, the dry weight of the embryo, and the density of the embryo solids. The fifth is a kinetic measurement i.e. the weight of embryos as a function of drying time. These values were determined for untreated embryos and for embryos subjected to the three steps involved in permeabilization–dechorionation, exposure to alcohol, and exposure to alcohol alkane mixtures. The procedures for the permeabilization steps and the measurements of static and kinetic embryo weights are detailed in the companion paper (Schreuders *et al.*, 1996), and consequently only the essentials are summarized here.

The experiments were performed on 15-h embryos, the stage that has been found optimal for cryopreservation (Mazur *et al.*, 1992a). The procedures for rearing flies and staging the embryos are detailed in Cole *et al.* (1993) and in the companion paper (Schreuders *et al.*, 1996).

The permeabilization procedure was performed using the method described in the companion paper (Schreuders *et al.*, 1996). Additional information is available in Mazur *et al.* (1992a, 1992b) and Cole *et al.* (1993).

In brief, the embryos, sandwiched between two polycarbonate (PC) membrane filters, were dechorionated by exposure to 50% Clorox solution (2.6% sodium hypochlorite) for a total of 2.5 min. Following the Clorox solution, distilled water was passed over the embryos to remove residual sodium hypochlorite. Because the aqueous solutions used in the dechorionation are not miscible with the alkanes used in the permeabilization of the vitelline membrane, the water on the surface of the embryos was removed by exposing the embryos to isopropanol, which is miscible with both water and alkane. If the embryos were to be examined following this step, the isopropanol was followed by a water rinse. Otherwise, air was allowed to pass around the embryos to evaporate the majority of the isopropanol. The embryo's permeabilization was completed by exposing them to *n*-heptane containing 0.3% butanol. The permeabilization process was quenched by washing away the butanol/heptane solution with pure heptane.

Static and kinetic dehydration measurements were made on the embryos after each step of the permeabilization (untreated, dechorionated, isopropanol treated, and heptane/butanol treated). After completion to the desired step, the filter sandwich was removed and transferred to a small petri dish containing 1–2 ml of either water (untreated, dechorionated, and isopropanol treated embryos) or isotonic D-20 *Drosophila* cell culture solution (Zalokar and Santamaria, 1977) (fully permeabilized embryos). Thus, prior to the initiation of drying, the embryos were at their fully hydrated normal volume.

Determination of embryo weights at full hydration, at steady state, and after complete dehydration

Embryo weight at 100% hydration. The embryo weight at 100% hydration was the weight of the embryos at time zero of drying, corrected for the weight of any residual surface water present on either the embryos or the PC filter. Time zero was taken to be the time of the initiation of the 30-s exposure to a flow of room air or the initiation of the brief blotting, both of which removed the bulk of the surface water. Corrections for any residual surface water were made by back-extrapolating to zero time from two weights determined over the first 1–5 min

(see Schreuders *et al.*, 1996 for details). This technique could not be used for the heptane/butanol treated embryos because their rate of water loss in air was so high. Instead, the 100% hydration weight of these embryos was computed by adding their dry weight to the average weight of the water present in the dechorionated embryos.

Embryo weight at steady state. The embryo weights for the dechorionated, isopropanol treated, and heptane/butanol treated embryos were determined at steady state with room air by allowing the embryos to dry for at least two days in room air (average relative humidity approx. 56%). At the end of this period the weight of the embryos was determined. The steady-state weight for the untreated embryos was not determined as part of the dynamic drying experiments. Instead, the weight was calculated from the average properties presented in the companion paper. Using these data, the steady-state weight of the embryos was 1.07 times the dry weight.

Embryo weight at 0% hydration. After the steady-state weights had been determined, the embryos were placed back into the petri dish and the dish placed into a vacuum desiccator. Two to five days later, the samples were weighed. This weight was the dry weight of the embryo.

Estimation of the density of the embryo solids

Calculation of the intraembryonic water concentration requires values for the densities of water and embryo solids (Equation 4). The former was obtained from standard tables (Diem and Lentner, 1970). The density of embryo solids was bracketed by determining whether fully dried embryos rose or sank in various liquids of known densities. The three fluids used were ethylene glycol (#E-9129, Sigma Chemical Company, St Louis, MO) (ρ=1.11 g/cm^3), glycerol (#GX0190-1, EM Science, Gibbstown, NJ) (ρ=1.26 g/cm^3), and 3 chloro-1,2-propanediol (#10,727-1, Aldrich Chemical Co., Milwaukee, WI) (ρ=1.48 g/cm^3) (Weist, 1982).

Embryo preparation. Samples of 12–14-h embryos were split into two groups. The first group was left untreated and the second group of embryos was dechorionated. Monolayers of the embryos, on polycarbonate filters, were dried in a vacuum desiccator for a minimum of 3 days and stored under vacuum until the density determinations.

Estimation procedures. Three procedures were used to determine whether the dry embryos were more (or less) dense than a given test liquid.

Dry loading methods

3–5 μl of dried embryos were placed into the bottom of Microfuge tubes and centrifuged for approx. 30 s to move all the embryos to the bottom of the tube. One hundred microliters of the test liquid was then layered over the embryos, taking care to avoid visible bubbles of air.

The embryos were subsequently centrifuged at approx. 8,000 ***g*** for 60 s using a Beckman Microfuge B (Palo Alto, CA). The tube was then opened and its contents degassed in a vacuum desiccator for approx. 60 s after which the embryos were centrifuged for 60 s and degassed a second time. After a third centrifugation (total centrifugation time 3 min), the location of the embryos was determined visually. If the embryos were at the bottom of the tube, they were assumed to be more dense than the test fluid. If the embryos rose to the fluid's surface, the experiment was repeated using the wetting method described below.

A number of runs were made using the above protocol, but with the embryos layered over the comparison fluid, rather than the reverse. The results using this second method were no different from those derived using the first.

Wetting method

Because the glycerol and the 3 chloro-1,2-propanediol wet the embryos with difficulty, we were concerned that entrapped air might have reduced the apparent density of the embryos. To circumvent this possibility, we performed measurements in which the embryos were wetted using ethylene glycol before the glycerol or chloro-propanediol was added. Both test liquids are miscible in ethylene glycol at the levels used. The procedure was as follows.

3–5 μl of the dried embryos were placed into the bottom of microfuge tubes and 100 μl of ethylene glycol was then layered over the embryos, taking care to minimize the formation of air bubbles. The embryos were then centrifuged for 60 s. The tube was opened and placed into a vacuum desiccator for approx. 30 s to remove any residual air. The embryos were then centrifuged for an additional 60 s. The dried embryos were denser than the ethylene glycol and, consequently, moved to the bottom of the tube. As much of the ethylene glycol supernatant as possible was then removed using a syringe. If the embryos were disturbed, the tube was centrifuged for an additional 30 s and most of the remaining supernatant removed. The total volume of the embryos and residual ethylene glycol was estimated to be 4–7 μl.

400 μl of glycerol or chloro-propanediol was then layered over the embryos and the embryo pellet was suspended in that test fluid by gentle stirring. At this point the embryos were slightly translucent, indicating good wetting. Furthermore, any residual ethylene glycol was well mixed into the comparison fluid. The tube was centrifuged for two minutes and the position of the embryos noted. The centrifugation was then continued for an additional minute, after which virtually all of the embryos had moved towards either the top or the bottom of the tube.

Kinetics of water loss from the embryos

Two different procedures were used to determine the rate of water loss as a function of time. The details are given in the companion paper (Schreuders *et al.*, 1996).

The first protocol was used for untreated, dechorionated, and alcohol treated embryos. A second protocol was used to determine the rate of water loss in the heptane/butanol treated (fully permeabilized) embryos. This second protocol minimized water loss prior to measurement, a necessity since the permeability of these embryos was substantially increased.

Untreated, dechorionated, and isopropanol treated embryos. Embryos (50–150) were loaded onto a pre-weighed 13 mm PC membrane filter. After loading, the exact number of embryos on each filter was determined and the filters were floated on water in a small petri dish. The embryo-laden PC filters were individually taken out of their petri dishes and the bulk of their surface water removed by passing room air through the filter. After this, the PC filter was transferred onto a Cahn G-2 electrobalance (Ventron Instruments Corp., Paramount, CA) and weighed. Time zero was taken as the point where the disc was placed on the vacuum filter. The weight of the filter plus embryos was determined at regular intervals with more points taken during periods where the rate of water loss was high. After 5–6 h, the PC filters in their petri dishes were put in a vacuum desiccator and dried to a constant weight.

Heptane/butanol treated embryos. Fifty embryos were loaded onto pre-weighed 13 mm PC filter discs and floated on D-20 in a small petri dish to prevent water loss prior to the experiment. One PC filter was taken out of its petri dish and blotted with 2–3 strokes on a piece of bibulous paper. Afterwards, the filter and embryos were transferred onto the measuring pan in the Cahn electrobalance and weighed. Time zero was taken as the point where the disc was taken off the bibulous paper. The PC disc was weighed once a minute and the times of the measurements recorded. After 40 min, the weight loss approached zero. The disc was then placed into an empty petri dish, the lid put on, and put into a vacuum desiccator. After this, the procedure was followed for each of the other PC discs. The discs were taken out periodically and weighed. The dry weight of the embryos was that at which no more weight loss was observed.

RESULTS

The experimentally determined model inputs

Determining the kinetic constants using Equation 15 required knowledge of several traits of the *Drosophila* embryos. These traits include the density of the embryo solids, the embryo's water concentration at full hydration and at steady state with room air, and the weight of the embryo solids. The results of determining the densities and the concentrations are discussed below. The dry weights were gravimetrically assessed during each run.

Embryo solids density. The densities for the embryo solids were estimated using vacuum dried, untreated, and dechorionated embryos. Estimates were not obtained for the permeabilized or the alcohol treated embryos. These densities were presumed to be within the same range as the dechorionated embryos.

The dried untreated embryos sank in ethylene glycol independent of whether they were loaded at the top or at the bottom of the centrifuge tube. Furthermore, they appeared to be well wetted by the liquid, as demonstrated by their changing from a white to a translucent state. Sinking was indicative of an embryo density greater than 1.11 g/cm^3. When the embryos were centrifuged in glycerol, using either top or bottom loading, they floated. However, they did not assume the translucent appearance, suggesting that there was air entrapped in the irregular shape of the dried embryo. Therefore, the *wetting* method was used. The dried embryos sank with this technique indicating a density greater than 1.26 g/cm^3. The dried untreated embryos rose to the surface of the chloro-propanediol (ρ=1.48 g/cm^3) using both the *bottom loading* and the *wetting* techniques. Similar results were obtained for the dried dechorionated embryos; they sank to the bottom in both ethylene glycol and glycerol and rose to the surface in chloro-propanediol. In all cases, the dried embryos exhibited a translucency indicative of good wetting. Thus, the density of solids in both intact and dechorionated embryos was between 1.26 and 1.48 g/cm^3. The value used in transport modeling was the midpoint of these two values (1.37 g/cm^3).

The water concentrations

Water concentration in the air

Using steam tables (Himmelblau, 1974), the concentration of water in the air (22°C, relative humidity approx. 56%) was computed to be 1.064×10^{-5} mg/mm^3.

Embryo water concentrations at 100% hydration and at steady state with room air

Two embryo water concentrations appear as constants in Equation 15; the concentration at full hydration (C_i^0) and the concentration in steady state with room air (C_i^*). These concentrations were computed for embryos at each step of the permeabilization protocol using Equation 4, the densities of the embryo components, and the embryo weights.

The water concentrations of the fully hydrated embryos were calculated directly from data gathered for the individual runs of intact, dechorionated, and isopropanol treated embryos. The water concentrations for the embryos treated with heptane/butanol were computed by assuming that the amount of water in the embryos of each run was the same as the mean water weight in the dechorionated embryos and by using the dry weights of each run's embryos. The means of the resulting water concentrations (±SE) at full hydration for the intact, dechorionated, isopropanol treated, and heptane/butanol treated embryos were 0.799±0.002, 0.742±0.016, 0.827±0.001 and 0.814±0.002 mg/mm^3, respectively. This variation in embryo water concentration occurs because different amounts of solids and water are lost at

each step of the protocol (Table 2, Schreuders *et al.*, 1996).

The steady-state water concentration of the embryos was based on the embryo's steady-state weights. This weight was determined experimentally for each experiment involving the isopropanol treated embryos. For the intact, dechorionated, and fully permeabilized embryos, a given run's steady-state weight was computed by multiplying the measured embryo dry weight by the ratio of the mean steady-state weight to the mean dry weight for embryos. These means are for a number of runs and are found in Table 2 in the companion paper (Schreuders *et al.*, 1996). The computed weight percentages of water in the steady-state embryos are 6.7, 5.4, 5.8 and 4.6% for intact, dechorionated, isopropanol-treated, and heptane/butanol-treated, respectively. These data agree well with the 7% water found in yeast at the same relative humidity (56%) (Koga *et al.*, 1966). Based upon these weights, the mean water concentrations (± standard error for the alcohol treated embryos) at steady-state with room air for the intact, dechorionated, isopropanol-treated, and heptane/butanol-treated embryos were 0.088, 0.077, 0.066±0.004 and 0.058 mg/mm^3, respectively.

Embryo water concentrations as a function of time

The intraembryonic water concentration also appears in Equation 15 as a function of time. This concentration is computed using Equation 4. These concentrations were strongly non-linear with respect to the weight of water present in the embryo, as shown in Fig. 1. This was due to the presence of the two volumes (water and solids) in the denominator of the equation. At low water concentrations small changes in amount of water produced large changes in the concentration, because changes in water content produce only a minimal change in the embryo volume. At high water concentrations, the concentration was relatively insensitive to changes in the amount of water present. Under these conditions, the solids fraction contributed little to the volume of the system and decreases in the weight of water were matched by corresponding decreases in the volume.

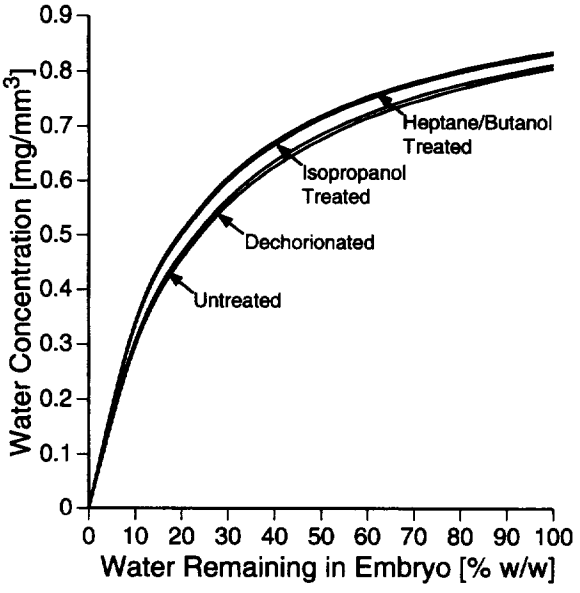

FIGURE 1. The concentration of water in the embryos as a function of the weight percentage of the water remaining in the embryo. The calculations are based on average properties for each permeabilization step.

The fit of the model to the experimental data

The mathematical form of Equation 15 is such that plots of time vs $f(C_i^t)$ are linear, where $f(C_i^t)$ is the right-hand side of the equation. k_i, the kinetic constant for movement of water from the embryo to the air, is the slope of the line. The first step in the analysis was to determine the portion of the experimental data over which the plots of $f(C_i^t)$ vs time were linear. The next step was to obtain the best linear fit of Equation 15 to $f(C_i^t)$ and, from the slope of the resulting line, obtain values of k_i for each experimental run.

Regions within the experimental data and their linearity. The data in Fig. 2(A) show the experimentally derived values of $f(C_i^t)$ vs time for representative drying runs on untreated, dechorionated, isopropanol-treated, and heptane/butanol-treated embryos. We have excluded from these plots those points in the first few minutes, where the data are confounded by the presence of extra-embryonic water. When the extra-embryonic water is present, the slope is artifactually high, as a result of the high rate of evaporation of surface water, and the value of $f(C_i^t)$ is negative.

The solid lines in Fig. 2(A) represent the results of a linear regression performed over the linear portion of the data. These regressions were performed using Excel (Macintosh version 4.0, Microsoft Corporation, Redmond, WA). At low water concentrations (high elapsed times), the rate of water loss (and, consequently, slope of the best fit) progressively decreased. These departures from linearity begin to occur when $f(C_i^t)$ rises to value of about 0.013. This corresponds to a water concentration (C_i^t) of 50% of full hydration or 0.39 mg/mm^3. From Fig. 1, we see that a halving of the water concentration corresponds to a loss of 80–90% of the embryonic water. On the basis of these results, the fitting of Equation 15 to the experimental data and the computation of k_i was carried out from $C_i^t = C_i^0$ (excluding the region where extraembryonic water was present) to the region $C_i^t = 0.5 \cdot C_i^0$.

The computation of k_i *for each step.* This section of the text considers effects of the various stages in the permeabilization on the curves generated by application of Equation 15 to the experimental data. These results are summarized in Table 1.

Untreated embryos

The earliest weight measurements in each of these data sets displayed the obvious presence of surface water on the embryo/filter assembly. However, after 2.1–6.0 min, this surface water had evaporated and all subsequent data were included in the analysis. The plots of the transfor-

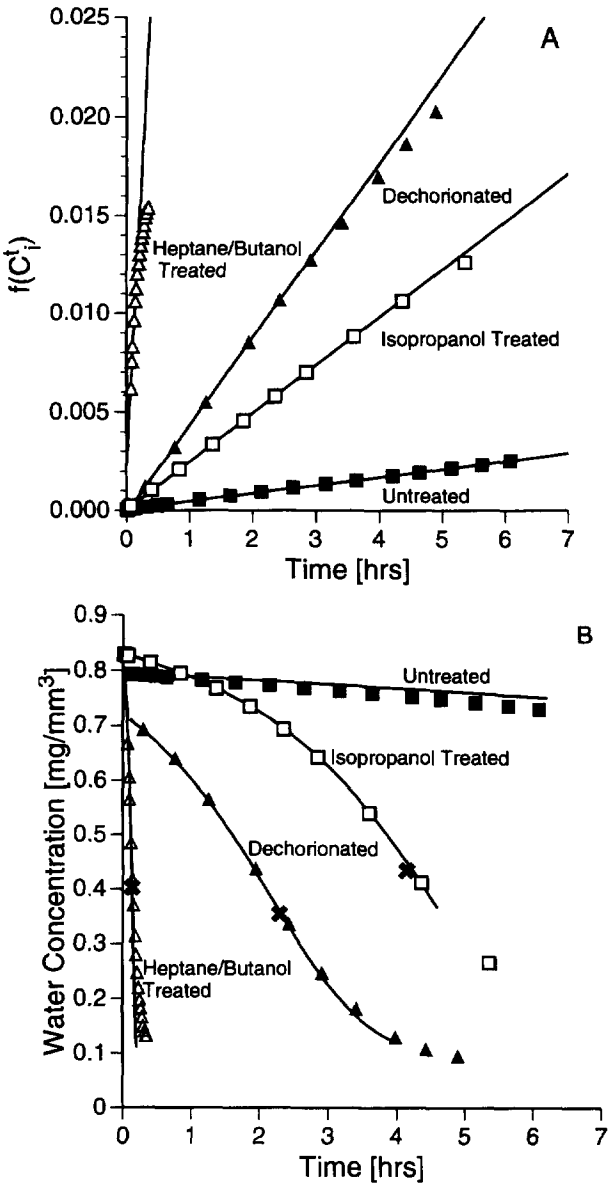

FIGURE 2. The data points in (A) are the experimental values of $f(C_i^t)$ for representative runs, where $f(C_i^t)$ is the right-hand side of Equation 15. The solid lines are the best fit linear regression over intraembryonic water concentrations ranging from C_i^0 to $0.5 \cdot C_i^0$ (which corresponds to $f(C_i^t)=0$ to approx. 0.013). (B) shows the same data sets and fitted curves with the ordinate expressed as the concentration of intraembryonic water. The x's on the fitted curves represent an intraembryonic water concentration equal to $0.5 \cdot C_i^0$.

med data vs time consisted of 12–17 observations in each run and were very linear over the entire experimental data set. A typical set of the transformed data is shown in Fig. 2(A). It should be noted that these runs lasted 5–6 h and did not extend out to the nonlinear region resulting from low water concentrations. The correlation values (r^2) ranged from 0.984 to 1.000. The slopes of the regressions (k_i) ranged from 4.06×10^{-4} to 5.35×10^{-4} with a mean of 4.87×10^{-4} mm³/embryo·h.

Dechorionated embryos

Dechorionation removes the surface irregularities of the *Drosophila* embryos and only minimal surface water is retained. The surface water for these, and the embryos following subsequent treatments, evaporated before the first weighing. Data on dechorionated embryos were collected over a range of intraembryonic water concentrations ranging from fully hydrated to just over 13% of full hydration. As shown in the representative plot Fig. 2(A), there was some deviation from linearity at the higher elapsed times. Following the elimination of points in the low water concentration region of the data ($f(C_i^t)>0.013$), 4–9 points remained in each data set for analysis. The $f(C_i^t)$ vs time plots of these remaining data were linear over this entire data set, with the values of r^2 ranging from 0.981 to 1.000. In all but one of the runs, the values were over 0.995. The slopes of the regressions (k_i) ranged from 1.71×10^{-3} to 5.31×10^{-3} with a mean of 3.69×10^{-3} mm³/embryo·h (Table 1).

Isopropanol-treated embryos

Concentration data gathered in these experiments ranged from fully hydrated to just approx. 7% of fully hydrated. However, only the data for water concentrations greater than 50% of C_i^0 (2.7–4.5 h elapsed drying time, depending on the run) were evaluated. With this restriction, 10–12 observations remained in each data set. As shown in the typical data set in Fig. 2(A), the transformed data was linear over the remaining observations, with values of r^2 for the transformed data vs time ranging from 0.998 to 1.000. The slopes of the regressions (k_i) ranged from 2.18×10^{-3} to 3.31×10^{-3} with a mean of 2.80×10^{-3} mm³/embryo·h.

Heptane/butanol-treated embryos

When $f(C_i^t)$ vs time was plotted for the entire heptane/butanol-treated experimental data set, nonlinearities were apparent in many of the data sets. This was primarily due to the high permeability of the vitelline membrane surrounding the embryo. The water concentrations quickly reached 32.0 to 19.5% of the initial water concentration. However, when the analysis was restricted to minimum water concentrations $\geq$50% of the estimated C_i^0, the transformed data were linear, with r^2 ranging from 0.985 to 0.995. The slopes of the regressions (k_i) ranged from 2.61×10^{-2} to 7.55×10^{-2} with a mean of 4.73×10^{-2} mm³/embryo·h.

Table 1 also gives values of k_a (the constant for the movement of water from the air into the embryos) (computed using Equation 13) and the time for the water concentrations to fall to 50% of normal after the several permeabilization steps. These values are analyzed in the DISCUSSION.

Comparison of the experimental data and the fits. To be considered successful, a model must be able to recreate the experimental data in simulation. In this specific case, meeting this criterion of validation required computation of the concentration of water within the embryo (using the evaluated k_i) so as to be able to compare the fit with the experimental data. However, C_i^t can not be completely isolated in Equation 15. Therefore, Equation

TABLE 1. Average kinetic constants describing the rate of water loss from *Drosophila* embryos

Treatment	k_i $\left[\frac{mm^3 \cdot 10^6}{embryo \cdot h}\right]$	k_a $\left[\frac{mm^3}{embryo \cdot h}\right]$	Time to 50% concentration (h)
Untreated	487 ± 20	4.02 ± 0.17	23.59
Dechorionated	3688 ± 303	23.63 ± 2.19	2.56
Isopropanol treated	2800 ± 109	17.44 ± 1.22	3.83
Permeabilized	47,320 ± 9320	258.53 ± 50.92	0.23

Average kinetic constants (±SE) describing water loss from *Drosophila melanogaster* embryos at various stages in the permeabilization process. The time required for the embryos to dry to 50% of their initial water concentration is shown for each of the four treatments.

15 was rearranged into the form: $C_i^t = g(C_i^t)$. In this form, C_i^t can be determined numerically. [Note that $g(C_i^t)$ and $f(C_i^t)$ are different functions]. The range of intraembryonic water concentrations during an experiment is bounded by $C_i^* < C_i^t < \rho_w . C_i^t$ and was calculated using this range and a golden section search (Press *et al.*, 1992) which we implemented in Fortran-77. The search minimized the absolute value of the error function $C_i^t - g(C_i^t)$ for specified times and required the input of values for ρ_w, ρ_s, W_{si}, C_i^0, and C_i^*. A computed C_i^t was considered acceptable when the error function was less than 1×10^{-6}. This usually required 30–35 iterations of the search routine. Typically, the search algorithm was stable for times greater than 0 and for $C_i^t \geq 0.2 \cdot C_i^0$.

Using the data and results from Fig. 2(A), the water concentration was back-calculated for each step of the permeabilization process. Concentrations are presented in Fig. 2(B) for the entire range that the back-calculation algorithm was stable. The × designates $C_i^t = 0.5 \cdot C_i^0$. For $C_i^0 \geq C_i^t \geq \sim 0.4 \cdot C_i^0$, the agreement between the fits and the experimental data is quite good, demonstrating that the model adequately handles the wide range of permeabilities present. As C_i^t becomes less than approx. $0.4 \cdot C_i^0$, the quality of the fit decreases.

Results based on averaged embryo properties. Once fits had been performed on *individual* drying runs and the kinetic constants determined for each run, drying runs were simulated using the *average* values of k_i (from Table 1), the *average* values for C_i^*, C_i^0, and W_{si} [from Table 2 of the companion paper (Schreuders *et al.*, 1996)], and the search algorithm described in the previous section. The results of these simulations are shown in Fig. 3(A–D), along with the data gathered during the drying experiments. The prediction curves are only shown for water concentrations greater than 50% of the initial water concentration.

In general, the agreement between the predicted embryo weights and the experimental data is good. However, as could be expected, there was some variation between the model's predictions using the average embryo properties for the entire data set (which included a number of runs whose kinetics were not followed) (Table 2, Schreuders *et al.*, 1996) and the more limited data sets involving kinetic experiments. For instance, in the simulation of water loss from a dechorionated embryo [Fig. 3(B)], the difference between the experimental data and the fit is the result of differences in the embryo's mean dry weight between the two data sets. For the fully permeabilized embryos, the difference between the predicted embryo weights and the experimentally determined values [Fig. 3(D)] is probably due to a combination of several factors; namely, the lack of an accurate estimate of the initial water content, the inability to remove all of the surface water without removing substantial intraembryonic water, and, possibly, decreases in the embryo temperature due to evaporative cooling.

DISCUSSION

The relationship between the permeability and the permeabilization step

The average values for k_i allow quantitative comparison of the effects of each step in the permeabilization protocol on the embryo's permeability. Furthermore, using the average values for k_i presented in Table 1, curves which predicted the embryo's weight as a function of time were generated. (k_a will be discussed shortly. It was not necessary for these predictive calculations, having been eliminated mathematically using Equation 13). Figure 4 shows the resulting curves for the kinetics of weight loss in an untreated embryo and an embryo following each of the three steps of the permeabilization protocol. The curves were computed over the entire range for which the model was numerically stable. A second method for comparing the predicted rate of water loss is t_{50}. This is the time required for a 50% reduction in an embryo's initial water concentration and was calculated using the mean values for k_i and the mean embryo weights from the kinetic experiments. Note that a reduction to 50% water concentration corresponds to the loss of 80–90% of the intraembryonic water.

The effects of the treatments on k_i. In the first step of the permeabilization protocol, the chorion was chemically removed from around the embryo, exposing the vitelline membrane. This dechorionation brought about a

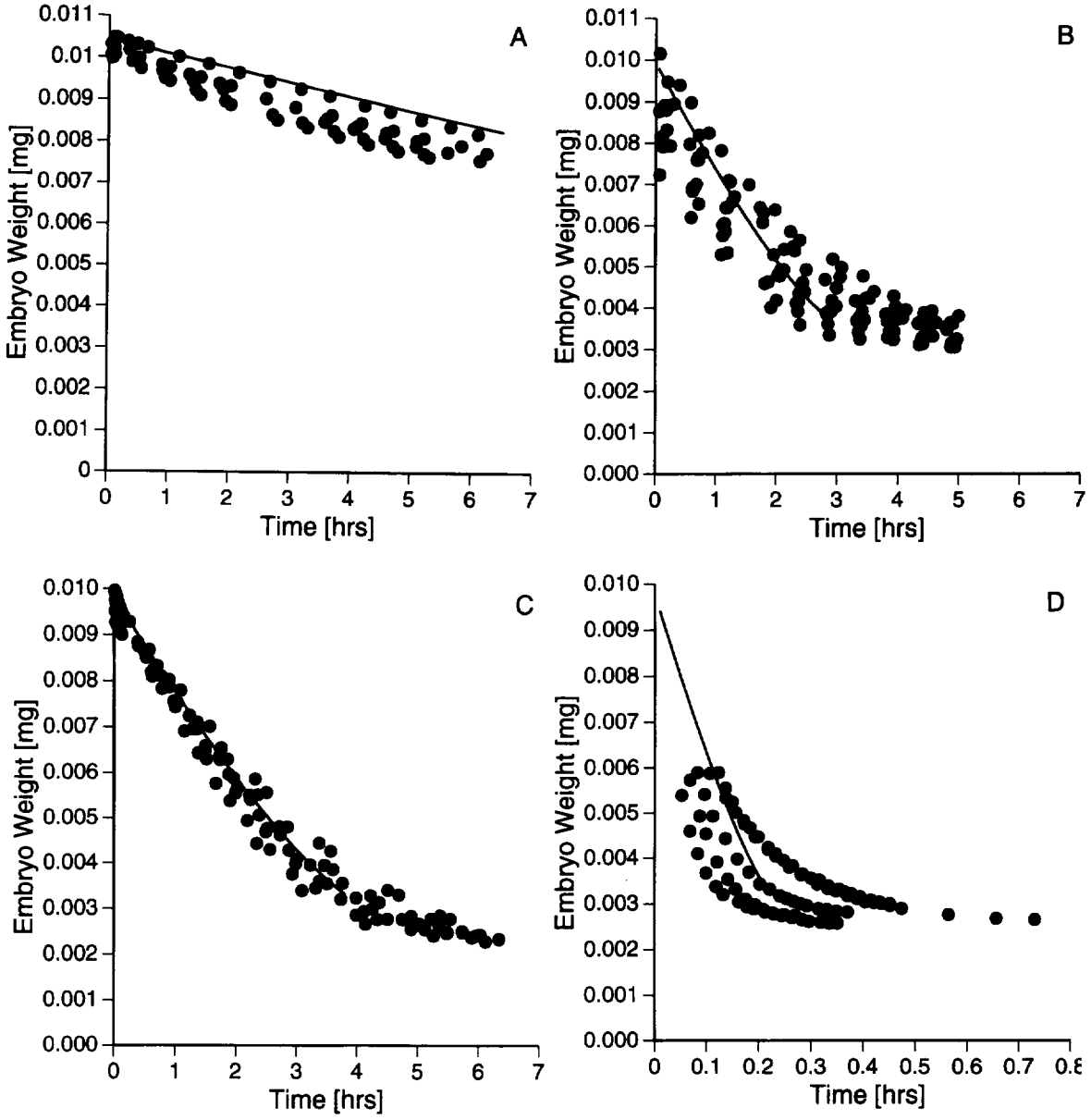

FIGURE 3. A comparison between experimental weights during air drying of (A) intact, (B) dechorionated, (C) isopropanol treated, and (D) heptane/butanol treated embryos (shown as (·)) and the weights predicted by the model (shown as solid lines). The computations by the model used the average kinetic constants for each treatment and the average values for C_i^*, C_i^0, and W_{si} [from Table 2 of the companion paper (Schreuders *et al.*, 1996)].

7.6-fold increase in the mean value of k_i (from 4.87×10^{-4} to 3.69×10^{-3} mm^3/embryo·h), when compared with the intact embryos. The computed effect of this greatly increased k_i on embryo drying rates is shown in Fig. 4. The corresponding change in t_{50} was almost an order of magnitude reduction in elapsed time, i.e. t_{50} for the dechorionated embryos was 2.6 h, while that of the untreated embryos was 23.6 h (Table 1).

The next step in the protocol, the isopropanol rinse, was not performed to remove material from the embryo, but rather to act as an intermediary between the aqueous solutions used in dechorionation and the alkane solutions used in permeabilizing the vitelline membrane. The rinse, in fact, decreases the embryo's permeability by a small (but significant) amount. This is shown as a decrease in the kinetic constant k_i from 3.69×10^{-3} (dechorionated embryos) to 2.80×10^{-3} mm^3/embryo·h (following the rinse), a factor of 0.76. The effect of this decrease in k_i on the computed drying kinetics is shown in Fig. 4. It corresponds to an increase in t_{50} from 2.6 to 3.8 h. Possible reasons for the increase are discussed in the companion paper (Schreuders *et al.*, 1996).

The final stage of the permeabilization protocol, treatment of the embryos with heptane/butanol to remove the waxy layer of the vitelline membrane, resulted in a 16.9-fold increase in k_i (from 2.80×10^{-3} mm^3/embryo·h in the isopropanol-treated embryos to 4.73×10^{-2} mm^3/embryo·h). t_{50} decreased to 0.23 h.

In summary, two steps in the permeabilization protocol resulted in increases in the embryo's permeability:

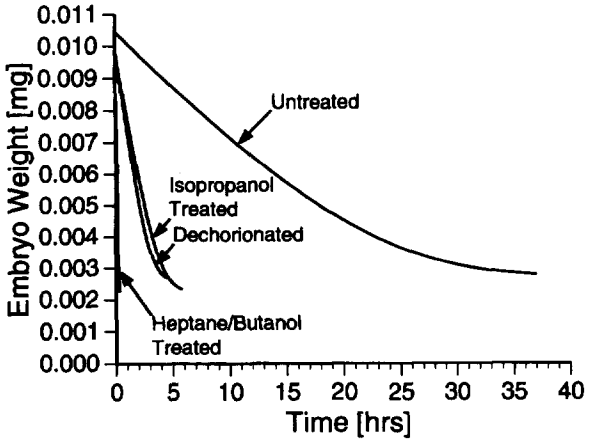

FIGURE 4. Predictions by the model of the comparative effects of the permeabilization steps on the kinetics of water loss during air drying.

dechorionation and the heptane/butanol-treatment. There was a slight decrease in permeability due to the isopropanol-treatment. Overall the embryo's k_i increased almost 100-fold. Similarly, t_{50} decreased by almost 100-fold. The final result was a viable embryo which was permeable to both water and the cryoprotectant ethylene glycol.

Applicability and limitations of the kinetic constants

As has been noted above, the range over which this model is applicable includes embryo water contents ranging from 100–20% weight fraction or below of the original water content. At lower contents, there was increasing deviation between the predictions of the model and the experimental values. Some of this deviation may result from the assumptions used in computing fully hydrated embryo weights (described in the METHODS). Three additional possible sources are: the assumption of a constant value for each k_i or k_a, the assumptions implicit in Equation 13, and inaccuracies in the estimation of the embryo solids' density.

The assumption of constant k's. We have assumed that the values for k_i and k_a are constants. As is evident from the decreases in the slope of the plots of the data sets in Fig. 2(A), this assumption is not valid at low water concentrations. At water concentrations below approx. 50% of fully hydrated, the value of k_i (and, consequently, k_a) decreases. This is probably due to interactions between the solids and the water within the embryo or because the vitelline membrane is no longer as accessible to the water within the embryo. The complications from the changes in these kinetic constants can be avoided by applying the model only within the region of constant k (our choice). The alternative would be to treat k_i as a variable, a choice that would greatly add to the complexity of Equation 14 and its solution, Equation 15.

The assumptions implicit in Equation 12 and Equation 13. In Equation 6, the net flux is described by two kinetic "constants", k_i and k_a. In Equation 15, which is the model used to calculate k_i and compare predicted vs experimental drying kinetics, k_a does not appear. It is eliminated by Equations 12 and 13. Equation 12 is based on the fact that, when an embryo is in steady state with the water vapor in room air, the fluxes of water into and out-of the embryo are equal. The algebraic elimination of k_a raises the potential problem that, when the intraembryonic water concentration is the steady-state value, the values of the k's are probably not the same as the k's at higher water concentrations. This problem is minimized by the assumption underlying Equation 13 that the two steady-state kinetic "constants" differ from those determined over the linear region of $f(C_i^t)$ vs t by the same ratio. Based on this assumption, Equation 12 becomes Equation 13, allowing the elimination of k_a from the equations. The validity of this assumption is supported by the fact that $f(C_i^t)$ vs t remains linear through the loss of 80–90% of the intraembryonic water.

The effect of the embryo solids' density on k_i. Another possible source of error in the evaluation of k_i was error in the estimation of the density of the embryo's solids, since the density was bracketed, rather than determined. Fortunately, the model was relatively insensitive to the effects of solids density. For instance, when a typical drying curve for a dechorionated embryo was analyzed using the range of possible solids densities, the respective values for k_i were 1.959×10^{-3} (ρ=1.26 g/cm^3), 1.921×10^{-3} (ρ=1.37 g/cm^3) and 1.889×10^{-3} (ρ=1.48 g/cm^3). Hence, use of the midpoint of the density range (ρ=1.37 g/cm^3) resulted in a maximum possible error in k_i of less than 2%, an indication that the approximation was adequate for this application.

The relationship between k_i and k_a

The drying of embryos in air involves the transport of water across a phase change boundary and transport across this boundary is an asymmetric process. In irreversible thermodynamic models, the asymmetries between the gas phase and the liquid phase are accounted for by modifying the concentration term. The result, the chemical potential, is then used to describe the gradient acting as the driving force. In our model, we have chosen to describe the gradient in terms of differences in the unmodified concentrations. The asymmetries in the transport between the phases are, instead, compensated for by assigning different values to the two k's: k_i is the kinetic constant for the movement of water from the embryo to the air; k_a is the constant for the reverse. The relationship between the constants is determined by the fact that, at a steady state, the fluxes across the phase change interface are equal, and at the steady state, from Equation 12, the ratio of the kinetic constants is reciprocally related to the concentrations of water in the two phases.

One way to test this approach to handling the asymmetry of water transport in embryos is to consider an ideal binary aqueous solution in equilibrium with the water vapor in air. From Raoult's law, the mole fraction of water in such a solution, and its activity, are numerically equal to 1/100th of the relative humidity of the air above the solution; i.e. the mole fraction of water in a

solution in equilibrium with air at 56% r.h. is 0.56. The mole fraction can be converted to the concentration of water at equilibrium if we specify the molecular weight of the solute and know its density. Here, we assume the density to be that of the dried *Drosophila* embryos (1.37 mg/mm^3). For solutes with molecular weights of 100, 250, 500, 750, and 1000, the calculated concentrations of water in the solution after equilibration with air at 56% r.h. is 0.239, 0.112, 0.059, 0.040, and 0.030 mg/mm^3 (or g/cm^3). (The equilibrium concentrations are relatively insensitive to the r.h. in the range of 50–60%. For example, when the solute molecular weight is 500, the values are 0.047, 0.059 and 0.069 mg/mm^3 at r.h. 50, 56, and 60%).

The measured concentrations of water in embryos equilibrated at 56% r.h. (C_i^*) ranged from 0.088 mg/mm^3 for intact embryos to 0.058 mg/mm^3 for heptane/butanol-treated embryos. Thus, the concentrations of water in the embryos after equilibration at 56% r.h. correspond to the equilibrium concentrations of water in an ideal binary aqueous solution in which the solute has a molecular weight of 400–500 Da.

The interior of an embryo is, of course, not a binary solution, but a multicomponent solution. The concentration of water in a multicomponent solution equilibrated at a given r.h. will depend on the number average molecular weight of the components. In other words, the water content of embryos equilibrated at 56% r.h. is consistent with a number average molecular weight of 400–500 Da for the intraembryonic solutes. Those are reasonable numbers considering that the interior of the embryo consists of a large number of low molecular weight ions, a moderate number of moderate molecular weight amino acids, fatty acids, and nucleotides, and a small number of high molecular weight proteins, nucleic acids, and lipids. The agreement between the water concentration in an equilibrated binary solution and that in the equilibrated embryo is also reasonable when one considers that the binary solution is assumed to behave ideally in accordance with Raoult's law, whereas the intraembryonic solution certainly departs substantially from ideality at low water concentrations.

We noted in Equation 12 that the ratio of the kinetic constants k_a/k_i is equal to the ratio of the concentration of water in the solution at steady state (C_i^*) to the concentration of water in air at the given r.h. (C_a). The latter has a value of 1.06×10^{-5} mg/mm^3 at 22°C and 56% r.h. For the embryos, the ratios of k_a to k_i ranged from 8270 for untreated embryos to 5450 for heptane/butanol-treated embryos (see Table 1). If we apply Equation 12 to ideal binary solutions with solute molecular weights of 250 or 500 Da, the ratios are in the same range, i.e. 10,500 and 5600, respectively.

In conclusion, the kinetics of the efflux of water from *Drosophila* embryos during exposure to air seems quantitatively consistent with viewing them as packets of a solution of 400–500 MW solutes in water bounded by kinetic barriers, the effectiveness of which changes as the eggshells are subjected to the several steps involved in permeabilization.

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