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The kinetics of autolysis in osmotically stressed sea urchin eggs

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Abstract

We have measured the kinetics of death from hyperosmotic stress in unfertilized sea urchin eggs in an attempt to dissect the two conflicting influences of temperature lowering below the freezing point: as more ice forms during temperature lowering, the rate of injury increases rather than decreases. Two models are examined. An Arrhenius model, in which the osmotic stress imposed by extracellular ice formation is envisaged as reducing the "activation energy", provides a qualitative model but fails to fit the data in detail. In contrast, a Johnson–Mehl–Avrami nucleation model, in which the osmotic stress triggers an autolysis that is essentially independent of temperature or degree of osmotic stress above a threshold value, fits the data well. Two separate decay processes were seen, for which the time constants were 269s and 658 s and the Avrami exponents were 7 and 1 respectively. The latter is an ordinary first-order attrition, though its independence from temperature distinguishes it from an Arrhenius model. The nucleation model is difficult to furnish with a simple physical representation.

INTRODUCTION

It has long been known [1] that perishable materials may be preserved longer at low temperatures and that if the temperature is low enough, the term of preservation is indefinite. This science of refrigeration or cryogenics concerned with the response of living material to the frozen state has been termed cryobiology [2]. While cryobiologists have enjoyed some marked successes at low temperature preservation, they have been acutely aware that there exists an intermediate range of temperatures, from just below freezing to perhaps −80°C, which causes an accelerated rate of injury, a range through which the cryopreserved tissues must pass twice,

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Dedicated to Professor Joseph H. Flynn in honour of his 70th birthday.

during freezing and during thawing [3]. This accelerated rate has generally been considered a consequence of the formation of ice within the tissue and its effect upon osmotic or electrolytic stress if the ice is extracellular, and upon cell architecture if the ice is intracellular [4]. Lower subfreezing temperatures produce more rapid and severe damage; yet at very low temperatures the rates of virtually all chemical processes become so slow that cryopreservation is possible. Thus, lowering the temperature below the freezing point initiates two contradictory influences on the stability of the material to be cryopreserved. Most cryobiological successes have been achieved by a careful and empirical balancing of these opposing tendencies by control of cooling and warming rates and have required, in addition, the presence of molar or greater concentrations of a cryoprotectant, a chemical additive which acts to limit the amount of ice formed and the rate at which ice propagates.

In this paper, we attempt to dissect and analyze separately the two contrasting effects of temperature using unfertilized sea urchin eggs as a cell type. As a biological model, these offer a number of advantages. They are well studied and a great deal is known about their response to various stimuli and stresses. Most importantly for our work, they are large and easily visible cells which remain spherical as they shrink under the influence of hyperosmotic solutions, simplifying the interpretation of the stress-strain relationships. In sea water or in isotonic solutions and at temperatures to which they are adapted, they persist for several days without sign of change, but eventually die. They are naturally resistant to considerable freezing stress, or its equivalent in hyperosmotic stress, in the absence of artificial cryoprotectants. When they eventually succumb to excessive freezing or hyperosmotic stress, they exhibit a convenient "end point": the cells rapidly darken and then disintegrate, a process termed "black cytolysis" [5]. The relationship between freezing, osmotic stress and injury in sea urchin eggs has been examined in earlier papers [6,7], and an example of the complexity of the contrasting effects of temperature below freezing on specimen stability is shown in Fig. 1.

The first model we tested is the simplest we could contrive which takes into account both the decelerating and accelerating action of temperatures below freezing point. For the isopiestic effects of temperature, we have chosen the Arrhenius expression. For the effects attributable to freezing but independent of temperature, we have chosen osmotic stress as the active agent, postulating that it supplies an "activation energy". An analogous approach has been used to model failure of structural polymers [8].

An array of experiments varied in the details of their design and execution, but all consisted of a number of measurements of the time interval t between the application of an osmotic stress to the number of cells N_0 at a series of temperatures T above freezing and the number of cells N remaining after those intervals. The decrease in cell number with

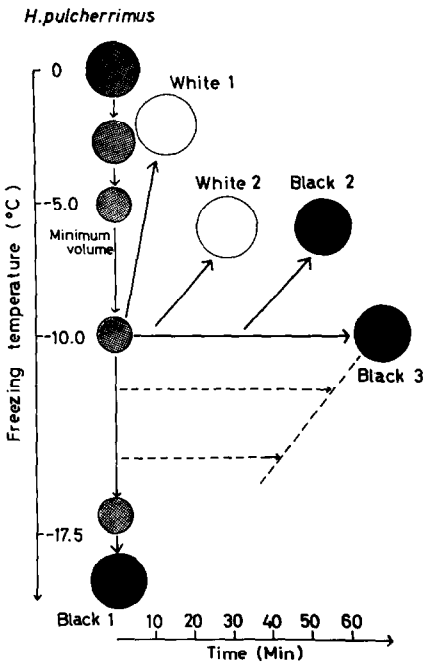

Fig. 1. The complex events of osmotic stress cytolysis in unfertilized sea urchin eggs exposed to freezing. Temperature lowering below the freezing point, because it increases the degree of osmotic stress, paradoxically reduces the length of time which the eggs resist stress. From ref. 5, with permission.

time was assumed to proceed at a first-order rate: $F = -\mathrm{d}N/N\ \mathrm{d}t$. The Arrhenius equation

$$\ln F = \ln A - \Delta H^*/kT \tag{1}$$

in which A is a constant entropy term, ΔH^* the "activation enthalpy" and k is the Boltzmann's constant, was modified for our purposes:

$$\Delta H^* = \Delta H_0 - \Delta H_s \tag{2}$$

where ΔH_0 is the activation enthalpy at zero (isomotic) stress and ΔH_s is the enthalpy stored as a strain within the cell. The magnitude or even the form of the strain is virtually undefinable in cells, but because the osmotic stress cannot exceed the chemical potential of the stressful solution, relative to isotonic

$$\Delta H_s \leqslant kT\ \ln(P/P_0) \tag{3}$$

and the strain would be coupled to the stress by an efficiency m, with a value between 0 and 1. Our complete initial model thus reads

$$\ln F = \ln A - \Delta H_0/kT + m\ \ln(P/P_0) \tag{4}$$

Alternatively, because the decay of individual cells might be autocatalytic, we also examined a model of the Johnson–Mehl–Avrami type [9]

$$\ln N_0/N = (t/\tau)^n \tag{5}$$

in which τ is a time constant in seconds and n is a constant, both to be determined empirically.

EXPERIMENTAL

The experiments reported here were all performed on *Strongyocentrotus purpuratus* obtained on three different dates from Bio-Marine Laboratories (Venice, CA) during the breeding season in February and in April. The water temperatures in their native environment are between 15 and 16°C. Sea water is, for the present purposes, 0.55 M NaCl. Unfertilized eggs were removed using classical KCl injection methods [10] and resuspended in artificial sea water (Instant Ocean: Aquarium Systems, Mentor, OH). For isothermal determinations, batches of eggs were suspended in test solutions made hyperosmotic with additional NaCl and a clock was started. Temperature control, accurate to about 0.1°C, was provided for the sea urchins and their isolated eggs by either mechanical refrigerators or ice baths. The microscope was equipped with a thermoelectrically controlled temperature stage of local manufacture and the same precision. For isopiestic determinations, cells were resuspended at a series of temperatures in each of the test solutions. Two measurement methods were used. In experiments which took long periods to complete, groups of cells were transferred to a microscope slide after specific time intervals, and the surviving cells per 100 cells were counted. In experiments in which a rapid decline in cell numbers was anticipated, smaller numbers of cells were placed under the microscope immediately after resuspension and viewed continuously. The time at which each egg in the field underwent osmotic lysis was noted. In all, 329 batches of eggs were measured, each batch containing between 10 and 200 eggs. Statistical analysis was done using the computer program RS/1, version 3 (BBN, Boston, MA).

RESULTS

Figure 2 shows representative sets of isothermal data on the loss of sea urchin eggs resulting from hyperosmotic stress. Two illustrations are given in order to cover the range of relatively low stress at low temperatures and of high stress at temperatures close to normal for the species. Casual examination of these data indicates that the loss of stressed cells as a function of time appears to approximate a first-order model and that increasing stress increases the rate of loss in an apparently exponential manner. When the goodness-of-fit was measured in these sets of data

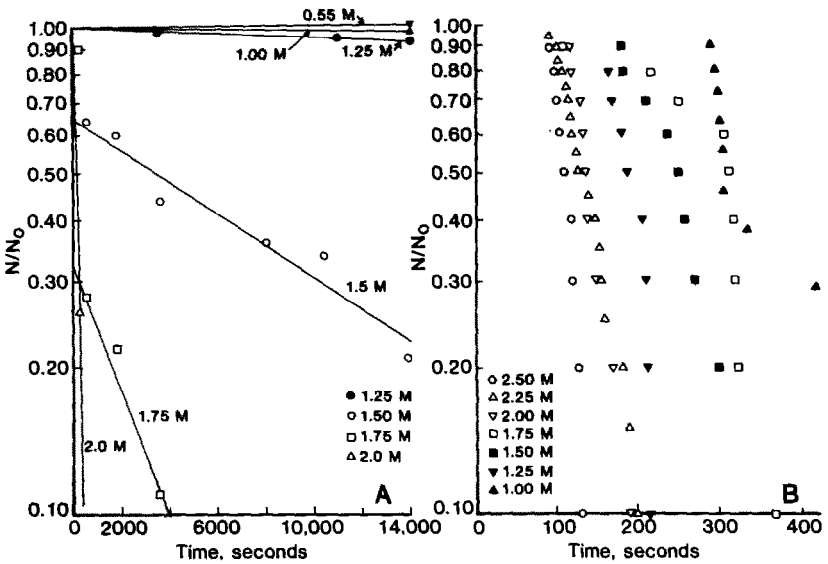

Fig. 2. Representative sets of data illustrating the loss of sea urchin eggs as a function of time under different levels of hyperosmotic stress. A. Exposure at 0°C to different concentrations. Note that while cell loss is semilogarithmic and increasing stress accelerates the process, the intercept at zero time is less than unity at concentrations above 1.5 M. B. Exposure at 15°C. Note that while higher osmotic stress makes decay more rapid, there is a delay at all concentrations before cells begin to decay at approximately the same rate.

individually, r^2 values ranged from 0.992 to 0.416. Only data sets with coefficients of correlation above 0.85, all but perhaps a dozen, were included in the analysis of the first model. Figure 2A shows a long experiment in which the cells surviving per 100 counted were recorded. A plot of estimated half-times for survival for these data at 0, 10, and 20°C showed good linearity and regressed on a zero stress (0.55 M) half-time of about 4 days. However, note that at hyperosmotic concentrations above 1.25 M, cell survival does not regress on 100% at zero time.

Figure 2B, a representative set of data from highly stressed cells whose time until lysis was brief enough to be observed directly under the microscope, shows other unexpected patterns. First, there is a delay of several minutes before black cytolysis begins and this appears to be somewhat affected by the degree of osmotic stress. Second, when cell loss does begin, it is quite linear but the rate of loss is not greatly affected by the degree of osmotic stress.

Estimates of the activation enthalpy ΔH^* when made from half-times for cell decay, show a regular decline from about 130 kJ at 1.5 M to under 10 kJ at 2.5 M, indicating predictability in the determination of m. However, when ΔH^* was determined from the slope of ln F as a function of $1/T$ for all sets of data in accordance with the first (Arrhenius) model,

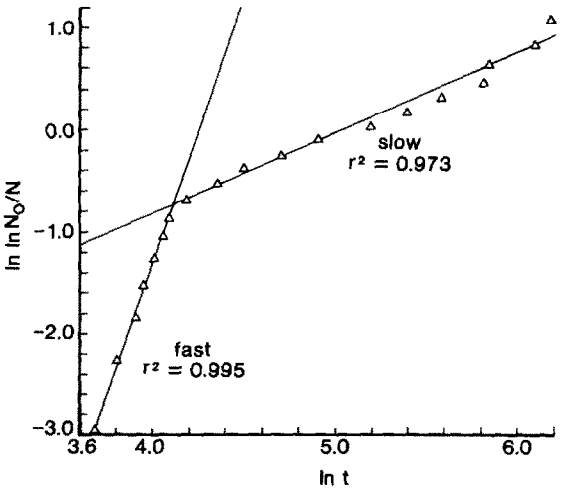

Fig. 3. A representative set of data analyzed according to the Johnson–Mehl–Avrami model. Note that there is an excellent fit to two slopes, representing a fast process and a slow process. See text for description.

no such pattern appeared; the coefficients of correlation were too low (0.2–0.7) to have significance and thus to allow any interpretation. Estimates of m by both methods ranged from 2 to over 12, but in no case were they significant ($0.10 < r^2 < 0.62$).

Because individual sets of data appeared to show such good linearity, we examined a second model of the Johnson–Mehl–Avrami type in which cell collapse begins from a nucleus. A representative set of data is given in Fig. 3. Note that when $\ln(\ln N_0/N)$ is plotted as a function of $\ln t$, the data set breaks into two straight sections with a fast and a slow decay rate. When the fast and slow data sets were analyzed separately, coefficients of correlation ranged from 0.790 to 0.997; of the 43 sets analyzed, only four had r^2 values below 0.900. There were, nevertheless, no consistent trends in either the isopiestic or isothermal values of either τ or n, allowing us to pool these data. The time constant τ for the slow process was 657 ± 182 s, and for the fast process, 267 ± 68 s (mean and standard error, number = 15). The respective values for the exponent n were 1.00 ± 0.17 (number = 14) and 7.32 ± 2.50 (number = 15). There were, in addition, 16 sets in which only the fast process was seen before the sample under observation ran out of living cells.

DISCUSSION

Bělehrádek [11] was perhaps the first to observe that, despite its great intuitive appeal and its considerable utility in chemistry, the Arrhenius expression is inappropriate as a generality for describing biological pro-

cesses. His advice has been routinely ignored. The expression does, of course, give reasonable predictions in certain specific instances and over sufficiently small temperature ranges, but biological processes are too complex and interrelated, and biological clocks too well temperature-compensated to permit such a simple model of a more extensive scheme. In our data, in Fig. 2, an Arrhenius model, modified to take osmotic stress into account, does predict the tendencies but fails careful analysis of what are manifestly precise data. There is evidence of competing processes: under conditions favoring slow kinetics (Fig. 2A) there is a considerable cell loss before the semilogarithmic process begins; under conditions of high stress (Fig. 2B) there is a considerable lag before the cells begin to decay at about the same rate, irrespective of the level of stress.

The decrease in the values of ΔH^* at higher osmotic stresses was predicted by the model. However, the values of m in excess of unity were not: figuratively, osmotic stress acts as a trigger but the cell supplies most of the energy to destroy itself autocatalytically. Beyond a threshold value, it does not matter how hard the trigger is pulled. In retrospect, this should have been predictable, because an unfertilized sea urchin egg is a carefully designed metastable state looking for appropriate circumstances to decay, and black cytolysis in some of the many cases that we observed was almost a parody of the fertilization process.

While we have demolished the applicability of our first model, at least to unfertilized sea urchin eggs, we have no similarly appealing model to substitute in its place. A mathematical, as opposed to a physical, model which appears to fit our data well is the Johnson–Mehl–Avrami [9] formalism of nucleation kinetics for spherical crystals

$$Z = 1 - \exp\left[-4/3\pi \int_0^t I(\tau)\mu(\tau)^3 (t-\tau)^3 \, \mathrm{d}\tau\right]$$

$$= 1 - \exp(-(\pi/3) I \mu^3 t^4) \qquad (n = 4) \tag{6}$$

where $I(\tau)$ is the constant nucleation rate and $\mu(\tau)$ is the constant growth rate. Thus a mean value of n of about seven is problematical, because the typical value is 2–4. An assumption of the formulation is that nucleus growth is constant in its linear dimension: $\mathrm{d}r/\mathrm{d}t = \mu$. Thus the mass converted is instantaneously dependent upon t^3 and when integrated over the lifetime of the population of nuclei, dependent upon t^4. To achieve a dependency of t^7, one must integrate a function of t^6 by setting $\mathrm{d}r/\mathrm{d}t = \mu t$. Then the equation for mass or volume conversion is proportional to r^3 and its integral is a function of t^6.

In our search for a chemical mechanism corresponding to this model, we consider the general growth mechanism of a crystal of mass x (the constant has been normalized to unity for simplicity)

$$\mathrm{d}x/\mathrm{d}t = x^m \tag{7}$$

In ordinary crystal growth, $x = t^3$ and $dx/dt = x^{2/3}$; in other words, the growth of the spherical crystal in the melt is proportional to its surface area, a reasonable assumption. However, if we assume that $r = t^2$, then $dx/dt = x^{5/6}$. If the nucleus grows two-dimensionally, confined to a surface, then $r = t^3$ and x is still equal to t^6. When growth is dependent uniformly upon the whole crystal mass or volume, then the solution approaches an exponential growth given by $dx/dt = x^1$, which for diffusion to a spherical surface makes no sense.

However, for the collapse of a well-crafted spherical or disk-like structure, this is a reasonable finding. The rate of collapse increases as the volume of damaged material increases, but not exactly in proportion if $m < 1$. It is as if one has an expanding phase of structural breakdown which passes through a region with the highest probability of failure in the newly recruited elements at the interface, but with some additional probability of failure distributed through the entire volume, a probability which decreases as the interface moves away. More formally, if the probability density of collapse is distributed as $1/r$, then the integral over the volume of the structure is $4\pi \int r\, dr$, or the probability (and thus the rate) is simply proportional to the area. If the probability density is uniformly distributed, we have

$$4\Pi \int r^2\, dr = (4\pi/3)r^3 \tag{8}$$

so that the rate is simply proportional to the mass of damaged material. To obtain an $x^{5/6}$ dependency we must postulate an $r^{-1/2}$ probability density function, or

$$4\Pi \int r^{3/2}\, dr = (8\pi/5)r^{5/2} \tag{9}$$

Thus the integrand is $r^{3/2}$ instead of r^2 (exponential, bulk growth rate dependency) or r (interface-dependent growth). On a logarithmic scale, our measured dependency falls neatly half-way between a bulk process and an interfacial process.

This leaves unresolved the sudden change from fast to slow kinetics in most data sets. The slow process seems to begin at the end of the fast process, because there is so little interference. Consider at a time $t \ll \tau_1$, τ_2:

$$N/N_0 = \exp -(t/\tau_1)^7 \approx 1 - (t/\tau_1)^7 \tag{10}$$

and

$$N/N_0 = \exp -(t/\tau_2) \approx 1 - (t/\tau_2) \tag{11}$$

Because $\tau_2 \approx 3\tau_1$, then $N/N_0 \approx 1 - (t/\tau_1)^7$ and $N/N_0 \approx 1 - (t/3\tau_1)$. These become equal at $t \approx 0.85\tau_1$, or roughly 200 s. At shorter times the contribu-

tion of the fast process rapidly becomes negligible. Likewise, at $t > 0.85\tau_1$, the fast process increases much more quickly than the slow process. Thus, if the slow process were operating at short times it would be the only process we could measure and if the fast process did not cease abruptly, the slow process would never be manifest.

Perhaps, in a certain fraction of the cells, the osmotic stress causes the cytoskeleton and adjoining membrane to form lesions that reach a critical size and then collapse like a house of cards. This would represent the fast process. In the remaining fraction, the lesion would not reach critical size until stochastic forces uniformly distributed in time contributed to further breakdown. The result would be a Poisson process with a lag independent of temperature or the magnitude of the stress.

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