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# THE HIGH-ORDER KINETICS OF CYTOLYSIS IN STRESSED RED CELLS

*R. J. Williams, A. G. Hirsh, H. T. Meryman and T. A. Takahashi*[1]

AMERICAN RED CROSS HOLLAND LABORATORY, 15601 CRABBS BRANCH WAY, ROCKVILLE, MD 20855 USA
[1]JAPANESE RED CROSS, SAPPORO, JAPAN

The rate of cytolysis in osmotically stressed unfertilized sea urchin eggs was analysed using a version of the Johnson-Mehl-Avrami equation, and fit with high precision ($r^2 > 0.90$) if the data sets were divided into two sectors. The slow process was first-order but the Avrami coefficient, *n*, for the initial fast reaction was 7. Suspecting that this might be a peculiarity of cells which are primed for climactic behaviour, we examined the process in red cells, whose decay is known to be of first or lower order at low temperatures if protected from excessive osmotic stress. Human red cells subjected to 'thermal shock', in which osmotically stressed cells are cooled below +12°C, show a pattern almost identical to the stressed sea urchin eggs except that *n* of the rapid process exceeded 10. Based on the geometrical implications of such a high *n*, we believe that this phenomenon reflects a stress failure in the cytoskeleton and has important ramifications in cryopreservation.

**Keywords**: cytolysis, cytoskeleton, erythrocyte, hemolysis, kinetics, osmotic stress

## Introduction

Though cryobiologists have had success in extending the *ex vivo* lifetime of human cells and tissues, from a few hours or days at ambient temperature to indefinite periods at liquid nitrogen temperature, there exists an intermediate range of temperatures below freezing but above the glass transition temperature within which lowering of temperature dramatically increases the rate of cell injury. This is generally attributed to the colligative effects of ice formation on the cells, which undergo an osmotic stress as a consequence of membrane semipermeability that allows water to exit the cells but excludes entry by most solutes. We have examined the rate of cell lysis in hyperosmotically stressed sea urchin eggs using an Arrhenius modes in which the 'activation energy' is a function of the strain. We presented evidence that injury does not follow Arrhenius kinetics over the range of temperatures and osmotic stresses used but that lysis was almost independent of either of these variables. Instead, the data fitted well ($r^2 > 0.90$) a

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*Akadémiai Kiadó, Budapest*

version of the Johnson-Mehl-Avrami equation: $\ln N_o/N = (t/\tau)^n$, where $N/N_o$ = the ratio of cells surviving, $t$ = time, $\tau$ = a time constant and $n$ = the Avrami constant. Because the Avrami constants measured were relatively high, these data also challenged the widely held assumption that the external membrane was the primary site of osmotic and freezing injury [1]. But the unfertilized sea urchin egg is a model of instability, a cell whose function has primed it for climactic behaviour. We decided to extend the experiments to a cell type which is a paradigm of stability, the human red cell.

We studied an unusual form of red cell hemolysis known as 'thermal shock' [2]. When human red cells are exposed to concentrations of salt in excess of 0.8 *M* at temperatures above 12°C and then cooled below this temperature at moderate rates, they undergo rapid lysis. The reverse is not true: cells first cooled and then hyperosmotically stressed show lysis which is indistinguishable from other forms of hyperosmotic lysis [3]. We chose this method because it allowed more rapid aquisition of data than the usual hyperosmotic lysis experiments which require months or years, especially at low temperatures, and because we had studied it extensively [3]. This paper reports our results, comparing them to the kinetics of lysis in frozen red cells protected with sufficient glycerol to reduce osmotic stress to within tolerated limits [4].

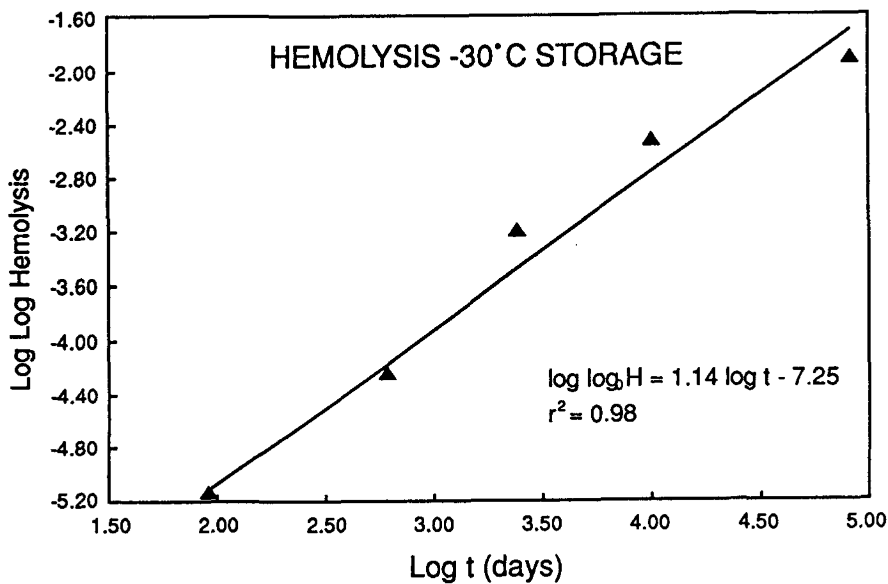
## Experimental

One ml aliquots of human red cells were obtained either from the American Red Cross Blood Services, Chesapeake and Potomac Region, or from volunteers on the laboratory staff. Cells were washed and centrifuged three times in buffered saline media and held in a refrigerator for up to two days before use. Ten μl samples were dispensed into 2 ml of 5.5% (w/w) NaCl solution (1.8 osmolar or 6 times physiological), mixed and allowed to stand for 10 to 90 minutes at room temperature (24°C) depending on the experimental design. From the settled cells at the bottom of the tube 10 μl were placed between 16 mm diameter coverslips in a Linkam THM 600 programmable thermal stage (Linkam, Carshalton Beeches, Surrey, UK) and cooled from 35° to 0°C at 35 deg·min$^{-1}$. The cells were videotaped through a Zeiss (Jena) Interphako microscope, at approximately 3200X magnification to the video screen. The tape was then reviewed and the times at which individual cells lysed was noted.

Tables thus compiled were analysed according to an Avrami model by computer (RS/1, version 3:BBN Software products, Cambridge, MA).

## Results

Figure 1 presents Avrami analyses of cytolysis in whole units of human red cells suspended in isotonic media containing 3.5 *M* glycerol and stored for a year



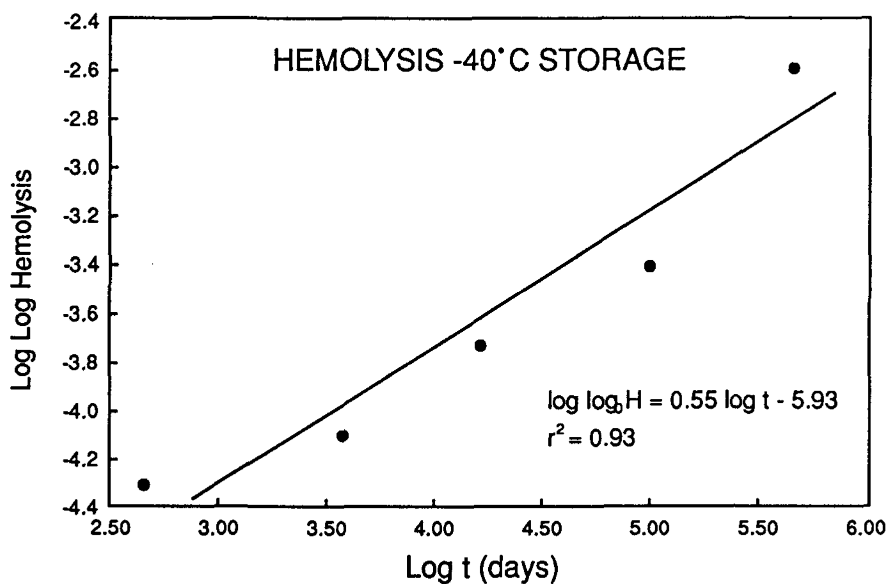
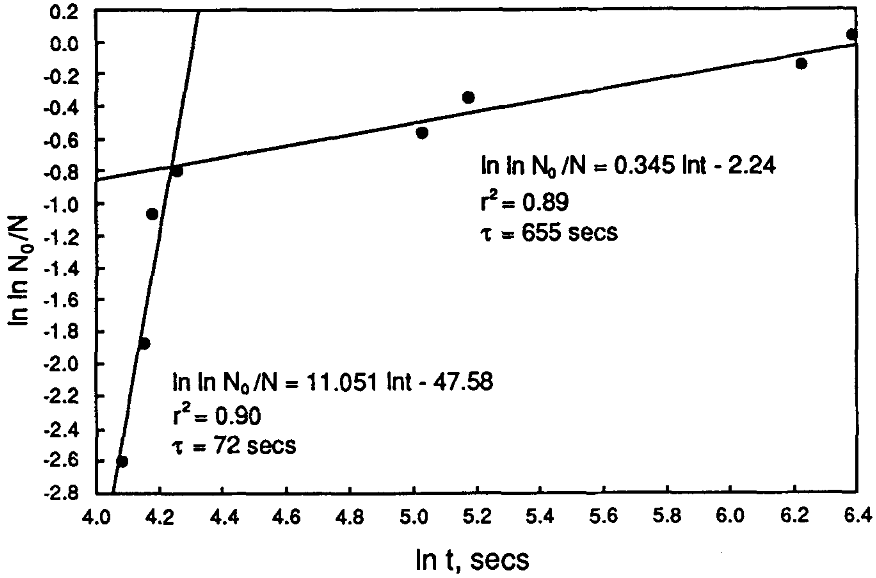




**Fig. 1** Johnson-Mehl-Avrami plots of the hemolysis of human red cells stored in solutions containing 3.5 *M* glycerol and frozen to –30° and to –40°C. The kinetics are first-order or less. However, the great disparity in values of $\tau$ over this 10 degree increment imply that these cells are becoming vitrified



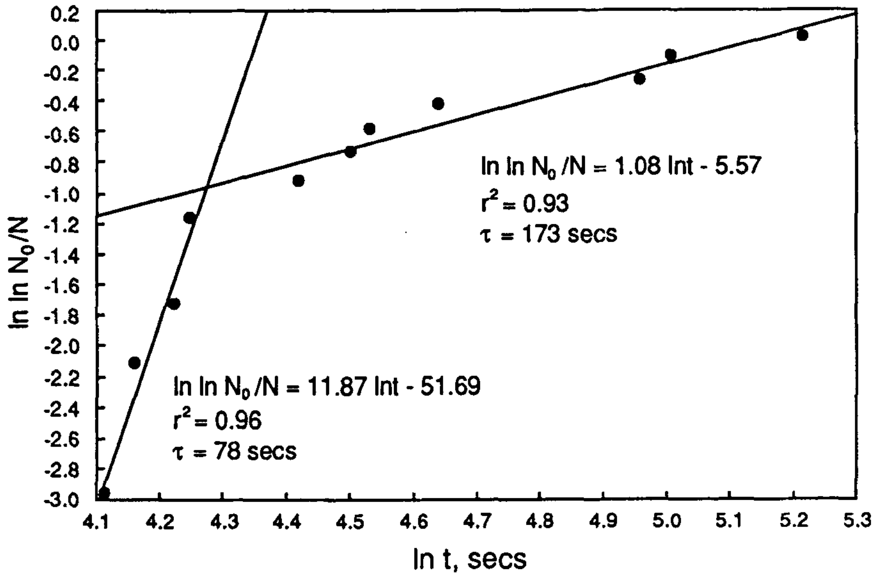




**Fig. 2** Johnson-Mehl-Avrami analysis of human red cells undergoing 'thermal shock' hemolysis after cooling to 0°C. The kinetics are biphasic, with a rapid portion having an Avrami constant, $n$, in excess of 10. This implies that cytolysis is a failure of the cytoskeleton rather than of the membrane alone

or more at sub-freezing temperatures [4]. Though extracellular ice was present in the samples, the glycerol reduced the amount of osmotic stress to within tolerated limits. In both cases, the Avrami coefficient, $n$, is one or less, as expected. Note the large difference in time constants between –30° and –40°C (580 *vs.* 48,000 days). This bespeaks Williams-Landel-Ferry rather than Arrhenius behaviour for the temperature-dependent $\tau$, that the cells are becoming vitreous over this temperature range. This, also, is the expectation based on our calori-metric measurements of these and other cells.

In contrast, the thermally shocked red cells (Fig. 2) show a biphasic lysis. This pattern is essentially the same as that seen in sea urchin eggs except that $n$ is higher, 10 or greater instead of 7, and $\tau$ considerably more rapid, 80 secs instead of 270 secs, for the fast process. The slow process was first-order or lower in both types of cells.

## Discussion

These data indicate that the high-order kinetics of cytolysis in osmotically stressed cells may be a general phenomenon and not restricted to unfertilized eggs, where it would be considered relevant to the physiology of cells whose function is to create an independent existence. Similar kinetics can be seen in red cells undergoing thermal shock hemolysis as well, even though lysis is first-order or less in red cells subjected to non-injurious osmotic stress. These data support our earlier conclusion [1] that the injury is a stress fracture which propagates from points of failure in numerous parts of the cytoskeleton. If the lesion had been a surface (membrane) phenomenon, as has generally been conjectured, the expected Avrami exponent would have been 4.

Details of exactly what structure fails are still not resolved. If the failure had been distributed uniformly throughout the bulk of the cell, the rate would have been doubly exponential in time, i.e., $n \to \infty$. Instead the kinetics show a range of Avrami coefficients from 7 to 10, which on a logarithmic scale is halfway between a surface nucleation and a volume nucleation.

Recent work on plant tissue culture cells has permitted a more accurate resolution of the specific sites of injury. Of the elements of the cytoskeleton, the spectrin component of red cells and the actin filaments of other cell types appear to be the most rigid. Frozen cells show great disruption of the actin filaments [5]. When cytochalasin D was used to partially depolymerize them, it was as effective a cryoprotectant as 5% dimethyl sulphoxide [6]. An analagous effect was observed when rabbit embryos were frozen and thawed in the presence of cytochalasin D [7].

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**Zusammenfassung** — Unter Anwendung einer Variante der Johnson-Mehl-Avrami Gleichung wurde die Cytolysegeschwindigkeit von osmotisch gespannten, unbefruchteten Seeigeleiern untersucht, wobei sich eine hohe Genauigkeit ergab ($r^2$>0.90), wenn die Datensets in zwei Sektoren unterteilt werden. Der langsame Prozeß war erster Ordnung, jedoch betrug der Avrami-Koeffizient n für die anfängliche schnelle Reaktion 7. In der Vermutung, daß dies eine Eigenheit von Zellen ist, die für klimaktisches Verhalten vorbereitet sind, untersuchten wir den Prozeß in roten Zellen, für deren Abnahme bei niedrigen Temperaturen die erste oder eine niedrigere Ordnung bekannt ist, wenn sie von überschüssigem osmotischem Einfluß geschützt sind. Werden menschliche rote Zellen einem "Thermoschock" unterworfen, bei dem osmotisch angespannte Zellen unter +12°C abgekühlt werden, so zeigen sie einen Kurvenverlauf, der mit dem von gespannten Seeigeleiern fast identisch ist, jedoch mit der Ausnahme, daß *n* des schnellen Prozesses über 10 liegt. Ausgehend von den geometrischen Folgerungen eines so hohen *n* Wertes, glauben wir, daß diese Erscheinung einen Spannungsfehler im Zellgerüst widerspiegelt und eine wichtige Bedeutung bei der Kryopräservation besitzt.