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RED CELL THERMODYNAMICS
A materials perspective

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Abstract

A cohort of human red cells of the same age persists in the circulation for about 110 days without access to repair but disappears over the next 10 days. Hyperosmotic stress accelerates the process exponentially. The kinetics are Avrami in all cases we have examined, with $n=2$. We have previously modelled this as a stress failure in a viscoelastic cytoskeleton, but because of the two dimensional long range order in the cytoskeleton, the data can also be interpreted as a state change in a crystalline material.

Keywords: Avrami exponent, erythrocyte, hemolysis, osmotic lysis, osmotic stress, red cells, spectrin

Introduction

The mammalian red cell is perhaps the best studied piece of life. Its protein sequences and how they interact with each other have been evaluated one amino acid at a time, its three-dimensional configurations resolved to 0.18 nm [1]. Still, the hard questions remain unanswered. Why does a "system" with such a high internal energy not rapidly decay into nothing in particular? Why can red cells persist for three months in the circulation, six weeks or more in a plastic bag in the refrigerator, but be destroyed in seconds in concentrated solutions in equilibrium with ice? This essay stems from a thermodynamic examination of the mammalian red cell, as the simplest model available, of why the cryopreservation of living materials has been so difficult. The approach was suggested by the writings of Gordon [2, 3] and Thompson [4]. All statements of fact not specifically referenced herein are available in standard texts; e.g., [5, 6].

Cell structure

The living cell is not only the unit of life but its residence. Evolution has given cell structure an unfathomable complexity of detail, but the basics are straightforward. Like any other modern domicile, the eucaryotic cell consists of two structural parts, an exterior cladding which keeps out the weather, and an internal structural framework which keeps the cladding from collapsing. The external amphiphilic lipid bilayer of a cell is passively permeable only to small, uncharged molecules,

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such as water, alcohols or ammonia. Since it is held together by hydrophobic forces, it has considerable compressive strength if it can be kept from buckling or shearing, but has by itself essentially no tensile strength. The cytoskeleton is a three dimensional array whose principal structural members are made from three proteins, and each is a polymer of polymers. All are highly "conserved" proteins; that is, their structure is largely identical in animals, plants and a group of bacteria whose ancestry has diverged over the last one billion or more years, a conservatism which bespeaks the demanding specifications on the details of their amino acid sequences.

Globular (G-) actin is a protein containing 374–385 amino acids which polymerizes under physiological conditions to filamentous (F-) actin, a double helix approximately 10 nm thick. The other structural protein is tubulin, which polymerizes into a helical tube about 25 nm in diameter. The few measurements available indicate that these are surprisingly stiff, approaching the moduli of green wood, and when the usual scaling rules are applied, would appear to make the cell quite durable. These proteins, often using intermediate proteins, form connections with proteins embedded in and often extending beyond the lipid bilayer membrane. The distance between these membrane proteins is perhaps 20 lipid pairs. The protein combination not only supports the bilayer, but provides pathways for specific larger molecules to traverse it. Additionally, there are proteins, known from their size as intermediate filaments, which form a network and are involved in keeping cell organelles, enzymes, etc. in appropriate locations but may contribute less to mechanical strength overall. The entire supramolecular protein assembly is held together by salt bridges of apposing charges on perhaps three to five pairs of amino acid residues near the ends of the linear polymers: as there are no covalent bonds in it, it is exquisitely responsive to ions in solution.

Red cell structure

Mammalian red cells are unique. Their progenitors begin as normal cells but depart fundamentally from this architecture during maturation, preserving essentially none of it. The maturing cells purge themselves of tubulin and intermediate filaments, the nucleus, and cytoplasmic structure generally. The structure they become cannot strictly be termed a living cell but rather a bag of concentrated protein solution. The envelope surrounding this is still a lipid bilayer, though it has become chemically distinctive. Certain of the lipids, notably phosphatidyl serine, appear to have specific affinities for the cytoskeleton [7], resulting in their asymmetrical distribution into the inner leaflet of the bilayer, but these are not well characterized. The cytoskeleton which is substituted during maturation is a network of triangles which can be treated as a two-dimensional surface. These interlock in six- and five-sided figures to resemble a geodesic dome affixed at its apices to the lipid membrane. More than any typical cell, the structure now has quite a regular and monotonous long-range order. To connect the transmembrane proteins the cells have substituted the linear structural element, spectrin.

Spectrin is a masterpiece designed to serve multifarious functions. Human α-spectrin is a linear array of 2429 amino acids (280 kDa) and β-spectrin of 2137

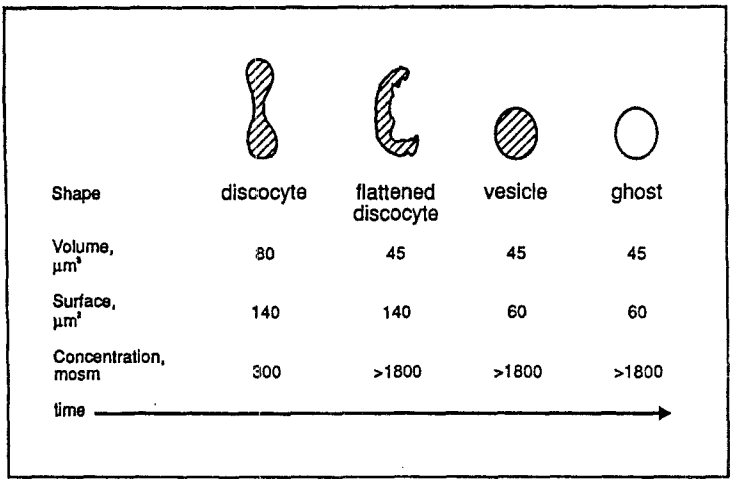

Fig. 1 Osmotic lysis in human red cells. When the isotonic red cell is exposed to 6× hyperosmotic media, the cell loses volume but maintains surface area. Subsequently, the cell loses surface area but maintains its (reduced) volume, becoming a vesicle. Finally, the vesicle loses its semipermeability and soluble hemoglobin leaks out, leaving a "ghost". One atmosphere ~100 kPa ~45 mosm

(246 kDa). Each contains a series of repeating, though not identical, 106 amino acid sequences, 22 in α- and 17 in β-. Each of these sequences is divided into three nearly equal α helices, which are Z-folded against one another, and held in place by precisely situated hydrogen bonds or salt bridges. The N-terminal (#1) residue of the β-chain is attached to the C-terminal end of the α-chain and two such heterodimers, and occasionally 4 or more, lie in antiparallel association. The free ends are attached to F-actin and thence to transmembrane proteins. In addition, on the β-chain between domains 15 and 16, there is a link to another transmembrane protein, ankyrin. The entire spectrin tetramer, stretched out, would be about 200 nm long, but entropic forces and some specific interactions have shortened it to about 70 nm [8]. Because of the weakness of the lipid bilayer in tension (≪0.3 mPa), the cell ruptures like a balloon if swelled to about twice its normal volume, but the length of the spectrin assemblies at the point of lysis would be only minimally increased. In contrast, the structure is resilient in compression (>3 mPa). The complexity goes on, but this will suffice for our arguments: why has this amount of metabolic energy been expended on a durable and sophisticated but lifeless vesicle?

Kinetics of cytolysis

The kinetics of red cell decay have been intensively studied. Of particular concern have been the lethal consequences of genetic variants such as sickle cell hemoglobin and hereditary spherocytosis, both probably fixed as antimalarial, and of exposure to hyperosmotic extracellular media, an unavoidable consequence of cryo-

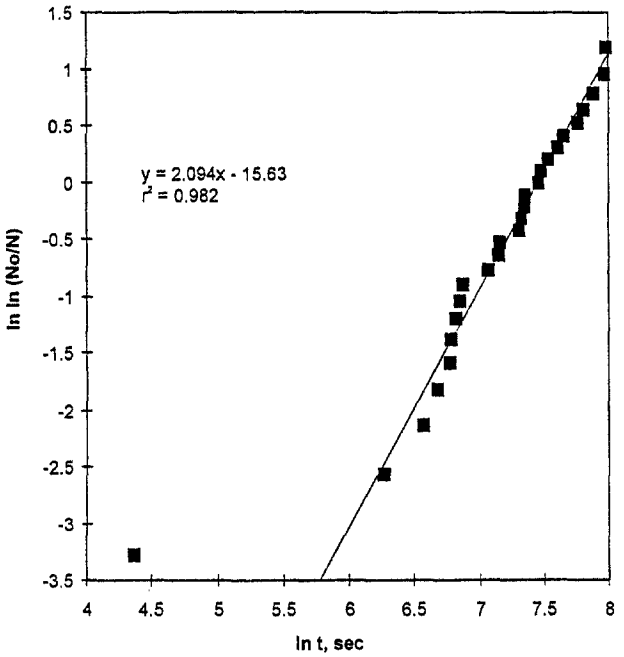

Fig. 2 The time course of lysis in a population of red cells observed in the microscope, at 1800 mosm (4 MPa) at 45°C. There is a lag period before cells begin to vesiculate (cf. Fig. 1), after which Avrami kinetics is well followed

preservation. As part of our efforts to improve the frozen storage of human red cells, we have studied the kinetics of typical cell and red cell collapse under hyperosmotic stress [9, 10]. An example of the latter is given in Fig. 1 and typical kinetics in Fig. 2. (from [10]). They can be modelled with considerable precision ($r^2 > 0.95$) by a Johnson-Mehl-Avrami equation:

$$\ln N_0/N = (t/\tau)^n$$

where N/N_0=the ratio of surviving cells, t=time, τ=the time constant and n=the Avrami exponent, reflecting the dimensionality of the decay process. The time constant, furthermore, can be treated as an Arrhenius function of temperature, in which the osmotic stress supplies part of the activation energy

$$\ln\tau = \ln A + \Delta H_0^*/kT - m \ln P/P_0$$

where A is a "constant", ΔH_0^* an intrinsic activation enthalpy and m a modulus, or efficiency, relating the strain of cytolysis to the osmotic mechanical stress, as a ratio of vapor pressures, P/P_0. A partial set of experimental values for τ, m and ΔH^* are presented in Table 1 (from [10]).

Table 1 Values of τ as a function of temperature and osmotic stress

Temperature/°C	Osmotic stress, mosm					Modulus of coupling, 'efficiency'
	1800	2200	2400	2600	2800	
45	1632±95	1080±11	546±164			0.51
35		2145±992	2898±265			0.45
25	8417	8075±1300	6280±673			0.13
15			58247	4831		
5				23643		
	Time constant/s					
	-38.6	-47.1	-72.1	-91.4		
	Arrhenius activation energy/kJ mol^{-1}					

Values of τ as a function of temperature and cosmic stree. From these values have been estimated m and ΔH^*

Discussion and conclusions

The loss of cell integrity appears to involve an autocatalytic collapse of the two-dimensional cytoskeleton followed by a decay of the lipid bilayer into a spherical vesicle. A cohort of cells of the same age, following this hyperexponential decay pattern, persists until near the end and then disappears rapidly. However, because in vivo survival data have tended to be presented as regressions in an exponential decay model, numerical data have been hard to locate and testing of our alternative model has been difficult. Some early *in vivo* data [11] for rat erythrocytes can be fit to the Avrami model (r^2>0.88; n= 2.0±0.5), suggesting that these kinetics may be general. In every determination, the Avrami exponent has been reassuringly two.

It has been generally conceded that the distinctive shape of the red cell is an "equilibrium" shape and that its resemblance to an inside-out tennis ball is the result of the distribution of surface forces throughout the entire bilayer-protein structure [12]: when red cells are subjected to shear, their motion along a surface is a "tank tread", indicating that the indentations are a property of the whole, isometric cell and have not been molded in place during maturation. Because the evidence indicates that the lipid bilayer is under a small negative interfacial tension, i.e., compression, [13, 14], the interior must be the more compressed: when subjected to severe osmotic disruption, red cell vesicular "ghosts" reassemble as inside-out spherical vesicles [15]. Additionally, the internal osmolality in isotonic media appears to be lower than the extracellular milieu by as much as 45 kPa [16]. It is apparent that a red cell exists in what Prigogine would define as a metastable state very far from equilibrium. This, of course, is a universality of living matter.

When a reticulocyte is propelled into the circulation to become a red cell, it divests itself of the last traces of its former structure and becomes essentially cut off from the resources available to typical cells during the 110 to 120 days that it circulates. It can metabolize glucose to form adenosine triphosphate and certain other products, but it cannot synthesize the enzymes required for this. In typical cells, enzymes may be disassembled and resynthesized in hours or less, an aspect of adaption to the cell milieu; in red cells, the enzymes persist for four months without access to recruitment and their activity diminishes only toward the end. (There may be some chaperonins present to renature their fenderbenders and restore their native conformations.)

The red cell's existence is one of nearly unremitting compressive stress. It spends about 70% of its time folded, forced through capillary vessels which are half of its diameter. Estimates based on blood volume and flow indicate that a cell endures 1.6×10^5 cycles before it is eliminated from the circulation. This elimination has been variously proposed to result from a diminution of its elasticity to the point that it no longer passes through the selective filtration of the reticuloendothelial system, to the loss of binding and the randomization of phosphotidyl serine to the outer leaflet of the membrane which flags the cell for destruction, or, simply, to "wearing out" . No mechanism is established.

Attempts have been made to examine the structural defects of red cells. The ankyrin-spectrin bond has been investigated by binding studies, producing a value

for $k_D = 10^{-7}$ [17]. This implies that at equilibrium as many as two per cent of these bonds may be open at any time [18]. These estimates appear unreasonably high as they do not take other constraints into account. The ankyrin-spectrin association occurs in pairs of pairs, there are multiple salt bridges involved and the interactions with the lipid bilayer along the length of the spectrin and transmembrane proteins [18] cannot be ignored. In Monte Carlo simulations using a 5% figure, these defects coalesce and predict disaster in time periods much shorter than those observed [19].

While it is difficult to draw conclusions from these facts, they impose constraints regarding what red cells can endure without injury. The first is their relative susceptibility to tensile stress. Prof. Gordon has offered the analogous but opposite example for trees [3]. He asserted that because wood is strong in tension but not in compression, trees have compensated by bringing their tissues under considerable tension, so that when the wind blows, the wood on the leeward side is exposed not to compression but to reduced tension. Appropriately, red cells, which are exposed to compression, have incorporated a compression into their structure so that stress will not bring them under tension.

The other secret of red cell endurance would appear to be its utter isometry. Bonds can and probably do open in the ankyrin-spectrin bond, etc., but their specificity is so great that the choices available for reforming the bond are thermodynamically indistinguishable. Thus, there is no way for the red cell to release free energy until the structure becomes asymmetrical, as may occur in a fold with small radius and a tensile stress. We suggest that in red cells, as in other materials, compressive stress failure is the consequence of a tensile stress failure locally [2]. The propagation of the defects formed would allow a loss of free energy from the structure and terminate when the cell became a lipid vesicle filled with the wreckage of the cytoskeleton.

Thus, cells will have short life spans when their network of metastable states is compromised. In older red cells, elasticity is reduced (and the internal compression probably relaxed) so that they come under more tension during circulation. In hereditary spherocytosis, with its diminished spectrin content, the internal compression is probably reduced or absent and cells will rupture in the capillaries like balloons squeezed in the middle. In sickle cells, crystallization of the hemoglobin imposes rigidity and sharp corners. Experimental manipulation provides another important insight. Lysis, once begun, continues after the osmotic stress has been removed (unpublished). In experiments in which red cells which have been partially aspirated into micropipettes the protrusions on the cell surface return to normal, but if the suction is left for some minutes, the protrusion formed on the cell persists, and the cells do not recover [20], implying some form of dislocation or viscoelastic flow, and that some form of nucleation is involved.

We originally speculated that the injury could be treated as a stress failure in a viscoelastic composite [10] and that the Avrami kinetics from metallurgy worked because of a similarity in the mathematical constructs [21]. It now appears that the collapse of the red cell may be better represented as a state change in a crystalline cytoskeletal structure which proceeds spontaneously when nuclei become critical and that the Avrami kinetics analysis is appropriate. The Avrami dimensionality ex-

ponent, n, in such a circumstance, would be two. But the hard questions remain: why do cells with more typical architecture appear to follow the same rules? In the one typical cell we have examined [9] Avrami kinetics were obeyed, but $n>5$.

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