# ROLE OF PLASMA MEMBRANE IN THE THERMAL HEMOLYSIS OF MAMMALIAN ERYTHROCYTES

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High temperature is the main physical factor causing damage of tissues, or burns, due to thermal injury and electric shock [1]. At cellular level these burns cause necrosis and, in rare cases, apoptosis. On the other hand, hyperthermia is used as a method for healing tumors. However, the primary targets whose destruction by heat leads to cellular death are not well understood.

Temperatures between 45 and 58$^{o}$C cause thermal hemolysis of erythrocytes, i.e., out leakage of hemoglobin. Thermal hemolysis exhibits all the features peculiar to thermal necrosis of cells. Another type of hemolysis occurs at other non-physiological conditions, the so called eryptosis [2], which demonstrates the main requisites of apoptosis. In this regard, thermal hemolysis of human and mammal erythrocytes provides useful cellular model with biological and medical importance.

The aim of this review is to present the main problems in our understanding of thermal hemolysis of enucleated erythrocytes from human and mammals.

Erythrocyte membrane (EM) consists of lipid bilayer with intercalated integral proteins, and under-membrane network of peripheral proteins, mainly spectrin. Human EM contains three major proteins, spectrin (25-30 weight %), glycophorin (about 25 weight %), and the dimers and olygomers of the anion exchanger (about 25 weight %).

Barrier function of the plasma membranes is vital property and the extent of its deterioration under adverse conditions reflects the sensitivity of cells to physical injury. At high temperatures cell membranes undergo permeabilization [3,4] whose midpoint temperature, $T_g$, is frequently used as a measure for the thermal stability of cell membranes. It was found that a slight increase (1-2$^{o}$C) in $T_g$ is regularly accompanied by a significant (40-60 %) increase in the thermoresistence of various types of cells [5,6], including mammalian erythrocytes [7].

Thermal hemolysis is a temperature-activated process with activation energy ($E_a$) of 300±15 kJ/mol [8,9,10,11]. Of all temperature-activated processes (excluding the dehydration of ions), similar value of $E_a$ is displayed only by the thermal denaturation of proteins ($E_a$ between 300-350 and 800 kJ/mol) and the conformation changes in proteins ($E_a$ <300 kJ/mol). Based on this value of $E_a$ it could be precluded that the rate-limiting step of thermal hemolysis includes a conformational transition in a protein or in a group of similar proteins.

Ions flow mainly through the protein-mediated pathways of cell membranes and only a small portion of this flow is ascribed to the basal (residual) permeability, P. Despite its small value, P is enhanced at hyperthermia causing hemolysis. According to data obtained with human erythrocytes [12,13,14,15], the rise in P precedes thermal hemolysis, which demonstrates colloid-osmotic character in its initial stage. The increase in P, which underlies the hemolysis, was studied in various temperature intervals: 47-65°C [12], 46-54°C [13], 50-58°C [15] and 38-57°C [16; 17]. In each of these intervals P is a temperature-activated parameter ($P = P_o.\exp(-E_a/RT)$) with the same $E_a$ of 250 ± 15 kJ/mol. This indicates a single mechanism for thermal activation of P, which serves as the prime target of heat in thermal hemolysis over the entire range of 38-60°C. For the 50-58°C interval, the target was identified with the heat-induced, cytosole-independent transition in EM at $T_g$, which equals 61°C for human erythrocytes [14,15]. This transition enhances ion permeability eliciting colloid-osmotic lysis at constant temperatures [15] and during heating with constant rate [14].

The same mechanism of thermal hemolysis was found in mammalian erythrocytes [7,17]. As for human erythrocytes, the $E_a$ for the activation of P was again 250 ± 15 kJ/mol. The value of $T_g$, however, demonstrated species differences in respect to the sphingomyelin content of EMs. The

higher the EM content of sphingomyelin the higher the value of $T_g$, as well as the greater was the time, $t_{1/2}$, for half hemolysis, i.e., the resistance of erythrocytes against thermal hemolysis.

Electric admittance of a cell suspension presents information about the dielectric polarization and barrier function of the plasma membranes. Fig. 1 shows the temperature profile (thermogram) of the temperature derivative of admittance of a heated suspension containing erythrocytes or their resealed EMs. Peaks A and G on the thermogram revealed two independent thermally-induced transitions in EM. The top temperatures of both peaks were shifted upwards due to the heating rate. For slower heating rate (0.5°C/min) the peak temperatures tended to the values of 49.5°C and 61°C ($T_{go}$), respectively.

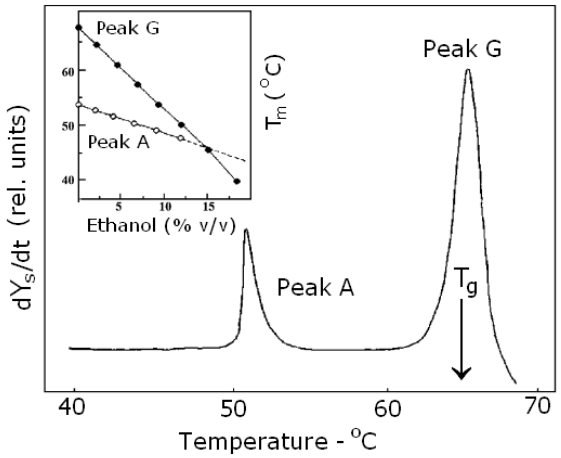


*Fig. 1. Derivative thermogram of the admittance, $Y_s$, of suspension containing human erythrocytes or isolated EMs, resealed with 150 mM NaCl. The suspension medium is isotonic solution of 60 mM NaCl and sucrose. Hematocrit, frequency and heating rate are 0.07, 10 kHz and 2.0°C/min, respectively. Peak A corresponds to the denaturation of spectrin and peak G to the permeability transition in EM at $T_g$. Insert shows the dependence of peak temperatures on the concentration of ethanol in suspension.*

A-peak corresponds to the threshold decrease in capacity of EM, associated with the denaturation and dephosphorylation of spectrin [18,19] at 49.5°C [20,21]. This peak is independent on the transmembrane gradient of ion concentration. The corresponding transition in EM did not affect the temperature activation of ion permeability and thermal hemolysis. The G peak depicted the collapse of the gradient of ion concentration due to the permeability transition, induced in EM at 61°C ($T_{go}$) [14,17,22]. The $T_g$ permeability transition represented the inactivation of the primary target of heat during the temperature activation of ion permeability and thermal hemolysis [7,17].

Deuterium oxide, $D_2O$, and glycerol are universal thermal stabilizers of proteins and protectors of cells against hyperthermia. Hemolysis of erythrocytes, exposed to a temperature of 55°C, is subdued by $D_2O$ [23]. In this study the substitution of 90% of water by $D_2O$ in erythrocyte suspension increased $T_g$ with 1.1 ±0.2°C [24]. Similar increase in $T_g$ (1.0 - 1.5°C), accompanied by a significant increase (50-60%) in the time, $t_{1/2}$, of half hemolysis was determined in the presence of glycerol (10-20 % v/v) and after incorporation of sphingomyelin in the membranes of erythrocytes [25]. These data support the involvement of conformational change in EM protein during the permeability transition at $T_g$ and in thermal hemolysis.

Direct investigation of the $T_g$ transition in EM, however, is impeded by the foregoing heat denaturation of spectrin, which is not involved in this transition, but induces concomitant changes in the structure and dynamics of EM. This difficulty was circumvented by using the different sensitivity of the $T_g$ transition and spectrin denaturation to the presence of ethanol, whereat the $T_g$ is reduced 3.5 times greater than that of spectrin denaturation (Fig. 1). Thus, in the presence of 18 % (v/v) ethanol the permeability peak is reduced to $T_g$ = 39.5°C that is 6°C in front of the spectrin denaturation peak [14,26,27,28]. Erythrocytes, incubated at 39.5°C (reduced $T_g$) for 3 min in isotonic medium containing 18% ethanol, 50 mM NaCl and sucrose as osmotic protector, became irreversibly permeabilized for NaCl, but retained their diffusion barrier to sucrose. This effect was called irreversible sparing permeabilization of EM by heat, in contrast to the thermal permeabilization of EM at 61°C whereby spectrin is denatured. After the removal of ethanol and hyperthermia the induced permeabilization persisted, which confirms the involvement of an irreversible conformational change of protein in it. However, if the incubation was conducted a few

°C lower than the $T_g$ (for example at 37°C instead of 39.5°C), the induced permeabilization was transient as cooling and the removal of ethanol restored the diffusion barrier to ions [26]. This result is in line with the data of Prinsze et al. [13] that the high level of EM ion permeability, induced at hyperthermia, is restored to normal one upon cooling. This result suggests that the initial stage of the permeability transition at $T_g$ in EM includes an initially reversible change in membrane protein, followed by the stage of irreversibility.

EMs of sparingly permeabilized erythrocytes were isolated and studied in comparison to intact EMs and to the control EMs of erythrocytes, processed as explained for the sparingly permeabilized ones, but at a lower temperature (27$^o$C). Micro calorimetric and SDS-PAGE profiles of sparingly permeabilized EMs were identical to those of intact and control EMs [26,27,28]. This result testifies for the absence of irreversible protein denaturation and preservation of the secondary and tertiary structure of membrane proteins during the $T_g$ transition. This conclusion is consistent with the reports [20,21] evidencing for the lack of strong calorimetric effects, lack of intermolecular S-S aggregation and invariability of optical activity (helical content) of EM in the 55-61°C interval.

In contrast to PAAGE the method of DSC was able to distinguish mild structural changes taking place at $T_g$ in the integral band 3 protein, the anion exchanger, responsible for the anion transport in EM. DIDS (4,4'-Diiso-thiocyanato stilbene-2,2'-disulphonate) is membrane impermeable, covalent amino reagent, which at low concentrations (<50 μM) binds specifically to the anion exchanger of EM, producing a strong inhibition and thermal stabilization of this protein [29]. In intact EMs the anion exchanger denatures at 67°C and this denaturation temperature is increased step-wisely by 13°C following the treatment of EMs by DIDS. In both permeabilized EMs and in DIDS-treated permeabilized EMs, however, the denaturation temperature of the band 3 protein was reduced by 2.5$^o$C in respect to that in intact and in DIDS-treated intact EMs, respectively [27, 28]. In addition, the ability of DIDS to increase step-wisely the denaturation temperature of band 3 protein by 13°C was fully preserved in permeabilized EMs [27]. These results evidenced that the structural changes in band 3 at $T_g$ had a milder, pre-denaturation character in respect to the irreversible thermal denaturation.

FNPS (4,4'-difluoro-3,3'-dinitrodiphenylsulfone) is highly lipophilic, bifunctional and membrane impermeable reagent capable to covalently bind and cross-link pairs of amino groups, distanced apart in less than 9 nm. At low concentrations (10-100 μM) FNPS was able to bind and cross-link the proteins of sparingly permeabilized EMs, mainly the anion exchanger, while it was not able to react with the proteins of intact and control EMs [26]. The above results indicate that an EM protein, mainly the anion exchanger, sustained a mild, initially reversible and later irreversible, pre-denaturation transition at $T_g$ causing its association and clusterization.

Fluorescent marker N-(3-pyrene) maleimide binds to the SH-groups of proteins and reports for the self-association and clusterization of these proteins. The marker forms excimers when two pyrene groups get closer and superimposed to each other at a distance of 4 to 10 Å. Excimerization of the marker, bound to EMs, increased by 67% after the sparing permeabilization of membranes indicating a substantial increase in the clusterization of membrane proteins. The maximum efficiency for energy transfer from the tryptophan residues of membrane proteins to pyrene, located in the proximal lipid milieu of annular lipids decreased from 0.95 for intact EM [30] and 0.92 for control EM to 0.70 for the permeabilized EMs [28]. This result again evidenced that the EM transition at $T_g$ was accompanied by association and clusterization of EM proteins whereat a part of their annular lipids were removed.

Similar results were obtained with continuously heated suspensions of isolated EMs, pretreated by the highly lipophilic maleimide derivatives N-(3-Pyrenyl)maleimide and 3-maleimido-proxyl (spin-label) [28]. Both reagents bind to the SH-groups of EM proteins, primarily spectrin and the anion exchanger [31] as the glycophorin does not contain SH-groups [32]. The temperature profile of the N-(3-pyrene) maleimide excimerization decreased indicating threshold increase in the molecular dynamics at the denaturation temperatures of spectrin (49.5°C) and anion

exchanger (67°C). Surprisingly, excimerization was greatly increased in the intermediate (58-62°C) interval around $T_g$. These results suggest subsidence of the molecular dynamics close to the SH-groups of some EM proteins, predominantly those of the anion exchanger at $T_g$.

EPR spectrum of isolated EM, spin-labeled with 3-maleimido-proxyl contains two peaks with amplitudes corresponding to the number of strongly immobilized (S amplitude) and weakly immobilized (W amplitude) spin-labels, covalently bound to the SH-groups of EM proteins, mainly spectrin and the anion exchanger [33]. The temperature dependence of the S amplitude and of the W/S ratio just coincided with the above commented temperature profile of the excimerization of fluorescent probe [28]. This result again showed a decrease in the number of strongly immobilized binding sites about the denaturation temperatures of both spectrin and the anion exchanger and an increase in this number (i.e., protein immobilization) at $T_g$.

Thus, microcalorimetric, electrophoretic, spectrofluorometric and EPR data on the EM permeability transition at $T_g$ all testified that despite the absence of gross protein unfolding, this transition involved moderate changes in the structure (clusterization) and dynamics (immobilization) of certain integral proteins, most likely the anion exchanger. Inasmuch as the EM transition at $T_g$ concerned mainly the dynamics of EM proteins it can be viewed as a dynamic, a second order transition. This type of transition could explain the activation of basal ion permeability and hemolysis at high temperatures. This view is consistent with the data of other authors showing that increasing the temperature from 25°C to 45°C results in substantial oligomerization of the dimers of the EM anion exchanger [34]. The oligomerization of the anion exchanger is a temperature-dependent, reversible process up to 45°C. Based on our results it can be presumed that the oligomerization becomes irreversible close to $T_g$.

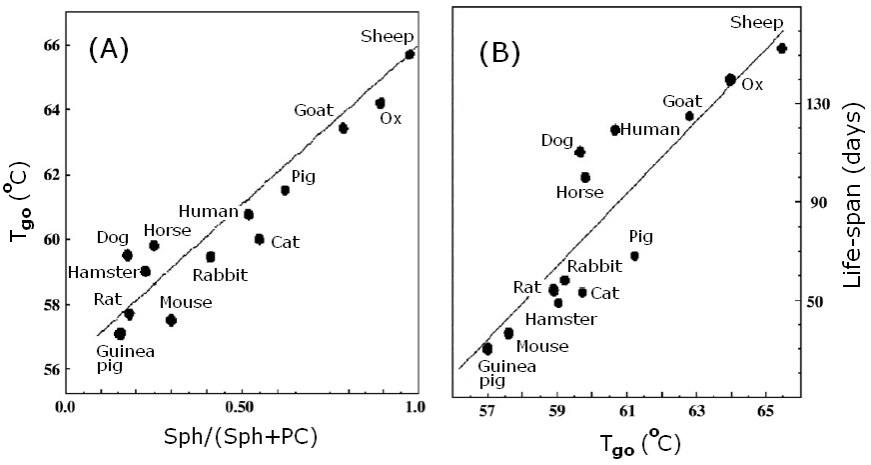


*Fig. 2. Species differences in the thermal resistance (panel A) and in the life-span (panel B) of mammalian erythrocytes. Thermal resistance is represented by the inducing temperature, $T_g$, of the permeability transitions in EM, which activates basal permeability above 37°C [7,17].*

The above presented mechanism for thermal activation of ion permeability and hemolysis is consistent with our findings and the data of others, which all relate this mechanism with the fluidity of lipid bilayer. The sphingomyelin content of mammal EMs displays strong species differences; the increase in the sphingomyelin content correlates the increase in viscosity [35] and in $T_g$ of EMs (Fig. 2) [7, 17]. Conversely, the presence of n-alkanols decreases the $T_g$, i.e., the resistance against thermal hemolysis, proportionally to the decrease in membrane viscosity and to the reduction of the order parameter of lipid bilayer [36]. It can be assumed that the clusterization of the anion exchanger originates from the saddle splay and curvature instability in the lipid bilayer of EM and the impaired lipid to protein complementarity consequent to increased fluidity at hyperthermia [37].

The following studies were designed to clarify whether the changes in the anion exchanger are simply accompanying or directly involved in the thermal stability of EMs ($T_g$) and in the resistance of erythrocytes against thermal hemolysis ($t_{1/2}$). The thermal stability of proteins and thermal resistance of cells is increased by a small number of factors - polyhydric alcohols and sugars [38], osmolytes [39], DNA and amino acids [40], some divalent cations [41], heavy water

and mild static pressure [42], some non-steroidal anti-inflammatory drugs and plant extracts [43]. However, they all have non-specific action and can not identify specific cellular protein responsible for the changes in $T_g$ and $t_{1/2}$.

The specific and covalent binding of DIDS to the anion exchanger of human erythrocytes, conducted under optimal conditions (15 μM, 4°C, 2 mM EDTA, 100 mM sucrose for 10 min), increased both the thermal stability, $T_g$, of EM by about 2.5°C and the resistance of erythrocytes against thermal hemolysis, $t_{1/2}$, by 65 % [24]. These results are interpreted in favor of the direct involvement of the pre-denaturation restructuring and clusterization of the anion exchanger in thermal stability of EM and in the resistance of erythrocytes against thermal hemolysis.

The above conclusion has broader biological significance as the EM anion exchanger represents a subclass of the so called AE family of membrane proteins which carry out DIDS-inhibitable exchange diffusion of anions and maintain the intracellular pH and the concentration of $Cl^-$ in the cells of vertebrates [44]. This fact stipulates studies, similar to the above presented, on the animal, including tumor, cells in view of the possible involvement of the anion exchanger in the thermal sensitivity of these cells.

Another result of biological significance is the strong positive correlation ($r = 0.87$) between the thermal resistance ($T_g$) of mammalian erythrocytes and their life-span (fig. 2). The following considerations indicate this correlation could be causal. Aged erythrocytes are removed *in vivo* mainly through opsonized phagocytosis in the spleen. Phagocytosis is triggered predominantly by the appearance of changed and clusterized anion exchanger proteins on the outside surface of aged cells. Disturbed biochemical and biological processes are usually suspected as reasons for the alteration of anion exchanger and its clusterization. Another reason would be the belonging of body temperature to the temperature interval in which the EM passive ion permeability is activated by the temperature – induced conformation changes in anion exchanger [16,17]. With the time going these conformation changes would increase the number of dimers to olygomers ratio resulting in the appearance of aggregates recognized as markers for aging.

In conclusion, the results obtained with mammalian erythrocytes suggest that the permeability transition of EM at $T_g$ (the destruction of heat target responsible for thermal hemolysis) involved initially reversible pre-denaturation change in the conformation and dynamics of the anion exchanger followed by clusterization and immobilization of its dimers, activation of basal ion permeability, accelerated aging and hemolysis. These results shed light on the mechanisms of cell and plasma membrane damage at acute hyperthermia and can be used for targeted correction of the heat resistance and prevention of thermal injury of cells.

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