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THE PHYSICO-MATHEMATICAL THEORY OF HUMAN ERYTHROCYTE HYPOTONIC HEMOLYSIS PHENOMENON

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Abstract

A physico-mathematical model of human erythrocyte hypotonic hemolysis has been developed in this work. It quantitatively describes the kinetics of the phenomenon occurring when cells are suspended in an hypertonic aqueous solution of permeable non-electrolyte.

Keywords: human erythrocyte, physico-mathematical model, hypotonic hemolysis, cell swelling.

INTRODUCTION

A thermodynamic approach for the description of one of the main membrane characteristics - membrane permeability, has been developed independently from the conception of membrane structure. The key work in this direction was done by Kedem and Katchalsky (8). A number of methods for determination of thermodynamic coefficients, which describe membrane properties, have been developed based on the irreversible thermodynamics equations. Mazur *et al.* (13) presented three main experimental approaches for permeability coefficient determination: (a) by following cell volume change; (b) by measuring the amount of substance penetrating into the cell using a chemical or an isotopic method; (c) by measuring the time required for hemolysis in hypotonic medium. All these methods have a number of experimental difficulties. Furthermore, calculation of coefficients assumes a certain approximation and simplification of theoretical models, which in turn may lead to systematic errors in their determination.

For the determination of permeability coefficients, the time of 50% hemolysis in aqueous solution is usually measured. To obtain the values of permeability coefficients, the erythrocyte volume change in time is calculated using the Kedem-Katchalsky equations at different values of permeability coefficients. That permeability coefficient at which the calculated time required for the cell to reach a critical volume coincides with the experimentally determined time of 50% hemolysis, is supposed to be the unknown value. Hemolysis is assumed to occur immediately when the cell reaches a certain critical size. Or, in other words, the time when hemolysis begins is assumed to coincide with the time required for the cells to reach a critical volume due to swelling (13,19). Moreover, in the majority of studies for measuring erythrocyte membrane permeability coefficients for electrically neutral substances, the transmembrane pressure difference, and its related isotropic strain, was not directly taken into account.

Saary and Beck (16) showed the assumption, that hemolysis occurs immediately when an erythrocyte reaches a certain fixed critical volume, not to be valid. The time t_h, during which hypotonic hemolysis in erythrocyte suspension reaches 50%, was shown to consist of two items:$t_h = t_k + t_s$, where t_k represents the time required for an erythrocyte to reach a critical volume. It can be determined by numerical solution of Kedem-Katchalsky equations and depends on the initial erythrocyte volume. The contribution of the spherical time period t_s was said to be due to the delay in hemolysis caused by potassium leakage from cells, after which hemoglobin is released from the erythrocyte (16). An alternative explanation is that the pore formation in membrane is an activated process and requires some finite period of time to occur. In the light of the ideas proposed (7,9) the assumption of immediate hemoglobin release is quite dubious. Otherwise, the time necessary for hemoglobin to leave an erythrocyte during hypotonic hemolysis would be of the same order of magnitude, or even higher, than the time required for macroscopic pore formation in the membrane. Moreover, the spherocyte volume at which hemolysis begins is not a fixed value but depends on the kinetics of the transport processes between the cell and the environment during hypotonic hemolysis (16). The uncertainty of the critical spherocyte volume is confirmed by the variation in its value from 1.5 to 1.9 of the initial volume for human erythrocytes according to different authors (3,5,14,6,17,21).

This work aims to eliminate the disadvantages of the previous method for measurement of human erythrocyte permeability coefficients for electrically neutral substances. To this aim, a theoretical model was developed for measuring the time required for 50% hemolysis. It introduces into consideration the concept of the so-called "spherical period" and dependence of critical erythrocyte volume on membrane transport characteristics.

THE HYPOTONIC HEMOLYSIS MODEL

The change of the cell volume and the intracellular concentration of the permeable solute with the time in hypertonic aqueous solution of the permeable substance is described by the ordinary differential equation system (See *Appendix 1*):

$$\begin{aligned} \frac{dy}{dt} &= \frac{1}{\tau_0}\left[\sigma(\hat{\pi}-\hat{\pi}')+\frac{1-\alpha}{y-\alpha}-\frac{\Delta p}{\pi_0}\right] \\ \frac{d\hat{\pi}}{dt} &= -\left[\frac{1}{\tau_1}(\hat{\pi}-\hat{\pi}')+\sigma\hat{\pi}\frac{dy}{dt}\right]\frac{1}{y-\alpha} \end{aligned} \qquad [1]$$

where t - the time, $y \equiv V/V_0$ - the relative cell volume, V and V_0 - the actual and initial cell volume respectively, σ - the cell membrane reflection coefficient for the permeable substance, π and π' - the intracellular and extracellular osmotic pressure of the permeable substance respectively, α - the nonsolvent volume fraction, Δp - the membrane pressure fall difference, π_0- the osmotic pressure of nonpermeable substances at the initial moment, which is equal to 8Pa under normal conditions

$$\hat{\pi} = \pi/\pi_0, \quad \hat{\pi}' = \pi'/\pi_0, \quad 1/\tau_0 \equiv \gamma \cdot L_p \pi_0, \quad 1/\tau_1 \equiv \gamma \cdot K$$

γ - the initial cell surface-volume ratio, L_p - the membrane filtration coefficient, K - the membrane permeability coefficient for the substance dissolved in the extracellular medium.

When water molecules transfer across the cell membrane, the cell membrane reflection coefficient correlates with filtration and permeability coefficients in the following way (59):

$$1-\sigma=\varepsilon\cdot c_0\cdot\vartheta \qquad [2]$$

where $\varepsilon=\tau_0/\tau_1$, $c_0=\pi_0/RT$, R - the gas constant, T - the absolute temperature, ϑ - the molar volume of the permeable substance. As follows from the equation [2], the cell membrane reflection coefficient is equal to 1, when K=0, i.e. when the substance dissolved does not penetrate through the cell membrane.

If the initial cell volume is $V_0 \le (S_0)^{3/2}/6\sqrt{\pi}$, where S_0 - the initial cell surface area, then until the moment when the relative cell volume is within the interval

$$1 \le y \le y_s = (S_0)^{3/2}/6\sqrt{\pi}\,V_0 \qquad [3]$$

the cell membrane pressure difference Δp can be neglected, hence the cell membrane is not subjected to an isotropic strain. But when the cell form becomes the spherical one and the relative cell volume exceeds the value of y_s, then the cell membrane is being strained and inside the cell an excessive pressure is being created. It is equal to (7)

$$\frac{\Delta p}{\pi_0} = C\left[(y/y_s)^{3/2}-1\right] \qquad [4]$$

where $C = 4\Gamma\sqrt{\pi}/\sqrt{S_0}\pi_0$, Γ represents the isothermal strain coefficient of the cell membrane. The human erythrocyte average values are (in the Ringer`s solution) for $S_0=136.9\times10^{-12}m^2$ and for $V_0=104.2\times10^{-18}m^3$ (1), therefore $y_s=1.4458$.

A rupture of erythrocyte membrane is known to occur under a very small relative change of the membrane surface area (4) and, hence, under a very small increase of the cell's relative volume $y=y_s$. That is why describing the cell volume change in the frames of $y_s<y<y_p$, we can suppose that

$$(y_p - y_s)/\!/\ y_s << 1 \quad \text{and} \quad \Delta p/\pi_0 = 2C(y/y_s - 1)/3 \qquad [5]$$

where y_p is the cell relative volume at which the macroscopic pore forms in the isotropically strained membrane. The inequality [5] is only strengthened, if in denominator y_s is changed to the bigger value y, and in numerator y_p is changed to the less value y. Therefore $(y-y_s)/y << 1$, if $y_s<y<y_p$.

If the ratio of the whole cell volume to the extracellular liquid volume in cell suspension is considerably lower than 1, then we can assume π' value to be unchanged. Therefore the concentration of the substance dissolved in the extracellular medium is practically unchanged during the redistribution process between the cells and extracellular medium.

τ_0 and τ_1 values have time dimensions and are characteristic periods during which chemical equilibrium at the cell membrane are being set correspondingly for the solute (water) molecules and dissolved substance, respectively, usually $\varepsilon<<1$ and hence $\sigma\sim1$ (11).

The nonlinear equation system [1] does not have an accurate analytical solution and therefore is usually solved numerically by PC. This causes difficulties both in analysis of the corresponding numerical calculations and their comparison with the experimental data.

However, answers more effective and more suited to the analysis equation system can be obtained by the method of asymptotic solution of singularly excited systems (12). In order to obtain this solution, taking into account the correlations and determinations above, let us present the [1] equation system in the following way:

$$\varepsilon\frac{dy}{d\hat{t}} = \sigma(\hat{\pi}-\hat{\pi}') + \frac{1-\alpha}{y-\alpha} - \frac{2}{3}\cdot\frac{C(y-y_s)}{y_s}$$
$$\frac{dz'}{d\hat{t}} = -\frac{z'}{y-\alpha} + \hat{\pi}' + \varepsilon\vartheta\pi_0\frac{dy}{d\hat{t}} \qquad [6]$$

where the dimensionless "slow" time $\hat{t} = t/\tau_1$ and the new variable $z'=(y-\alpha)\pi$ are introduced. Since the small parameter ε is figured as a factor at the derivative $\frac{dy}{d\hat{t}}$, then the asymptotic solution of the equation system [1] on times $\tau_0 << t$ can be found using the method of singularly excited systems mentioned above. Assuming in [6] $\varepsilon=0$, after simple transformations the system of the generating equations can be obtained. This system is one order lower than the initial one

$$\sigma\cdot z + 1 - \alpha = (y-\alpha)\left[\sigma\cdot\hat{n}' + \frac{2}{3}\cdot\frac{C(y-y_s)}{y_s}\right]$$
$$\frac{dz}{d\hat{t}} = \frac{(1-\alpha)\cdot\hat{n}'}{\sigma\cdot z + 1 - \alpha} \qquad [7]$$

where $z=z'/RT$ and $\hat{n}'=\hat{\pi}'=\frac{n'}{n_0'}$, n' and n_0' are the actual and initial extracellular concentration of the permeable substance. Solution of the equations [7], represents the solution of equations [6] in non-singular region (at times of τ_0 order) under the initial condition of $z(0)=0$ and is as follows:

$$\hat{t}-\hat{t}_s = 2(y-y_s) - \frac{2C'(y_s-\alpha)+3\sigma\cdot\hat{n}'}{4C'}\times\ln\left\{1-\frac{2C'}{3(1-\alpha)}\left[(y-\alpha)^2-(y_s-\alpha)(y-\alpha)\right]\right\} +$$
$$+\frac{\left[2C'(y-\alpha)^2+3\sigma\cdot\hat{n}'(y_s-\alpha)+12(1-\alpha)\right]}{4\sqrt{C'}\sqrt{C'(y_s-\alpha)^2+(1-\alpha)}}\times$$
$$\ln\left\{\frac{\sqrt{C'(y_s-\alpha)^2+(1-\alpha)}+2\sqrt{C'}(y-y_s/2-\alpha/2)}{\sqrt{C'(y_s-\alpha)^2+(1-\alpha)}-2\sqrt{C'}(y-y_s/2-\alpha/2)}\times\frac{\sqrt{C'(y_s-\alpha)^2+(1-\alpha)}-\sqrt{C'}(y_s-\alpha)}{\sqrt{C'(y_s-\alpha)^2+(1-\alpha)}+\sqrt{C'}(y_s-\alpha)}\right\} \qquad [8]$$

where $C'=C/y_s$ and $t_s = \frac{\left[(\sigma\cdot\hat{n}')^2(y_s-\alpha)^2-(1-\alpha)^2\right]}{2\gamma\cdot K(1-\alpha)\sigma\cdot\hat{n}'}$ - the time during which the cell becomes spherical.

For the erythrocytes of human peripheral blood, $\gamma=S_0/V_0=1.3138\times10^6 m^{-1}$ at the average values of the membrane surface area and cell volume mentioned above. Besides, $\alpha=0.42$ for these cells.

The solution [8] describes the cell relative volume change from the time when the cells come into contact with a hypertonic aqueous solution of permeable substance until the cell

becomes spherical. In the non-singular region, i.e. for the time exceeding τ_0, this solution approximates to the accurate solution of the input equation system [1] with the accuracy of the $O(\varepsilon)$ order terms.

When $y > y_s$ the cell membrane is subjected to an isotropic strain. A macroscopic pore formation is known to be energetically favorable under a rather strong strain. The average time of such a pore formation in the case of steady state is (See *Appendix 2*)

$$\langle t_p \rangle = As^{-1/2} \exp\left(\frac{\pi\gamma_0^{\,2}}{kTs}\right) \qquad [9]$$

where s - the isotropic strain of the membrane, k- Boltzman`s constant, $\gamma_0$ - the linear strain coefficient of the pore bound. The pre-exponential multiplier A doesn't depend on the membrane strain s. According to Hook`s law and [5]

$$s = \frac{2}{3}\Gamma(y/y_s - 1) \qquad [10]$$

Therefore [9] can be presented as:

$$\langle t_p \rangle = A\left[\frac{2\Gamma}{3y_s}\right]^{-1/2}(y - y_s)^{-1/2} \exp\left(\frac{3\pi\gamma_0^2}{kTR_s\pi_0 C'(y - y_s)}\right) \qquad [11]$$

The experimental data for the human erythrocyte membrane subjected to the isotropic strain, obtained by the method of drawing a single erythrocyte into a micropipette with a calibrated diameter under the fixed pressure difference (15) compared with [11] lead to the following values of the coefficient in this formula: $A' = 0.01$, $3\gamma_0^2/kTR_s\pi_0 = 0.08676$. At the temperature of 25°C this corresponds to the value of $\gamma_0 = 10^{-11}$N (this value coincides with the linear strain of the pore boundary in lipid bilayers).

Formula [11] cannot be used directly for the description of erythrocyte hypotonic hemolysis since the cell volume and corresponding membrane isotropic strain (when $y > y_s$) does not remain constant in this case but continuously increases. However, a macroscopic pore can be formed in membrane, if the time, during which the relative cell volume remains in the interval of dy values, is equal to the average time of a macroscopic pore formation corresponding to an y value within this interval by formula [11]. This statement can be presented in the analytical form in the following way:

$$\int_{y^*}^{y_p} \frac{dt}{dy} dy = \frac{1}{y_p - y^*} \cdot \int_{y^*}^{y_p} \langle t_p \rangle dy \qquad [12]$$

where y_p - the cell relative volume, when a membrane pore forms, y^* - the cell relative volume, which corresponds to the solution of the equation $t(y) - t_s = \langle t_p \rangle(y)$. $\langle t_p \rangle(y)$ is determined by [8]. Let $t(y) = \langle t_p \rangle(y) = t^*$ and t_p - the moment in time, when a membrane pore forms. Then, simple calculations lead to the following algorithm for determination of the whole time, during which a macroscopic pore forms in the membrane of the cell placed into the hypertonic water solution of permeable substance. The relative cell volume y^* and corresponding t^* - the time

during which the right side of equation [8] divided by $\gamma \cdot K$ is equal to that of the equation [11]. Then, the time during which the macroscopic membrane pore forms is determined as follows:

$$t_p = \frac{\left[(\sigma \cdot \hat{n}')^2 (y_s - \alpha)^2 - (1-\alpha)^2\right]}{2\gamma \cdot K(1-\alpha)\sigma \cdot \hat{n}'} + \frac{2(y^* - y_s)}{\gamma \cdot K} - \frac{2C'(y_s - \alpha) + 3\sigma \cdot \hat{n}'}{4\gamma \cdot KC'} \times$$
$$\times \ln\left\{1 - \frac{2C'}{3(1-\alpha)}\left[(y^* - \alpha)^2 - (y_s - \alpha)(y^* - \alpha)\right]\right\} + \qquad [13]$$
$$\frac{\left[2C'(y-\alpha)^2 + 3\sigma\hat{n}'(y_s - \alpha) + 12(1-\alpha)\right]}{4\gamma \cdot K\sqrt{C'}\sqrt{C'(y_s - \alpha)^2 + (1-\alpha)}} \times$$
$$\times \ln\left\{\frac{\sqrt{C'(y_s - \alpha)^2 + (1-\alpha)} + 2\sqrt{C'}(y - y_s/2 - \alpha/2)}{\sqrt{C'(y_s - \alpha)^2 + (1-\alpha)} - 2\sqrt{C'}(y - y_s/2 - \alpha/2)} \times \right.$$
$$\left. \times \frac{\sqrt{C'(y_s - \alpha)^2 + (1-\alpha)} + 2\sqrt{C'}(y_s - \alpha)}{\sqrt{C'(y_s - \alpha)^2 + (1-\alpha)} - 2\sqrt{C'}(y_s - \alpha)}\right\} +$$
$$+ \frac{\langle t_p \rangle^*}{1 + \frac{1}{2}\langle t_p \rangle^* \gamma \cdot K} \frac{3\pi\gamma_0^2\left[3(1-\alpha) - 2C'(y^* - y_s)(y^* - \alpha)\right]}{kTR_s\pi_0 C'(y^* - y_s)(y^* - \alpha)\left[3\sigma n' + 2C'(2y^* - y_s - \alpha)\right]}$$

RESULTS OF THE MODEL EXPERIMENTS

Figures 1-3 show the plots of function [13] under the different values of parameters given in the developed model. As follows from the correlations obtained, the time for the macroscopic pore formation increases proportionally to the concentration of the substance dissolved in the extracellular medium (Fig.1). Since the π' parameter is set by the experimentator it is easy to verify this fact empirically. The experiments carried out for determinating the time t_p by the small angle light scattering method, confirmed this theoretical prediction in the region of $\pi' < 8$.

When the extracellular solution concentration was higher, the deviation from the dependence predicted by the theory may be explaned by the direct chemical interaction of high concentration solutions with the membranes. Obviously, the change of the permeable substance concentration affects primarily the cell spherification time t_s, while the duration of the stage when the erythrocyte membrane is subjected to the strain deformation does not change considerably. As predicted by the model, the change of erythrocyte volume at which the macroscopic pore forms is kinetic, and it decreases upon a rise of extracellular concentration.

This is caused by the fact, that the increase in the membrane pressure difference leads to an increasing of the rate of the cell volume change, and this in its turn leads to the cell "slipping" up to the values of $y - y_s > y_s$, at which the time of a macroscopic pore formation becomes short. The t_p rise with decreasing of the γK parameter (Fig.2) is mainly caused by the increasing time taken for the cell to reach a spherical form, and is due to the decrease in erythrocyte permeability coefficient for the permeable substance dissolved. The rise in relative erythrocyte volume at which a macroscopic pore forms with an increase in the γK parameter is also conditioned by the "slipping" effect mentioned above, because the rate of the cell volume change is inversely proportional to γK. The decrease of t_p in the more rigid membrane, i.e.

under higher values of C′ is explained by the fact that the membrane appears under a lower isotropic strain necessary for the membrane pore formation in the more rigid membrane, hence the membrane pore forms during a shorter period of time (Fg.3).

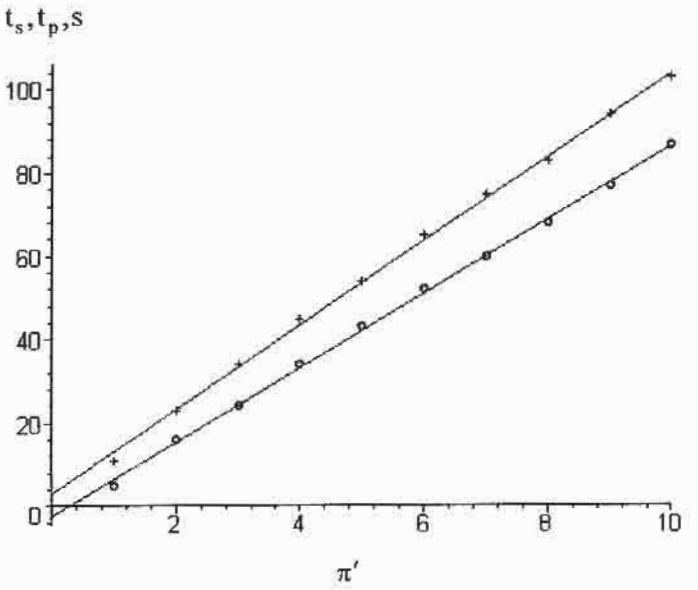

Figure 1. Dependence of the spherification time (ooo); the time, at which a macroscopic pore forms in the cell membrane (xxx), upon the osmotic pressure of the extracellular solution.
α=0.4; σ=0.95; C′=0.25
T=298K; γK=0.1s^{-1}.

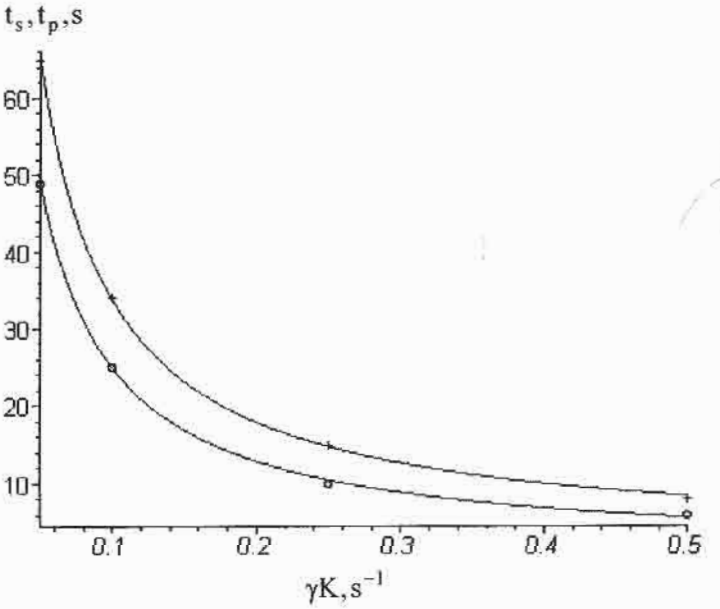

Figure 2. Dependence of the spherification time (ooo), the time, at which a macroscopic pore forms in the cell membrane (xxx), upon the $\gamma \cdot K$ parameter.
α=0.4; σ=0.95;C′=0.25;
T=297K; $\hat{\pi}' = 3$.

According to (7,9) a part of intracellular content is ejected from the cell through the macroscopic pore formed in the isotropically strained membrane under the influence of a residual hydrostatic pressure difference. Therefore, the pressure inside the cell rapidly falls to the critical value and the cell relative volume decreases to the volume which coincides with $y = \left(\frac{1}{6}\sqrt{\pi}\right)(S_0)^{3/2}$ within a close approximation. At this moment the pore heals as it becomes thermodynamically unfavorable (free deformation energy without the pore has the lower value

than that with the membrane pore). However, the difference of transmembrane concentrations of permeable substance does not disappear. The permeable substance continues to penetrate and correspondingly the cell volume increases again until a new pore forms by the mechanism explained above. Consequently, erythrocyte hemolysis is a periodic process.

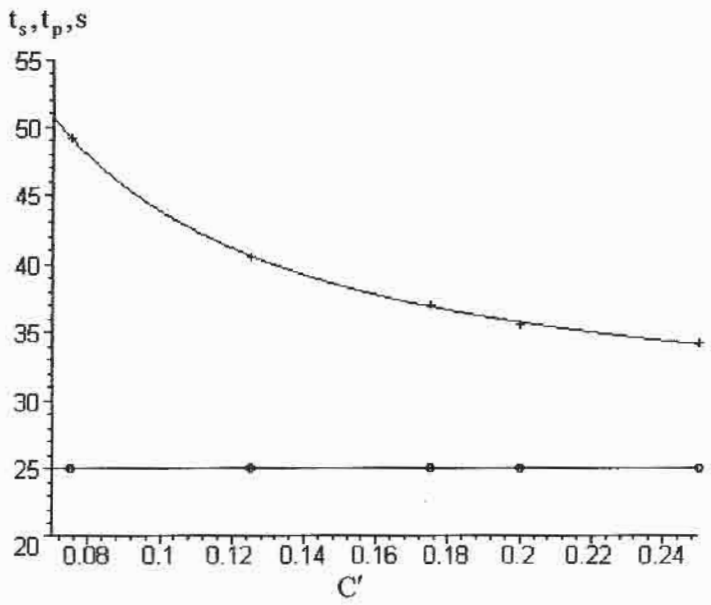

Figure 3. Dependence of the spherification time (ooo), the time, at which a macroscopic pore forms in the cell membrane (xxx), upon the C′ parameter.
α=0.4; σ=0.95; $\hat{\pi}' = 3$
T=298K; γK=0.1s^{-1}.

The following designations are introduced: i-stage of hemolysis represents the hemolysis process from the moment of i-pore closing to the moment of (i+1) - macroscopic pore formation in the membrane. The actual values at the i-stage of hemolysis will be marked by the upper index in the brackets (i). The values at the moment of macroscopic pore formation further will be marked by the lower index p. Therefore the moment of i -pore formation is designated as $t_p^{(i)}$, and the relative volume in this moment as $y_p^{(i)}$. The time intervals during which the intracellular solution is being ejected through a pore (7) are negligibly short in comparison with the duration of the i-stage of hemolysis, hence these time intervals can be assumed to be equal to zero.

Assuming the intracellular content to be homogeneously distributed inside the erythrocyte and taking into account the fact that a part of intracellular solution is ejected through a macroscopic pore as a whole, the following corelations apply between the molar quantity of permeable and nonpermeable substances (M) and the nonsolvent volume fraction (α) before and after i-pore formation:

$$M_p^{(i)} = M^{(i)}\left(t_p^{(i)}\right) = M^{(i-1)}\left(t_p^{(i)}\right)\frac{y_s}{y_p^{(i)}}$$
$$\alpha_p^{(i)} = \alpha^{(i)}\left(t_p^{(i)}\right) = \alpha^{(i-1)}\left(t_p^{(i)}\right)\frac{y_s}{y_p^{(i)}}, \quad i = 1,2,3... \qquad [14]$$

From the first equation of [14] it follows that the concentration of the substances dissolved inside the erythrocyte does not change due to ejection of the intracellular solution through a membrane pore. Since the content of nonpermeable and nonsolvent substances

inside the cell changes only at the moments of membrane pore formation, then the following equations for the actual values at the i -stage of hemolysis can be obtained:

$$M^{(i)} = M(0)\frac{y_s^i}{\prod_{k=1}^{i-1} y_p^{(k)}}, \quad \alpha^{(i)} = \alpha\frac{y_s^i}{\prod_{k=1}^{i-1} y_p^{(k)}}, \quad i = 1,2,3,... \qquad [15]$$

where M(0) represents the initial content of normally nonpermeable intracellular substances. Therefore, the reduced concentration $\hat{n}^{in}$ or reduced osmotic pressure $\hat{\pi}^{in}$ of nonpermeable intracellular substances between the moments of i and (i+1) pore formation is as follows:

$$\hat{n}^{in} = \hat{\pi}^{in} = \frac{(1-\alpha)\dfrac{y_s^i}{\prod_{k=1}^{i-1} y_p^{(k)}}}{y - \alpha\dfrac{y_s^i}{\prod_{k=1}^{i-1} y_p^{(k)}}}, \quad i = 1,2,3,... \qquad [16]$$

Thereto the initial concentration of intracellular permeable substances at the i-stage of hemolysis is equal to the final one at the previous stage.

$$A^{(i)} = \frac{y_s^i}{\prod_{k=1}^{i-1} y_p^{(k)}}, \quad x^{(i)} = y^{(i)} - \alpha \cdot A,$$

$$x_s^{(i)} = y_s^{(i)} - \alpha \cdot A^{(i)}, \quad \tilde{x}^{(i)} = \tilde{y}^{(i)} - \alpha \cdot A^{(i)},$$

$$y^{(i)}\left(t_p^{(i)}\right) = \tilde{y}^{(i)}, \quad i = 1,2,3,...$$

At the i-stage of hemolysis with the designations accepted above the membrane transfer equations in the water solution of the electrolyte penetrating into the cell are:

$$\sigma\hat{\pi}^{(i)} - \sigma\hat{\pi}' + \frac{(1-\alpha)A^{(i)}}{x^{(i)}} - \frac{2}{3}C'\left(x^{(i)} - x_s^{(i)}\right) = 0$$

$$x^{(i)}\frac{dx^{(i)}}{d\hat{t}}\left(4C'x^{(i)} + 3\sigma\hat{\pi}' - 2C'x_s^{(i)}\right)dx^{(i)} = 3(1-\alpha)A^{(i)} - 2C'x^{(i)}\left(x^{(i)} - x_s^{(i)}\right) \qquad [17]$$

$$i = 1,2,3,...$$

The solution of the second of these equations is:

$$\hat{t}^{(i)} = \hat{t}_p^{(i-1)} - 2\left[x^{(i)} - x^{(i)}\left(t_p^{(i-1)}\right)\right] - \frac{3\sigma\hat{\pi}' + 2C'x_s^{(i)}}{4C'} \times \ln\left[\frac{3(1-\alpha)A^{(i)} - 2C'x^{(i)}\left(x^{(i)} - x_s^{(i)}\right)}{3(1-\alpha)A^{(i)} - 2C'x^{(i)}\left(t_p^{(i-1)}\right)\left(x^{(i)}\left(t_p^{(i-1)}\right) - x_s^{(i)}\right)}\right] +$$

$$+\frac{3\sigma\hat{\pi}'x_s^{(i)} + 2C'x_s^{(i)}x_s^{(i)} + 12(1-\alpha)A^{(i)}}{4\sqrt{C'}\sqrt{2C'x_s^{(i)}x_s^{(i)} + 12(1-\alpha)A^{(i)}}} \times \ln\left[\frac{\sqrt{C'x_s^{(i)}x_s^{(i)} + 6(1-\alpha)A^{(i)}} + 2\sqrt{C'\left(x_s^{(i)} - x_s^{(i)}/2\right)}}{\sqrt{C'x_s^{(i)}x_s^{(i)} + 6(1-\alpha)A^{(i)}} - 2\sqrt{C'\left(x_s^{(i)} - x_s^{(i)}/2\right)}} \times \right. \qquad [18]$$

$$\left. \times \frac{\sqrt{C'x_s^{(i)}x_s^{(i)} + 6(1-\alpha)A^{(i)}} + 2\sqrt{C'\left(x_s^{(i)}\left(t_p^{(i-1)}\right) - x_s^{(i)}/2\right)}}{\sqrt{C'x_s^{(i)}x_s^{(i)} + 6(1-\alpha)A^{(i)}} - 2\sqrt{C'\left(x_s^{(i)}\left(t_p^{(i-1)}\right) - x_s^{(i)}/2\right)}}\right]$$

Of course at the initial moment of i-stage of hemolysis, the quasi-stationary equilibrium is infringed. Actually in this moment $\hat{\pi}^{(i)}\left(t_p^{(i-1)}\right) = \hat{\pi}_p^{(i-1)}$ and $y^{(i)}\left(t_p^{(i-1)}\right) = y_s$, but at the end of the previous stage the same equations satisfy the following condition:

$$\hat{\pi}^{(i-1)}\left(t_p^{(i-1)}\right) = \hat{\pi}_p^{(i-1)} \text{ and } y^{(i-1)}\left(t_p^{(i-1)}\right) = y_p^{(i-1)}$$

However, the quasi-stationary state can be considered to be restored very quickly during the time of about τ_0 due to the redistribution of water between the cells and the environment, but not the solutes. Therefore, for this new quasi-stationary condition, which determines the initial condition for the reduced equation system at the i-stage of hemolysis, the intracellular content both of nonpermeable and permeable substances remains invariable during the time of relaxation to a new quasi-stationary state. Hence, for the determination of a new quasistationary state we can assume

$$\hat{n}^{(i)} = \hat{\pi}^{(i)} = \frac{\hat{\pi}_p^{(i-1)}\left(y_s - \alpha A^{(i)}\right)}{y^{(i)} - \alpha A^{(i)}}, \quad i = 1,2,3 \qquad [19]$$

That is why at the quasi-stationary equilibrium at the begining of the i-stage of hemolysis:

$$\frac{\sigma\hat{\pi}_p^{(i-1)}}{\tilde{x}^{(i)}} - \sigma\hat{\pi}' + \frac{(1-\alpha)A^{(i)}}{\tilde{x}^{(i)}} - \frac{2}{3}C'\left(\tilde{x}^{(i)} - x_s^{(i)}\right) = 0 \qquad [20]$$

where the relative volume corresponding to the quasistationary state is designated as $\tilde{y}^{(i)}$ and represents the initial value of $y^{(i)}\left(t_p^{(i)}\right)$, used in the solution of [18]. As follows from the [20] this initial value is equal to

$$\tilde{y}^{(i)} = y_s - \frac{3\sigma\hat{\pi}' + 2C'\left(y_s - \alpha A^{(i)}\right)}{4C'} + \sqrt{\frac{\left[3\sigma\hat{\pi}' + 2C'\left(y_s - \alpha A^{(i)}\right)\right]^2}{16C'^2} + \left(y_s - \alpha A^{(i)}\right)\left(y_p^{(i-1)} - y_s\right)} \qquad [21]$$

$$i = 1,2,3,\ldots$$

Therefore, the change of relative erythrocyte volume with time during the i-stage of hemolysis is completely defined by the equations [18] and [21]. The time during which i-pore forms in the erythrocyte membrane is:

$$t_p^{(i)} = t_p + \sum_{k=1}^{i-1} t_p(k) + \frac{\left\langle t_p^{(i)} \right\rangle^*}{1 + \frac{\gamma \cdot K \left\langle t_p^{(i)} \right\rangle^* 3\pi\gamma_0^2 \left[3(1-\alpha)A^{(i)} - 2C'\left(y^{(i)*} - y_s\right)\left(y^{(i)*} - \alpha A^{(i)}\right)\right]}{2kTR_s\pi_0 C'\left(y^{(i)*} - y_s\right)^2 \left(y^{(i)*} - \alpha A^{(i)}\right)\left[3\sigma\hat{\pi}' + 2C'\left(2y^{(i)*} - y_s - \alpha A^{(i)}\right)\right]}} \qquad [22]$$

$i = 1,2,3,..$

$y^{(i)*}$ represents the value of the relative cell volume when $t^{(i)} = \left\langle t_p^{(i)} \right\rangle = \left\langle t_p^{(i)} \right\rangle^*$, where $t^{(i)}$ is determined by the solution [18] together with the initial condition [21], and $\left\langle t_p^{(i)} \right\rangle$ is equal (7) to:

$$\left\langle t_p^{(i)} \right\rangle = A\left[\frac{2\Gamma}{3y_s}\right]^{-1/2} \left(y - y_s\right)^{-1/2} \exp\left(\frac{3\pi\gamma_0^2}{kTR_s\pi_0 C'\left(y - y_s\right)}\right)$$

The correlations obtained completely describe the hemolysis kinetics of a single erythrocyte in an hypertonic aqueous solution of a permeable substance. Obviously, we can assume the α parameter to be volumetric hemoglobin content.

Figures 4 and 5 show (according to the algorithm developed) the time dependences of the relative erythrocyte volume and the intracellular nonsolvent fraction (hemoglobin content) both at the stages of swelling and hemolysis at typical human erythrocyte values of the parameters used in the theory C', π' and $\gamma \cdot K$.

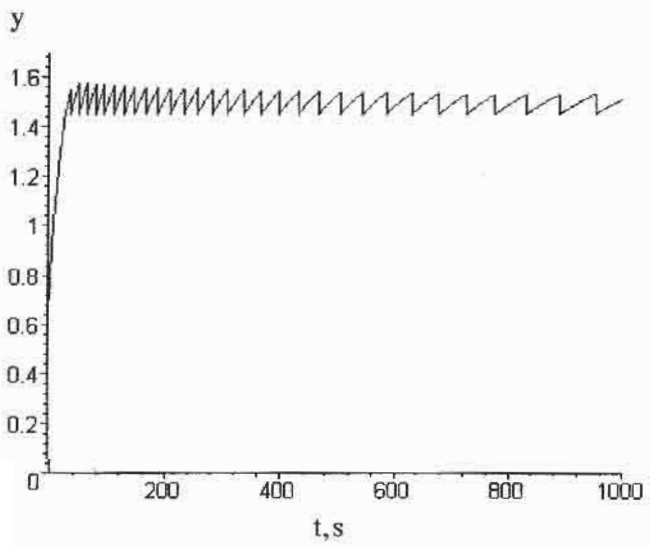

Figure 4 The time dependence of relative erythrocyte volume calculated under the following parameter values:
α=0.4; σ=0.95; T=298K;
$\gamma \cdot K$=0.1s^{-1}; C'=0.25; $\hat{\pi}' = 3$.

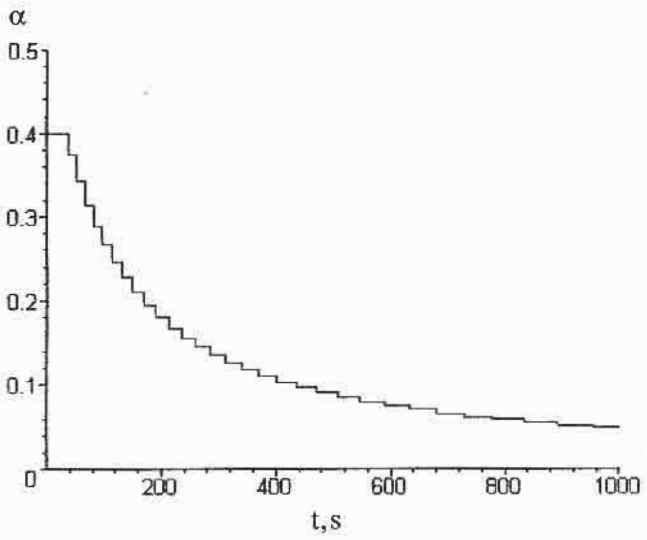

Fig.5 The time dependence of volumetric content of osmotically inactive intracellular substances calculated under the following parameter values:
α=0.4; σ=0.95; T=298K;
$\gamma \cdot K=0.1 s^{-1}$; C′=0.25; $\hat{\pi}' = 3$.

Figures 6 and 7 show the dependence of time during which half of the initial hemoglobin content is released from the cell upon the same parameters. As the data show, when the erythrocyte membrane permeability coefficient for a permeable substance decreases and C′ parameter (being directly proportional to the membrane isothermic strain modulus) increases, the frequency of pore formation in the cell membrane decreases. The amplitude of the average cell volume change also decreases during hemolysis.

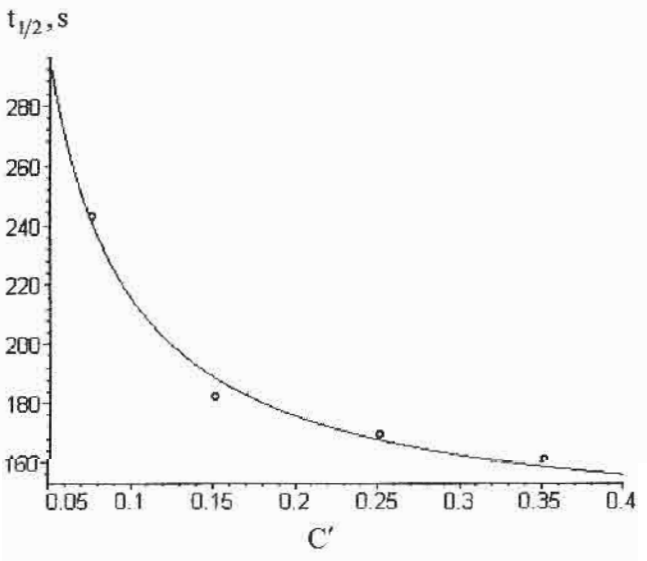

Figure 6. The dependence of time during which a half of hemoglobin content release from the cell upon the C′ parameter.

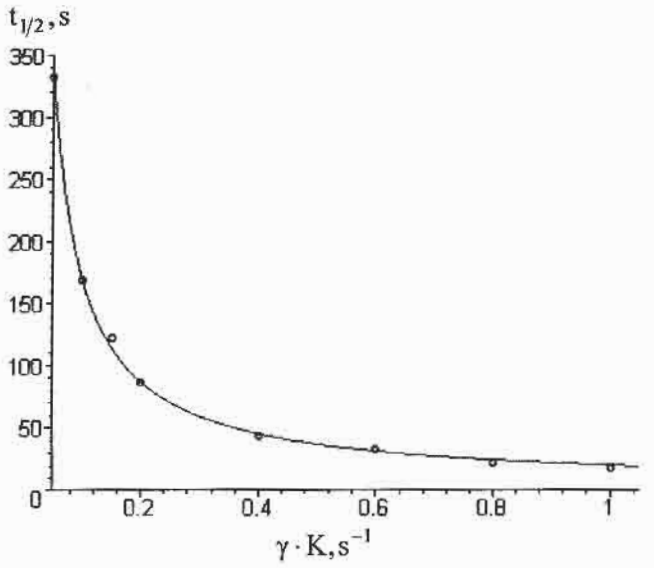

Figure 7. The dependence of time during which a half of hemoglobin content release from the cell upon the $\gamma \cdot K$ parameter.

The time during which the definite hemoglobin content is being ejected from the cell with an increase in the parameters K and C′ decreases, but rises proportionally to the osmotic pressure of the substance dissolved in the extracellular medium. The quantity of the intracellular solution ejected through the macroscopic pores, decreases with the rise of C′ parameter and increases with the rise of permeability coefficient K. During hemolysis, the frequency of intracellular content ejections, and volume changes caused by them, decrease.

DISCUSSION

The developed physical and mathematical model explains the process of hypotonic hemolysis as a whole, and in particular connects the time required for 50% release of hemoglobin with the cell membrane permeability coefficient for non-electrolyte. This theory, therefore, can be used for the experimental determination of cell membrane permeability coefficients for non-electrolytes.

According to Mazur *et al.* (13), the procedure for determination of the permeability coefficient is as follows: Firstly, the time of 50% hemolysis in the solution of the substance under investigation is determined experimentally. For this purpose, a small quantity of erythrocytes is added to the aqueous solution of the substance at a given concentration. Small samples are then taken from the suspension after certain periods of time, sedimented by centrifugation, and the hemoglobin concentration in the supernate determined by one of the standard methods. As a result, the time-dependence of hemoglobin content in the extracelullar medium during hypotonic hemolysis is determined. As hemoglobin release from an individual cell is supposed to occur by an "all or nothing" principle (13,19), the relative hemoglobin content in the supernate is related to the percentage of the hemolysed cells. The time, t, of 50% hemolysis is determined from this. Furthermore, the time-dependence of relative erythrocyte volume change at different values of the permeability coefficient is calculated using the Kedem-Katchalsky equations or their derivatives. Hemolysis of an individual cell is assumed to start as soon as the relative volume reaches a certain fixed critical value, and the time taken for an erythrocyte to reach this volume at different values of the permeability coeffient is calculated. The critical relative volume is set beforehand and is supposed to be

fixed as was mentioned previously. Different authors propose the following different values of critical volume: 1.67 (21), 1.74 (3), 1.78 (18), 1.79 (5), 1.8 (14), 1.9 (17). That value of the permeability coefficient at which the calculated time required for cells to reach a critical volume coincides with the experimentally determined time of 50% hemolysis, is supposed to be the unknown value.

As a consequence of the developed theory of hypotonic hemolysis, it follows that the time t of 50% hemolysis is not directly related to the time required for the cells to reach the volume when hemolysis starts. These times can significantly differ, because the process of hemoglobin release from erythrocytes occurs not instantaneously but over quite an extended period of time. This supposition of the model agrees with the published experimental data (2,16) whereby the direct microscopic observation the spherification time of human erythrocyte is shown to be essentially less than that of 50% hemoglobin release. Until now, this fact has been explained by the potassium leakage preceding hemoglobin release. The proposed model explains the effect otherwise by suggesting hemoglobin is released from cell gradually through periodical opening and closing of a pore (or pores).

This model permits the theoretical determination of the time of 50% hemoglobin release. Moreover, the critical volume at which hemolysis starts is calculated in the frames of the model and depends upon enviromental conditions, but is not a fixed value determined beforehand. Obviously, this distinction results in the membrane permeability coefficient defined from the model being higher than that obtained accoding to the other models. It follows from the fact that traditionally the time of 50% hemolysis is identified with a time during wich the cell reaches the critical volume. While in the proposed model the time of 50% hemolysis is given by the sum of the times at which the cell reaches the critical volume and time of gradual hemoglobin release up to 50%. So the time of 50% hemolysis is always higher than that required by the cells to reach a critical volume. Therefore, the permeability coefficients for electrically neutral substances measured by the previously applied method are underestimated. The lower the value of the permeability coefficient, the greater the error.

The model developed may be used for the description of osmotic lysis in other cell types. However, comparison of the theoretical dependence [9] to the experimental data (9) shows γ_0 to be equivalent to the linear tension in lipid bilayer. Therefore it is likely a macroscopic pore forms in the regions sparse in actin-spectrin filaments. From the theoretical point of view, the difference in cytoskeletal structure may affect the pore formation process quantitatively, since other cell types are characterised by different parameters, particularly by elasticity modulus and linear pore tension. However, the model may be applied for the other cell types.

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Appendix 1

Membrane transfer of electrically neutral permeable substance is recognized as being described by the Kedem-Katchalsky ordinary differential equation system (8):

$$\frac{d}{dt}V = L_p RT\left[\sigma_s\left(n_s - n_s'\right) + \left(n_n - n_n'\right) - \Delta p\right] \qquad [A1.1]$$

$$\frac{d}{dt}N_s^{in} = -k_s\left(n_s - n_s'\right) + \bar{n}_s\left(1-\sigma\right)\frac{d}{dt}V \qquad [A1.2]$$

where N_s^{in} represents moles of permeable substance inside the cell, $\bar{n}_s$ is a mean concentration of this substance inside and outside the cell, n_s and n_s' is a concentration of permeating substance inside and outside the cell, n_n and n_n' is a concentration of impermeating substance inside and outside the cell respectively. Other designations are the same.

The molar concentration of the s substance inside the cell is $n_s^{in} = \frac{N_s^{in}}{V - V_n}$, where V_n – the volume of intracellular nonsolvent fraction (V_n = const). Consequently,

$$\frac{d}{dt}N_s^{in} = \left(V - V_n\right)\frac{d}{dt}n_s^{in} + n_s^{in}\frac{d}{dt}V \qquad [A1.3]$$

Substitution of [A1.3] in [A1.2] gives:

$$\frac{d}{dt}n_s^{in} = \frac{1}{V - V_n}\left\{-k_s\left(n_s^{in} - n_s^{out}\right) + \left[\left(\bar{n}_s - n_s^{in}\right)\left(1-\sigma_s\right) - \sigma_s n_s^{in}\right]\frac{d}{dt}V\right\} \qquad [A1.4]$$

Since $\bar{n}_s - n_s^{in} = \frac{n_s^{out} - n_s^{in}}{2}$, therefore $\left(\bar{n}_s - n_s^{in}\right)\left(1-\sigma_s\right)\frac{d}{dt}V$ is the second order value of thermodynamic moving forces causing the substance transfer through membrane. This value can be neglected compared with the other additives in the frames of linear irreversible thermodynamics. Hence

$$\frac{d}{dt}n_s^{in} = -\frac{1}{V - V_n}\left[k_s\left(n_s^{in} - n_s^{out}\right) + \sigma_s n_s^{in}\frac{d}{dt}V\right] \qquad [A1.5]$$

Intracellular and extracellular osmotic pressures are $\pi = RTn_s^{in}$ and $\pi' = RTn_s^{out}$ correspondingly by definition. Taking into account the accepted designations, the equation [A1.5] results in the second equation of [1]. The first equation of [1] is equivalent to [A1.1].

Appendix 2

According to the elasticity theory, the free energy of the isotropically strained cell membrane is known to be as follows:

$$F_g = \frac{\Gamma}{2}\frac{(S-\tilde{S})^2}{\tilde{S}}$$

[A2.1]

where S and $\tilde{S}$ are the surface areas of unstrained and strained membraine. On the one hand the free deformation energy decreases for the value of $\frac{\Gamma}{2}\frac{(S-\tilde{S}+\pi a^2)^2}{\tilde{S}} - \frac{\Gamma}{2}\frac{(S-\tilde{S})^2}{\tilde{S}}$, but on the other hand it increases, since the pore formation requires an additional energy $2\pi a \cdot \gamma_0$, where γ_0 represents the energy of the pore edge length unit, i.e. linear pore tension. Hence, the free deformation energy of the isotropically strained membrane with the pore of, a, radius is equal to

$$F_{ga} = \frac{\Gamma}{2}\frac{(S-\tilde{S}+\pi a^2)^2}{\tilde{S}} + 2\pi a\gamma_0$$

[A2.2]

From [A2.2] it follows that under a low strain this energy increases monotonously as a function with increasing of the pore radius. Therefore acording to the thermodynamical principle of minimum free energy a pore formation is energetically unfavourable under a low strain. However, under a strong enough strain free deformation energy F_{ga} as a function of pore radius has a minimum at pore radius different from zero. When the minimum free deformation energy is less than that without a pore, then the pore formation will be inevitable. In this case the activation energy barier, ΔF, that the thermodynamical system has to overcome for the pore formation may be easyly determined by the calculations: $\Delta F = \frac{\pi\gamma_0^2}{S}$. As follows, this barier decreases with increasing of the isotropic strain.

According to the theory of the activation type processes, the average time of a thermodynamical system trasfer from one local minimum to the next one over the energy barier, ΔF, due to thermal fluctuations is proportional to the activation multiplier: $\langle t_p \rangle \sim \exp\frac{\Delta F}{kT}$.

The preexponential multiplier, used in [9] may be obtained by the more detailed transfer analysis of the thermodynamical system according to the Focker- Plank equation.

The formula [11] could be directly applied for determining the time of a pore formation in the isotropically strained erythrocyte membrane only in the case of an infinitively slow increase of the cell volume. Then, the time and relative volume y^*, at which a rupture occurs could be obtained as coordinates of the cross point of the plots $\langle t_p \rangle(y)$ and t(y), i.e. the cross point of the plots determined by [11] and [8].

However the rates of the cell volume change and the membrane strain (when $y>y_s$) are finite. Therefore, the strain which is energetically favourable for the pore formation is not sufficient for the actual pore formation. The time during which membrane is subjected to the necessary strain is required to be juxtaposed to the average time of pore formation $\langle t_p \rangle$ in the isotropically strained membrane under this strain. This statement can be presented in the analytical form as the equation [12].

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