CryoLetters **26** (1), 65-72 (2005)

EXPERIMENTAL DETERMINATION OF HUMAN ERYTHROCYTE MEMBRANE PERMEABILITY COEFFICIENTS FOR A SERIES OF AMIDES

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Abstract

Erythrocyte membrane permeability coefficients have been determined for a series of amides by a method based on the physical and mathematical modelling of hypotonic haemolysis process. The results show that penetration of the substances occurs by two alternative ways – through aqueous pores formed by proteins and by the direct dissolving of the molecules in membrane lipids. This conclusion can be confirmed by the correlation analysis between permeability coefficients of native erythrocytes and those pre-incubated with the monosodium salt of p-chloromercuribenzenesulfonic acid (pCMBS), and the partition coefficients of the substances in hydrophilic-hydrophobic phases. Penetration of substances through hydrophilic channels is limited by the sterical factor and diameter in particular. Permeability coefficients for erythrocytes pre-incubated with pCMBS increase in an accordance with the rise of the partition coefficients with correlation coefficient of 0.94, thereby indicating a lipid route of permeation of molecules through erythrocyte membranes.

Keywords: human erythrocyte, permeability coefficients, amides.

INTRODUCTION

Human erythrocytes have been shown to possess an enormously high permeability for aqueous molecules (2,6,11,12,16) as well as high permeability for small hydrophilic non-electrolytes (11,12,21,22). A bulk number of experimental data (3,11,12,18,19,21,22) testify that aqueous and small hydrophilic molecules permeate, at least partially, through human erythrocyte membrane by the ion-exchange integral protein of band III. Reagents such as the monosodium salt of p-chloromercuribenzenesulfonic acid (pCMBS) can block this kind of channel (15). Our previous report (7) concerns the permeability of human erythrocyte membranes for a series of diols. The aim of the investigation was to compare physical and chemical properties of the molecules in homologous series and structural isomers and elucidate the influence of these properties on membrane permeability. The investigations show that substances can penetrate by two different routes: through hydrophilic protein channels with fixed dimension, which can be blocked by pCMBS, and through lipid bi-layer. The evidence for the lipid pathway is based on the directly proportional dependence of membrane permeability for diols after erythrocyte incubation with pCMBS on partition coefficient of the penetrating substance between hydrophobic and hydrophilic phases. Inversely proportional dependence of permeability on the molecule volume is observed for

this pathway. Penetration through hydrophilic protein channels depends more critically on the molecular diameter. When the molecular diameter exceeds the value of 4 Å, the permeability coefficient obviously decreases for hydrophilic molecules. For example, this is evident for propanediol isomers and for the series of 'ethylene glycol – diethylene glycol – triethylene glycol'. The geometrical dimension of the molecules is a decisive factor that influences their permeability. In the case of the substances with higher hydrophobic ability, for example butanediols, a decrease in the permeability through protein channels exhibits a lesser percentage of blocking, while joint permeability increases at the expense of lipid permeability.

This study continues the investigation of human erythrocyte permeability for another set of substances – a series of amides.

MATERIALS AND METHODS

Chemicals

The following non-electrolytes have been chosen for investigation: acetamide, methyl acetamide, dimethyl formamide, dimethyl acetamide. All substances were of chemical pure standards and additionally purified. Acetamide was twice re-crystallised, firstly from the methanol and ethyl ether mixture and then from absolute dioxane, and dried in vacuum at 271 K. Methyl acetamide, dimethyl formamide and dimethyl acetamide were kept for 10-12 hrs on calcium oxide with periodical shaking, and supernatant was twice distilled at the residual pressure 3-4 mm Hg (17).

The geometrical parameters of the molecules were estimated based on Stewart models using the Hyper Chem Pro v.5.1 software.

Partition coefficients of the substances were determined in the system water/non-polar phase. N-octanol has been used as non-polar component. In contrast to oil, it is completely standardised. Measurement of the concentration of the substances in aqueous phase was performed with a refraction method (20) and the partition coefficients were determined by the following formula: $Kp=(C_1-C_2)/C_2\times V_1/V_2$, where C_1 represents the initial concentration of the substance in the aqueous phase; C_2 is the equilibrium concentration of the substance after mixing with n-octanol; V_1 is the volume of the aqueous phase in ml; V_2 - the volume of n-octanol in ml.

Blood samples

Erythrocytes were obtained from the blood bank supplied by the Kharkov State Blood Perfusion Station. The erythrocytes were isolated 2 days after blood collection in order to test blood for AIDS, hepatitis and other infections. Only uninfected blood was used in the experiments. The state of erythrocytes was controlled using light microscopy. After two days of storage at hypothermic conditions the erythrocytes had normal discoid shape and retained the ability to form rouleaux.

The aqueous protein channels were blocked using p-Cloromercuribenzenesulfonic Acid, Monosodium Salt (pCMBS, Sigma). The erythrocytes were incubated with 2mM pCMBS for 1 hour at 22°C (13). Native erythrocytes and erythrocytes after incubation with pCMBS were washed in phosphate-buffered saline (5 mM sodium phosphate, 150 mM NaCl, pH 7.4).

Theoretical basis of small angle light scattering method

Permeability coefficients were determined by a method elaborated by us, based on the physical and mathematical model of haemolysis in aqueous solutions of permeating substance

(5). The kinetics of hypotonic haemolysis were registered by small angle light scattering in cell suspension.

According to the accurate theory of light scattering by spherical particles, the intensity of the light scattered under a small angle θ by the spherical particle with radius R, is presented as (8):

$$J(\theta)=(2\pi/\lambda)^2(R^4/r^2)F(\rho,z) \quad [1]$$

where

$$\rho = 2x(n^{in}/n^{out} - 1),\ z = x\theta,\ x = 2\pi Rn^{out}/\lambda, \quad [2]$$

I_0 – the intensity of the reference beam, λ - the wavelength of the reference beam, which incidences in the solution with refraction coefficient of n^{out} where the sphere is placed, r – the distance between the scattering sphere and the photo-receiver.

As shown in (5), during hypotonic haemolysis an erythrocyte can be considered as a sphere pulsing in time from the moment when the cell volume exceeds the value of $S_o^{3/2}/6\sqrt{\pi}$, erythrocyte haemoglobin content changes spasmodically at the point, when a macroscopic pore forms in the erythrocyte membrane and eventually falls to zero. Thus, we can use equation [1] for the calculation of the intensity of light scattered by erythrocyte as a function of time during hypotonic haemolysis. In this case, membrane scattering could be neglected (14) and intracellular substances are assumed to be homogeneously distributed in the cell volume.

The dependence of the refraction coefficient on volumetric haemoglobin content α/y ($\alpha=V_n/V_0$ – volumetric content of osmotically inactive intracellular substances, V_n – volume of osmotically inactive substances, practically haemoglobin volume inside the cell, V_0 and V – initial and current cell volume, $y = V/V_0$) is as follows

$$n^{in} = n^{out} + (n_{Hb}-n^{out})[1-\exp(-k\alpha/y-\alpha)] \quad [3]$$

where n_{Hb} - haemoglobin refraction coefficient. It has a real value in the red spectrum region of visible light, as spectral haemoglobin bands are in the blue spectrum region. Proportionality coefficient k - is a fitting parameter, which could be determined by comparing the theoretically calculated intensity of the scattered light according to [1] with that experimentally determined.

For the device used ($\lambda = 10^{-6}$m, $\theta = \pi/20$) k = 9 in [3]. Extra-cellular refraction coefficient approximately equals that of water (4/3). Haemoglobin refraction coefficient n_{Hb}= 1.4 (9). Thus [3] could be presented as

$$n^{in}-n^{out}=0.05[1-\exp(-9\alpha/y-\alpha)] \quad [4]$$

Taking into account the above values and conditions, the parameters in the equation [2] in the case of light scattering under an angle of 9° are

$$\rho=0.05(4\pi R/\lambda)[1-\exp(-9\alpha/y-\alpha)],\quad `z=2\pi^2Rn^{out}/20\lambda \quad [5]$$

Obviously, $R = (3V_0y/4\pi)^{1/3}$. Furthermore, when $\lambda = 10^{-6}$m, during hypotonic haemolysis the parameters ρ, x and $(n^{in}/n^{out}-1)$ change in the following limits

$$0 \le \rho \le 2.75; \qquad 27.6 \le x \le 34.5; \qquad 0 \le (n^{in}/n^{out}-1) \le 0.03 \quad [6]$$

In the case of anomalous diffraction when x»1, $(n^{in}/n^{out}-1)$«1 and at intermediate values of ρ, the accurate Mie solution for the scattering by homogeneous sphere (8) can be accurately approximated by the

$$F(\rho,z)=\frac{\pi}{2}\frac{\rho^2}{2(\rho^2+z^2)^{3/2}}I_{3/2}^2\sqrt{\rho^2+z^2}+\frac{\rho^4}{z^4}[I_2(z)-\frac{1}{1\cdot 3}\xi I_3(z)+$$

$$+\frac{1}{1\cdot 3\cdot 5}\xi^2 I_4(z)-\frac{1}{1\cdot 3\cdot 5\cdot 7}\xi^3 I_5(z)+...]^2 \qquad [7]$$

where I_ν - represents ν-order Bessel function of the first kind, $\xi=\rho^2/z$ and the series in square brackets is convergent at any combinations of ρ and z. Substitution [7] to [1], taking into account [2] and [5], will give the intensity of light scattered by an erythrocyte under the angle of 9° towards the reference beam

$$J=J\left(\frac{\pi}{20}\right)=Cy\left\{\frac{1}{\left[1-\exp\left(-\frac{9\alpha}{y-\alpha}\right)\right]\left[1+\frac{\pi^2(n^{out})^2}{4\left[1-\exp\left(-\frac{9\alpha}{y-\alpha}\right)\right]^2}\right]}x\right.$$

$$\times J_{3/2}^2\left(\frac{0{,}2\pi}{\lambda}\left(\frac{3V_0y}{4\pi}\right)^{1/3}\left[1-\exp\left(-\frac{9\alpha}{y-\alpha}\right)\right]\left[1+\frac{\pi^2(n^{out})^2}{4\left[1-\exp\left(-\frac{9\alpha}{y-\alpha}\right)\right]^2}\right]^{1/2}\right)+$$

$$\frac{6{,}4\left(\frac{3V_0y}{4\pi}\right)^{1/3}\left[1-\exp\left(-\frac{9\alpha}{y-\alpha}\right)\right]^4}{\lambda\pi^4(n^{out})^4}\left[J_2\left(\frac{0{,}1\pi^2n^{out}}{\lambda}\right)\left(\frac{3V_0y}{4\pi}\right)^{1/3}-\right.$$

$$-\frac{1}{1\cdot 3}\xi J_3\left(\frac{0{,}1\pi^2n^{out}}{\lambda}\left(\frac{3V_0y}{4\pi}\right)^{1/3}\right)+\frac{1}{1\cdot 3\cdot 5}\xi^2 J_4\left(\frac{0{,}1\pi^2n^{out}}{\lambda}\left(\frac{3V_0y}{4\pi}\right)^{1/3}\right)-$$

$$\left.\left.-\frac{1}{1\cdot 3\cdot 5\cdot 7}\xi^3 J_5\left(\frac{0{,}1\pi^2n^{out}}{\lambda}\left(\frac{3V_0y}{4\pi}\right)^{1/3}\right)+\frac{1}{1\cdot 3\cdot 5\cdot 7\cdot 9}\xi^4 J_6\left(\frac{0{,}1\pi^2n^{out}}{\lambda}\left(\frac{3V_0y}{4\pi}\right)^{1/3}\right)-...\right]^2\right\} \qquad [8]$$

where $C=\frac{15}{2}\pi\frac{V_0}{\lambda r^2}I_0$

When the time dependence of the relative spherocyte volume y (at $y\geq y_s$) is known as well as cell haemoglobin content α , the time course of the intensity of the light scattered under the angle of π/20 towards the reference beam with the wavelength of λ=1 μm could be obtained.

Permeability coefficients determination

Permeability coefficients for non-electrolytes were determined as follows. At the initial moment, 3μl of washed packed erythrocytes were added to 3ml of aqueous solution of the penetrating substance, in the chamber of the device for measuring of the intensity of the scattered light. The time dependence of the light scattered by the cell suspension was registered at 9° towards the reference beam (λ= 1000nm). The intensity of the light scattered

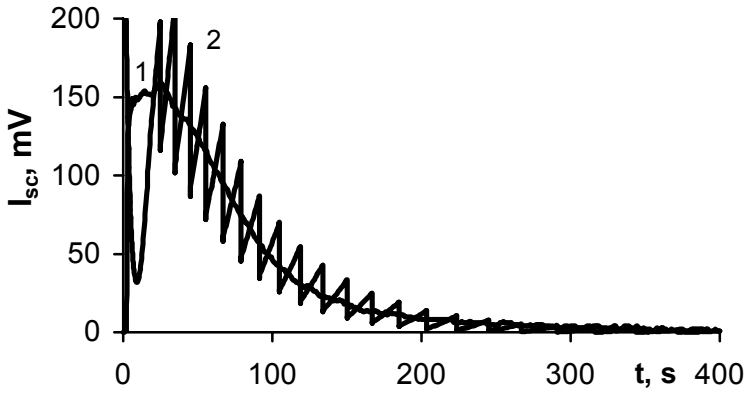


Fig.1. Coincidence of experimental (1) and theoretical (2) curves of light scattering intensity (I_{sc}) vs time (t, s) by erythrocyte suspension at hypotonic haemolysis

can be determined theoretically based on the physical and mathematical model of hypotonic haemolysis (5) and using Mie scattering theory (8). Therefore, we obtain the theoretical dependence of the light scattered by the cell suspension under a certain angle upon the concentration of haemoglobin in the cell during haemolysis. The value of permeability coefficient could be obtained by the coincidence of the experimental (Fig.1, plot 1) curve of the intensity of the light scattered by erythrocyte suspension and theoretical (Fig.1, plot 2) curve at the convenient value of this coefficient.

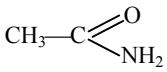
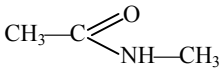
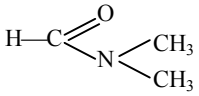
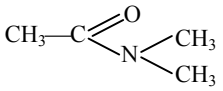
RESULTS

Following the described method, permeability coefficients of human erythrocyte membranes for the substances under investigation were determined at native conditions and after pre-incubation with pCMBS. Measurements were carried out at 20°C and the concentrations used were 1M.

The experimental results and calculated geometrical parameters of the molecules are presented in Table 1 as well as previously obtained (10) octanol/aqua partition coefficients (K_p), which reflect hydrophobic properties of the molecules.

The data obtained show that permeability coefficients of native erythrocytes for amides only slightly differ in the series. However, dimethyl formamide permeability is authentically (P=0.999) higher than that of acetamide and methyl acetamide. By analogy the permeability coefficient for dimethyl acetamide could be expected to increase according to its higher value of partition coefficient, but in fact it is even less than that for dimethyl formamide (P=0.95) (Fig.2, plot1). With reference to the molecular diameters presented in column 6 of Table 1, one could see that the permeability coefficient decreases, when molecular diameter exceeds 4Å, as permeability through aqueous pores decreases. This is

Table 1. Erythrocyte membrane permeability coefficients (P) at 20°C and partition coefficients (K_p) and geometrical parameters of penetrating molecules (D – diameter, L – length, V - volume)

Substance	Structural formulae	Permeability coefficients, $P \cdot 10^6$ m/s		Partition coefficient, K_p	Geometrical parameters of molecule		
		Native erythrocytes	PCMBS treated erythrocytes		D, Å	L, Å	V, $Å^3$
Acetamide		2.66±0.18	0.528±0.07	0.062	3.2	3.8	30.5
Methyl acetamide		2.64±0.2	1.37±0.23	0.113	3.3	4.9	42.4
Dimethyl formamide		2.96±0.1	1.96 ±0.13	0.233	3.9	4.1	44.0
Dimethyl acetamide		2.86±0.14	2.04±0.22	0.291	4.2	5.1	70.6

NB. Probability of difference between permeability coefficients of native erythrocytes and those treated with pCMBS is >0.999 for all the substances

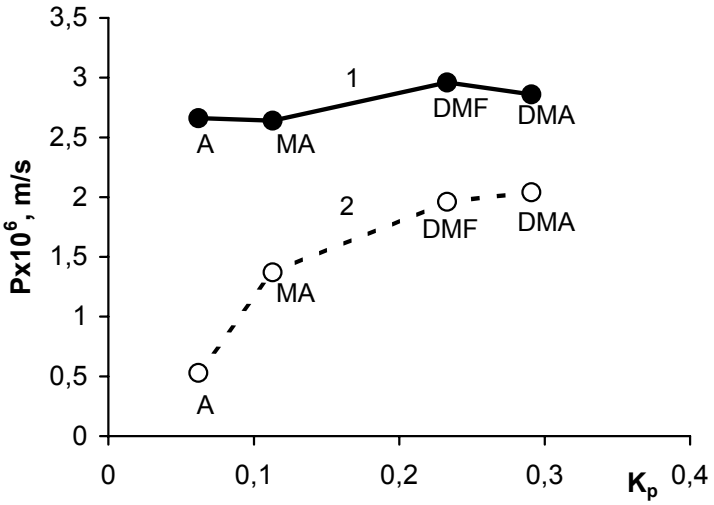


Fig. 2. Dependence of erythrocyte membrane permeability coefficients (P, m/s) at 20°C vs. partition coefficient (K_p) of the penetrating substances in the "*n*-octanol-water" system. 1 – native erythrocytes; 2 – pCMBS treated erythrocytes; A – acetamide, MA – methyl acetamide; DMF – dimethyl formamide; DMA – dimethyl acetamide.

confirmed by the permeability coefficients of the substances presented in columns 3, 4 of the table.

Permeability coefficients obtained for erythrocytes pre-incubated with pCMBS additionally confirm the suggestion about an alternative lipid permeability pathway in an erythrocyte membrane for the substances under investigation. Obviously, permeability coefficients for erythrocytes pre-incubated with pCMBS increase in an accord with the rise of the partition coefficients (Fig.2, plot 2), with correlation coefficient of 0.94. These results are in good agreement with those for butanediol isomers (7), where correlation coefficient for the same parameters is 0.996.The higher correlation coefficient for butanediols could be due to less structural differences in the series as compared to the amides.

The rate of amides permeability inhibition, caused by incubation with pCMBS, is in an accord with the molecular size, particularly its diameter. For dimethyl acetamide with the diameter of 4.2 Å it comes only to 28.5%. The correlation coefficient between the rate of amides permeability inhibition and diameters of the molecules is -0.87. It is worth mentioning that in erythrocytes pre-incubated with pCMBS the permeability coefficients for acetamide and ethylene glycol (7), with close geometrical parameters, almost coincide (0.528×10^{-6} m/s and 0.526×10^{-6} m/s respectively).

DISCUSSION

In this work erythrocyte membrane permeability coefficients for a series of amides have been determined by the method developed in our laboratory. The values obtained are in line with those obtained by other methods. For example, the permeability coefficient for acetamide $(2.68\pm0.18)\times10^{-6}$ m/s almost coincides with the published data, $(2.76\pm0.5)\times10^{-6}$ m/s (21). There is also good agreement between permeability coefficients for ethylene glycol measured by our method $(1.98\pm0.48)\times10^{-6}$m/s (7) and those reported by Toon and Solomon (21). At the same time we obtained a considerably higher (1.5 times) value of permeability coefficient for methyl acetamide, compared with those measured by Toon and Solomon (22).

The results testify that penetration of the substances occurs by two alternative ways – through aqueous pores formed by proteins and by the direct dissolving of the molecules in membrane lipids. This conclusion can be confirmed by the correlation analysis between permeability coefficients of native erythrocytes and those pre-incubated with pCMBS and the partition coefficients of the substances in hydrophilic-hydrophobic phases. Penetration of substances through hydrophilic channels is limited by the sterical factor and diameter in particular.

In contradistinction to our interpretation, Solomon (19) suggested that band III protein, which forms the channel, changes its conformation by interaction with pCMBS. As a consequence, the residual permeability of the substances as well as water could occur through the same pores, but is limited by the conformational changes in the channel. According to this preposition, the permeability of small molecules (in particular amides and diols) is subjected to less inhibition, than that of large molecules. In contrast, our results show higher inhibition of permeability in small hydrophilic molecules (in particular, acetamide, ethylene glycol, 1,2-propanediol) which have high permeability coefficients in native erythrocytes. At the same time, permeability coefficients for the substances which penetrate less efficiently through the membrane of native erythrocytes due to sterical limits (dimethyl acetamide, 1,3-propanediol, triethylene glycol), are subjected to less changes. This is also true for the substances with high values of partition coefficient between hydrophilic and hydrophobic phases (dimethyl acetamide, dimethyl formamide, 2,3- and 1,2-butanediol). Moreover, the data on permeability of artificial lipid bilayers for small non-electrolytes give additional evidence to our interpretation of the permeability process. According to the published data (4), permeability

of lipid bilayer membrane comprised of phosphatidylcholine and cholesterol for acetamide and 1,4-butanediol are 0.196×10^{-6} m/s and 0.2×10^{-6} m/s, respectively. This is in good accord with our data for the erythrocytes pre-incubated with pCMBS taking into account that permeability of membrane depends on its composition. These values are slightly less than ours due to the difference in the indexes of double bonds of phosphatidylcholine (0.96) and for common lipids (1.4) of human erythrocyte membrane (1).

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