ELSEVIER

Bioelectrochemistry and Bioenergetics 43 (1997) 97–103

BIOELECTROCHEMISTRY AND BIOENERGETICS

Determination of the inner surface potential of the erythrocyte membrane

Aleš Iglič [a,b,*], Milan Brumen [c,d], Saša Svetina [b,c]

[a] *Faculty of Electrical Engineering, University of Ljubljana, Tržaška 25, SI-1000 Ljubljana, Slovenia*
[b] *Institute of Biophysics, Medical Faculty, University of Ljubljana, Lipičeva 2, SI-1000 Ljubljana, Slovenia*
[c] *Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia*
[d] *Department of Physics, Faculty of Education, University of Maribor, Koroška cesta 160, SI-2000 Maribor, Slovenia*

Received 25 July 1996; revised 1 October 1996

Abstract

The time dependent uptake of 1-anilino-8-naphthalene sulphonate (ANS) by bovine erythrocytes was studied. Experimental results were compared with theoretical predictions in order to determine the inner erythrocyte membrane surface potential. The value of the inner membrane surface potential under physiological conditions was estimated as −35 mV. In addition, the number of ANS binding sites on the outer surface of the single bovine erythrocyte membrane was determined as 2.2×10^6. © 1997 Elsevier Science S.A.

Keywords: Membrane; Surface potential; Erythrocyte; Diffusion

1. Introduction

Some of the constituents of the biological membranes are ionized and therefore these membranes are electrically charged. The membrane charge, its spatial distribution and the spatial distribution of ions in the solution in the close proximity of both membrane surfaces determine the profile of electric potential across the membrane [1–5]. For the sake of simplicity the electric properties of the membrane are usually described in terms of the values of the electric potential at some characteristic points and the electric potential differences such as the membrane surface potentials at both membrane–solution interfaces, the membrane internal potential and the transmembrane potential [6–10].

The membrane surface potential is defined as the potential difference between the membrane–solution interface and the bulk-solution phase. It results from the charged groups at the membrane–solution interface. The inner and outer membrane surface potentials are important functional properties of the membrane. Among others they determine the partition of some biologically important charged membrane ligands between the bulk-solution and the membrane–solution interfaces and thus affect their binding to the membrane [3,11]. They also influence the transport of ions across the membrane [2,12].

The outer membrane surface potential can be estimated by different experimental methods such as electrophoresis [12–14], NMR methods [14], EPR methods [15,16] or fluorescence methods [12,14,17].

The inner membrane surface potential can not be measured directly. To estimate its value inside-out membrane vesicles must be prepared [16,18]. The inner membrane surface potential can also be estimated indirectly by studying the transmembrane transport of ions, where the necessary information is provided by the relaxation data [15,19,20]. For the erythrocyte membrane, preliminary such measurements suggest that cytoplasmic membrane leaflet has negative surface potential [16,21]. This is in accordance with the known asymmetric structure of the erythrocyte membrane. Namely, head-groups of aminophospholipids which are predominantly situated in the inner bilayer leaflet as well as the spectrin network attached to the bilayer at the inner side carry negative charge [22,23].

A comprehensive study of an indirect determination of the inner surface potential in the erythrocyte membrane is given in this paper. The inner bovine erythrocyte membrane surface potential is obtained by studying the time dependent uptake of 1-anilino-8-naphthalene sulphonate (ANS) by erythrocytes in the isotonic solution of sodium chloride and saccharose. A mathematical model of ANS uptake, including the dependence of influx of negatively charged ANS molecules on the surface potential difference, is constructed. In the model, the binding of ANS to

* Corresponding author. Tel.: +386-61-314127; fax: +386-61-1315127.

PII S0302-4598(96)05181-1

the outer surface of the membrane and to haemoglobin is also taken into account. From the comparison of the experimental results and the theoretical predictions the value of the inner surface potential is estimated.

2. Model and basic assumptions

2.1. System

When erythrocytes free of ANS are suspended in the isotonic solution with ANS added, a loss of ANS in the outer medium occurs. This is partly due to the binding of ANS to the erythrocyte membrane in the phospholipid head region which is almost an instantaneous process [24,25]. On the time scale of 1 h, a loss of ANS is a consequence of diffusion of ANS into erythrocytes [24] through the cell membrane which is permeable for ANS molecules [12]. In erythrocytes ANS partially binds to haemoglobin [26].

Our basic assumption is that the influx of negatively charged ANS molecules in the interior of erythrocyte is driven by the difference between the values of electrochemical potentials at the two membrane–solution interfaces. This difference arises due to the ANS concentration difference as well as due to the electric potential difference between the two membrane–solution interfaces, the later reflecting the whole electric potential profile across the membrane.

The electric potential profile at both membrane–solution interfaces is described in this work in the model of two electric double layers, separated by the erythrocyte membranes (Fig. 1). Indices i and o refer to the inner and outer membrane surfaces and to the inner and outer solution of the erythrocyte suspension, respectively. Here, ψ_i and ψ_o are the inner and outer electric potentials in the bulk solution while ψ_i^m and ψ_o^m are the electric potentials at the inner and outer membrane–solution interfaces. Potential differences $\Delta\psi = \psi_o^m - \psi_o$ and $\Delta\psi_i = \psi_i^m - \psi_i$ are defined as the inner and outer membrane surface potential, respectively, while $\Delta\psi_{Cl} = \psi_i - \psi_o$ is known as the transmembrane potential. The electric potential difference between the two interfaces $\Delta\psi_m = \psi_i^m - \psi_o^m$ can then be expressed as:

$$\Delta\psi_m = \Delta\psi_i - \Delta\psi_o + \Delta\psi_{Cl} \quad (1)$$

Symbols d_i and d_o in Fig. 1 denote the effective thickness of erythrocyte membrane spectrin network and glycocalyx, respectively, while d is the thickness of lipid bilayer part of the membrane.

Charged solute molecules are distributed in the vicinity of the membrane in accord with the electric potential profile (Fig. 1). Thus, as each ANS molecule bears a single negative unit charge, the molar ANS concentration at the membrane–solution interface (c_j^m) differs from that in the bulk solution (c_j):

$$c_j^m = c_j \exp\left(\frac{e_0 \Delta\psi_j}{kT}\right), \; j = \mathrm{i}, \mathrm{o} \quad (2)$$

where k is the Boltzmann constant, e_0 unit charge and T absolute temperature.

In the experimental and theoretical considerations presented in this work the ANS uptake by bovine erythrocytes is studied in erythrocyte suspension where the outer solution is always isotonic solution of sodium chloride and saccharose with ANS added. Changing the ratio of NaCl/saccharose in the outer solution of erythrocyte suspension at isotonic conditions would lead to different transmembrane potential $\Delta\psi_{Cl}$,

$$\Delta\psi_{Cl} = \left(\frac{kT}{e_0}\right) \ln\left(\frac{c_{Cl,i}}{c_{Cl,o}}\right) \quad (3)$$

and different outer surface potential $\Delta\psi_o$ [2,3], which would exert an effect on ANS uptake by erythrocytes. Here, $c_{Cl,i}$ and $c_{Cl,o}$ denote molar chloride concentration in the inner and outer solution, respectively. The outer surface potential $\Delta\psi_o$ at given external sodium chloride concentration can be calculated according to the theory of electric double layer from the Poisson–Boltzmann equation (see Appendix A), while the inner surface potential is taken to be constant during the variation of the NaCl/saccharose ratio in the outer solution. Namely, it was shown that the intracellular conditions are not affected significantly by changing the extracellular NaCl/saccharose ratio [27].

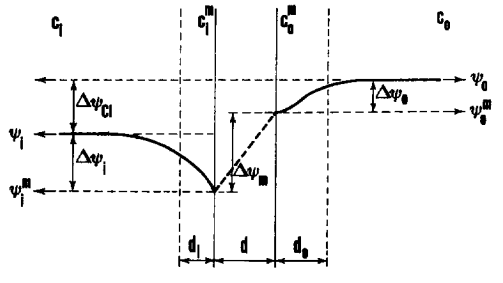

Fig. 1. Schematic presentation of electric potential profile on both sides of erythrocyte membrane and the corresponding ANS concentrations in erythrocyte suspension [2,4,7]. The meanings of the symbols are given in the text.

2.2. ANS binding

The magnitude of the negative inner surface potential is expected to be much higher than the magnitude of the negative outer surface potential [2,16,21] indicating a weaker binding of ANS to the inner bilayer leaflet. It was also demonstrated that phosphatidylethanolamine which is mostly placed in the inner bilayer leaflet of the bovine erythrocyte membrane [28,29] practically binds no ANS molecules [30]. Therefore, it is taken in this work that

negatively charged ANS molecules bind only to the outer side of the membrane.

The binding of negatively charged ANS molecules to the outer erythrocyte membrane surface is described here by the Langmuir adsorption equation [7,31]:

$$N_{MA}/N_{MT} = (c_o^m/K_A)/(1 + c_o^m/K_A) \quad (4)$$

where N_{MA} is the number of bound ANS molecules, N_{MT} is the total number of ANS binding sites on the single erythrocyte membrane surface and K_A is the dissociation binding constant for ANS binding to the outer membrane surface [17] in the absence of electrostatic interactions. It is taken that the binding depends on the ANS concentration at the membrane–solution interface.

The binding of ANS to haemoglobin is described by the first order saturation kinetics as:

$$N_{HbA}/N_{HbT} = (c_i/K_{Hb})/(1 + c_i/K_{Hb}) \quad (5)$$

where N_{HbA} is the number of ANS molecules bound to haemoglobin molecules in a single erythrocyte, N_{HbT} is the total number of ANS binding sites on haemoglobin molecules in a single erythrocyte, while K_{Hb} is the dissociation constant for the ANS binding to the haemoglobin. The ANS binding to haemoglobin depends on the bulk concentration of ANS. The dependence of K_{Hb} on pH is not taken into account. In the range of ANS used in this work, the factor c_i/K_{Hb} can be neglected compared to unity [26], therefore Eq. (5) can be written as

$$N_{HbA}/N_{HbT} = c_i/K_{Hb} \quad (6)$$

The total number of ANS molecules in erythrocyte suspension must be conserved

$$N = N_i + N_o + N_e(N_{MA} + N_{HbA}) \quad (7)$$

where N is the total number of ANS molecules in the system and it equals to the initial number of ANS molecules introduced in the outer solution at time $t = 0$, N_i and N_o are the numbers of free ANS molecules in the corresponding solutions and they change with time due to redistribution across the membrane, while N_e is the total number of erythrocytes in the suspension. By taking into account Eq. (6), the conservation equation Eq. (7) can be written as

$$c_i V_{ef} + c_o V_o = c_o(0) V_o \quad (8)$$

where V_{ef} is defined as the effective volume of the inner solution in the erythrocyte suspension,

$$V_{ef} = V_i(1 + N_e N_{HbT}/K_{Hb} N_A V_i) \quad (9)$$

V_o is the volume of the outer solution in erythrocyte suspension, V_i is the volume of the solution inside all erythrocytes in suspension, i.e. the volume of erythrocytes inner solution where the volume of haemoglobin is not taken into account, N_A is the Avogadro number, and $c_o(0)$ is the molal ANS concentration in the outer solution immediately after the rapid process of the ANS binding to the outer membrane surface and before the ANS influx occurs,

$$c_o(0) = c_T - N_e N_{MA}/N_A V_o \quad (10)$$

c_T is the initial total molar concentration in the outer solution before the fast process of ANS binding to the outer erythrocyte membrane surfaces. In the derivation of Eq. (8), it is taken into account that an amount of ANS molecules in the close proximity to the membrane where the concentration deviates from the bulk concentration is insignificant for the total amount of ANS in suspension. In addition, it is also neglected that the amount of ANS molecules bound to the membranes is decreasing due to the decrease of the outer ANS concentration with time.

2.3. ANS influx

The influx of ANS molecules (J) is described by the modified Goldman equation [2],

$$J = \frac{P e_0 \Delta\psi_m}{kT}\left(\frac{c_o^m - c_i^m \alpha_m}{1 - \alpha_m}\right) \quad (11)$$

$$\alpha_m = \exp\left(-\frac{e_o \Delta\psi_m}{kT}\right) \quad (12)$$

where P is the permeability of the erythrocyte membrane for the ANS molecules. Since in this work all the ANS concentrations are low it is taken [32] that the activity coefficient of ANS is equal to one.

By taking into consideration the continuity equation

$$J N_e A_e = V_o \frac{dc_o(t)}{dt} \quad (13)$$

the mass conservation equation (Eq. (8)) and the flux equation (Eqs. (11) and (12)), it is possible to derive the exponential dependence of the outer ANS bulk concentration on time,

$$c_o(t) = a\exp(-\beta t) + b \quad (14)$$

where

$$a = c_o(0)(1 - \vartheta) \quad (15)$$

$$b = c_o(0)\vartheta \quad (16)$$

$$\vartheta = 1/(1 + V_{ef}\alpha_o/V_o\alpha_m\alpha_i) \quad (17)$$

$$\beta = N_e A_e P \ln\alpha_m(\alpha_m\alpha_i/V_{ef} + \alpha_o/V_o)/(\alpha_m - 1) \quad (18)$$

$$\alpha_j = \exp\left(\frac{e_0 \Delta\psi_j}{kT}\right),\ j = i, o \quad (19)$$

The exponential decay on time of bulk ANS concentration in the outer solution described by Eq. (14) has been observed previously [24].

By taking into consideration Eq. (1) it can be concluded from Eqs. (14)–(19) that influx of ANS in the interior of erythrocyte can be viewed as being dependent on the

transmembrane potential $\Delta\psi_{Cl}$, both membrane surface potentials $\Delta\psi_i$ and $\Delta\psi_o$ and initial ANS concentration in the outer solution after the rapid process of the ANS binding $c_o(0)$, where the influence of the bound molecules on the outer surface potential $\Delta\psi_o$ must also be taken into account. In the derivation of Eqs. (14)–(19), the influence of the release of the bound ANS on the magnitude of the outer erythrocyte surface potential $\Delta\psi_o$ due to decrease of the outer ANS concentration with time is not taken into account.

3. Materials and methods

3.1. Drugs

Sodium chloride, potassium chloride, disodium phosphate, monosodium phosphate, calcium chloride, saccharose and dextrose were obtained from Merck (Darmstadt, Germany), while 1-anilino-8-naphthalene sulphonate (ANS) and citrate–phosphate–dextrose solution (anticoagulant) were purchased from Sigma (St. Louis, USA).

3.2. Preparation of red blood cells

Erythrocytes were centrifuged from fresh bovine blood anticoagulated with citrate–phosphate–dextrose solution. The blood was resuspended in the medium, 150 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 5.5 mmol l^{-1} Na_2HPO_4, 0.8 mmol l^{-1} $NaH_2PO_4 \times H_2O$, 0.5 mmol l^{-1} $CaCl_2 \cdot 6H_2O$, 5 mmol l^{-1} dextrose, pH ca. 7.4, and centrifuged. The supernatant was removed. The washing procedure was repeated three times. The final concentration of erythrocytes was about 80 vol% (determined by the haematocrit measurements in Ultracentrifuge Janetzki TH12, Janetzki Maschinbau, Leipzig, Germany). Erythrocytes were treated at room temperature throughout the preparation and further experiments.

3.3. The kinetics of ANS uptake by the erythrocyte

The time dependent uptake of ANS by the intact erythrocyte was experimentally determined by measuring the free ANS concentration in the outer solution of erythrocyte suspension (c_o) as a function of time. Six equal samples of the erythrocyte suspension with ANS added in the outer solution of the suspension were prepared as follows. The washed cells (1 ml 80 vol%) were resuspended in 5 ml of isotonic ANS–NaCl–saccharose solution, therefore the final volume of the outer solution V_o was 5.2 ml. Knowing the value of the mean bovine erythrocyte volume 58 μm^3 [33], the total number of erythrocytes in the suspension $N_e = 1.38 \times 10^{10}$ was estimated. The initial concentration of ANS in the outer solution before the ANS binding to the outer erythrocyte membrane surfaces (c_T) was varied in different experiments around the value 1.2×10^{-4} mol l^{-1}.

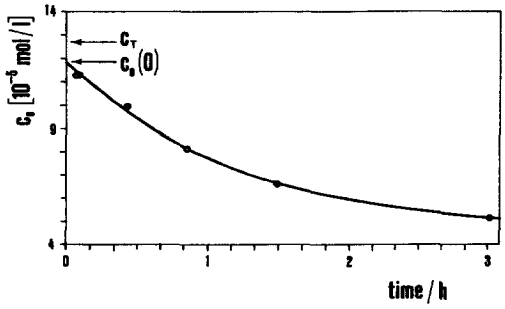

Fig. 2. Time dependence of free ANS concentration $c_o(t)$ in the outer isotonic NaCl/saccharose solution ([NaCl] = 150 mmol l^{-1}) of bovine erythrocyte suspension. Initial total ANS concentration in outer solution c_T was 12.7×10^{-5} mol l^{-1}. Experimentally determined values of $c_o(t)$ (points) were fitted utilizing Eq. (14), where the values of parameters $a = 7.3 \times 10^{-5}$ mol l^{-1}, $\beta = 2.34 \times 10^{-4}$ s^{-1} and $b = 4.57 \times 10^{-5}$ mol l^{-1} are obtained using the least squares method.

At selected time intervals the samples were centrifuged in Centrifuge Janetzki T23 (Janetzki Maschinenbau, Leipzig, Germany) in order to put down intact erythrocytes and membrane fragments produced by partial haemolysis [24]. The concentration of ANS in the supernatant (c_o) was determined from the optical density of supernatant at 370 nm in Shimadzu Multipurpose Recording Spectrophotometer (Shimadzu Seisakusho, Kyoto, Japan). Corrections due to the haemoglobin trace in the supernatant were determined from the optical density of the supernatant at 540 nm where the absorption in the supernatant is predominantly due to the presence of haemoglobin molecules. The measured values of c_o are plotted versus time and fitted by Eq. (14) (Fig. 2), where the parameters a, β and b were determined using the least squares method. The value of c_o at time zero was then determined as $c_o(0) = a + b$.

4. Results

4.1. Determination of the number of ANS binding sites

The number of ANS molecules bound to the outer surface of single erythrocyte membrane (N_{MA}) before ANS influx in erythrocytes was determined in order to estimate the total number of ANS binding sites on the outer surface of the single erythrocyte membrane (N_{MT}). Knowing the initial drop of ANS concentration in the outer solution of the erythrocyte suspension ($c_T - c_o(0)$), the number of bound ANS molecules before ANS influx, i.e., at time $t \cong 0$, may be obtained from Eq. (10):

$$N_{MA}(0) = N_A V_o (c_T - c_o(0))/N_e \quad (20)$$

In order to determine the value of $c_o(0)$ the outer bulk ANS concentration c_o in the intact bovine erythrocyte suspension was measured as a function of time (Fig. 2).

The value of $c_o(0)$ was then determined by extrapolation to time zero as it is described in Section 3.3.

The experiment presented in Fig. 2 was repeated 48 times. The mean value of $c_o(0)$ was 11.28×10^{-5} mol l^{-1}, while the mean value of the $(c_T - c_o(0))$ was 0.74×10^{-5} mol l^{-1}, where the sodium chloride concentration in the outer isotonic solution was 150 mmol l^{-1} in all cases. The volume of the outer solution V_o was 5.2 ml and the total number of erythrocytes in the suspension was $N_e = 1.38 \times 10^{10}$ as it was mentioned before. Therefore by using Eq. (20), the initial number of ANS bound to the outer surface of single erythrocyte $N_{MA}(0) = 1.67 \times 10^6$ at [NaCl] = 150 mmol l^{-1} was determined.

Using the values $N_{MA} = 1.67 \times 10^6$ and $c_o = 11.28 \times 10^{-5}$ mol l^{-1} with ANS binding constant of $K_A = 2.7 \times 10^{-5}$ [34] the total number of ANS binding sites on the outer surface of the single erythrocyte membrane was estimated from Eq. (4): $N_{MT} = 2.18 \times 10^6$, where it was taken into account that ANS concentration at the outer surface of the membrane c_o^m depends on the outer surface potential $\Delta\psi_o$ (Eq. (2)) which is influenced by the bound ANS molecules. Therefore, while calculating N_{MT}, the equations of the electric double layer theory were also considered (Appendix A).

4.2. Determination of the inner surface potential

The comparison of experimental results and theoretical predictions in order to obtain the value of the inner surface potential $\Delta\psi_i$ was carried out in terms of the dependence of the parameter λ:

$$\lambda = (dc_o/dt)_{t=0}/c_o(0) = a\beta/(a+b) \tag{21}$$

on the NaCl/saccharose ratio in the outer solution of the erythrocyte suspension, where the values of parameters a, b and β at given outer sodium chloride concentration can be experimentally obtained from the measurements of free ANS concentration in outer solution $c_o(t)$ (Fig. 2).

By inserting the expressions for a, b and β from Eqs. (15)–(19) in Eq. (21) the parameter λ can also be expressed as

$$\lambda = \frac{N_e A_e P}{V_o} \times \frac{[e_0(\Delta\psi_i - \Delta\psi_o + \Delta\psi_{Cl})/kT]\exp(e_0\Delta\psi_o/kT)}{1 - \exp[-e_o(\Delta\psi_i - \Delta\psi_o + \Delta\psi_{Cl})/kT]} \tag{22}$$

In order to theoretically determine the dependence of parameter λ on the outer sodium chloride concentration from Eq. (22), the dependencies of the transmembrane electric potential $\Delta\psi_{Cl}$ and the outer membrane surface potential $\Delta\psi_o$ on outer sodium chloride concentration are calculated while the inner membrane surface potential $\Delta\psi_i$ is taken as a free model parameter. The value of $\Delta\psi_{Cl}$ as a function of outer sodium chloride concentration is calculated using Eq. (3) for the intracellular chloride concentration $c_{Cl,i} = 128$ mmol l^{-1}. The dependence of $\Delta\psi_o$ on the outer sodium chloride concentration is determined according to the theory of electric double layer (Appendix A), taking into account the equations of ANS binding (Eq. (2), Eq. (4) and Eq. (10)) for $N_{MT} = 2.18 \times 10^6$, determined in the previous section, $c_T = 1.2 \times 10^{-4}$ mol l^{-1}, which is the mean value of c_T in all measurements given in Fig. 3, $K_A = 2.7 \times 10^{-5}$ [34], $V_o = 5.2$ ml and $N_e = 1.38 \times 10^{10}$.

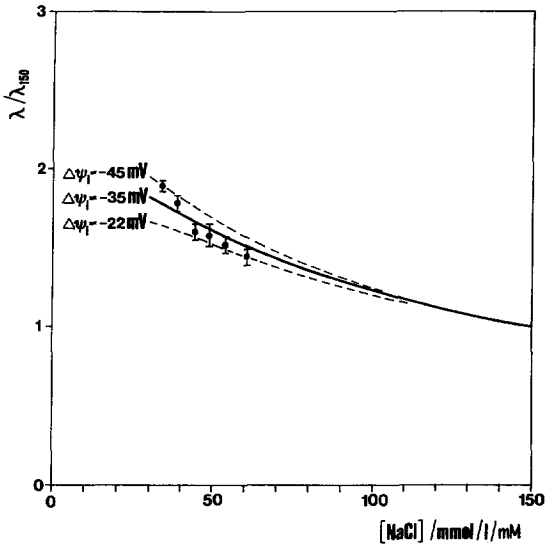

Fig. 3. Experimentally determined (points) and calculated (curves) dependence of the ratio λ/λ_{150} (Eqs. (21) and (23)) as a function of the outer sodium chloride concentration. The curves are calculated for the indicated different values of the inner membrane surface potential $\Delta\psi_i$.

However, the value of membrane permeability P in Eq. (22) can not be determined independently. Therefore in this work the dependence of the ratio λ/λ_{150} on the outer sodium chloride concentration is used for determination of $\Delta\psi_i$, where λ_{150} is the value of λ at sodium chloride concentration of 150 mmol l^{-1}. The ratio λ/λ_{150} can be expressed (Eq. (21)) as

$$\lambda/\lambda_{150} = \left(\frac{a\beta}{a+b}\right)\left(\frac{a\beta}{a+b}\right)^{-1}_{150} \tag{23}$$

In order to determine the value of the inner membrane surface potential $\Delta\psi_i$ the experimentally obtained dependence of the ratio λ/λ_{150} on the outer sodium chloride concentration (Fig. 3) was fitted using Eq. (22) where it is taken that the values of V_o and P are not changing as a function of the outer sodium chloride concentration [27,35]. The pH dependence of the λ/λ_{150} is not taken into account since the inner pH is not altered significantly with the outer sodium chloride concentration [27,35]. The best fit is achieved by an appropriate choice of the parameter $\Delta\psi_i$ (Fig. 3). From comparison of the experimental and theoretical values of λ/λ_{150} given in Fig. 3 the value of the inner membrane surface potential was estimated as $\Delta\psi_i \cong -35$ mV.

5. Discussion

The value of the inner membrane surface potential $\Delta\psi_i \cong -35$ mV determined in this work is smaller than the theoretically predicted value of more than -60 mV which was calculated on the basis of electrostatic model, using the realistic data of electric charges of the haemoglobin molecules, spectrin layer and inner phospholipid molecules of human erythrocyte [2]. This discrepancy may arise due to compositional and structural differences between the human and bovine erythrocytes.

On the basis of different experimental and theoretical results, it can be concluded that the magnitude of the outer erythrocyte membrane surface potential $\Delta\psi_o$ is of the order of 5 mV [2,16] which is much smaller than the magnitude of the inner erythrocyte membrane surface potential determined in this work. This may be explained partly by the fact that acidic lipid molecules in erythrocyte membrane are almost entirely localised in the inner half of the erythrocyte lipid bilayer, while the outer half of the erythrocyte lipid bilayer is practically neutral at physiological pH [22,28,29,36,37]. The other reason is that the negative charges of sialic acids in glycocalyx protrude into the solution phase and therefore the outer erythrocyte surface potential is smaller [13] in comparison to the situation where the glycocalyx sialic acids would be distributed in the plane. In addition, the number of negative elementary charges within the spectrin layer at the inner membrane surface [23] is a few times larger than the number of negative elementary charges within the glycocalyx [38], while its thickness is smaller than the thickness of glycocalyx [39,40].

The value of the inner surface potential of bovine erythrocyte membrane $\Delta\psi_i$ estimated in this work depends on the choice of the values of the model parameters as it is the number of sialic acids in glycocalyx N_{si}, the effective thickness of glycocalyx layer d_o, the dissociation constant for ANS binding to the outer membrane surface K_A and the permittivity of the outer solution ϵ. Namely, the variation of the parameters of the system may influence the calculated value $\Delta\psi_o$ and consequently also the estimated value of $\Delta\psi_i$. However, it can be shown that the variation of these parameters has no essential influence on the estimated value of $\Delta\psi_i$. This can be explained by the fact that the value of $\Delta\psi_o$ is several times smaller than $\Delta\psi_{Cl}$ and $\Delta\psi_i$ [2,16,35] and has therefore minor effect on the transmembrane transport of ANS and consequently also on the estimated value of $\Delta\psi_i$.

Acknowledgements

The authors wish to thank Dr. V. Kralj-Iglič for help in numerical calculations and M. Kozamernik for technical assistance.

Appendix A. Membrane electrostatics

The outer surface potential $\Delta\psi_o$ and the number of ANS molecules bound to the outer surface of single erythrocyte membrane N_{MA} at given sodium chloride concentration in outer solution of erythrocyte suspension ([NaCl]) are calculated by solving simultaneously the equations of ANS binding to the outer bilayer surface (Section 2.2) and the Poisson–Boltzmann equation describing the electrostatics of the outer solution extending in the positive x direction from the outer surface at $x = 0$ [2]:

$$\frac{d^2\psi(x)}{dx^2} = \frac{-1}{\epsilon\epsilon_o}\left(\rho_{GL} - 2e_0 N_A[NaCl]\,sh\left(\frac{e_0(\psi(x)-\psi_o)}{kT}\right)\right) \quad (A1)$$

where ϵ is the permittivity of the outer solution, ϵ_o is the permittivity of the free space and

$$\rho_{GL} = -N_{si}e_0/d_o A_e \quad (A2)$$

is the constant space charge density of glycocalyx in the domain of the outer solution from $x = 0$ to $x = d_o$ [2,13], where N_{si} is the mean number of negatively charged sialic acids in single erythrocyte membrane glycocalyx, d_o is the effective thickness of glycocalyx of single erythrocyte membrane, while A_e is the mean surface area of the single erythrocyte [38,39]. The boundary condition $(d\psi(x)/dx)_{x=\infty} = 0$ [41] and the choice $\psi(x=\infty) = \psi_o$ (see Fig. 1) are already incorporated in Eq. (A1). While solving the Eq. (A1) the outer membrane surface charge density due to the bound ANS [7,17,42]:

$$\sigma_{ANS} = -N_{MA}e_0/A_e \quad (A3)$$

is considered by including the boundary condition

$$(d\psi/dx)_{x=0} = -\sigma_{ANS}/\epsilon\epsilon_o \quad (A4)$$

where it is taken into account that the contribution of charged phospholipid head groups to the surface charge density of the outer membrane surface can be neglected [35]. Here, N_{MA} is the mean number of ANS molecules bound to the outer surface of single erythrocyte membrane. Additionally, the boundary conditions at $x = d_o$ (see Fig. 1) must also be taken into account [2]:

$$\left(\frac{d\psi(x)}{dx}\right)_{x=d_o} = -\left(\frac{8N_A[NaCl]kT}{\epsilon\epsilon_o}\right)^{1/2} sh\left(\frac{e_0(\psi(x=d_o)-\psi_o)}{2kT}\right) \quad (A5)$$

Therefore in order to determine the values of $\Delta\psi_o$ and N_{MA} at given sodium chloride concentration in the outer

solution the Poisson–Boltzmann Eq. (A1), subject to the boundary condition (Eq. (A4)), had to be numerically integrated from $x = d_o$ to $x = 0$ (see Fig. 1), taking into account the equations of ANS binding to the outer membrane surface (Section 2.2) and Eq. (A3) describing the dependence of the outer membrane surface charge density due to bound ANS (σ_{ANS}) on the number of bound ANS molecules N_{MA}. The Runge Kutta method of the 4th order was applied. The initial values of $\psi(x = d_o)$ was determined by an iterative procedure according to the boundary condition (Eq. (A4)), where the value of $(d\psi/dx)_{x=d_o}$ was determined from the Eq. (A5). The following values of model parameters were used in the described calculations: $d_o = 5.5$ nm [39], $N_{si} = 1 \times 10^7$ [38], $A_e = 95$ μm^2 [33], $\epsilon = 78.5$ and $T = 293$ K.

References

[1] G. Cevc, S. Svetina and B. Žekš, J. Phys. Chem. 85 (1981) 1762.
[2] R. Heinrich, M. Gaestel and R. Glaser, J. Theor. Biol. 96 (1982) 211.
[3] S. McLaughlin, Annu. Rev. Biophys. Biophys. Chem. 18 (1989) 113.
[4] J.C. Franklin, D.S. Cafiso, R.F. Fleweling and W.L. Hubbell, Biophys. J. 64 (1993) 642.
[5] V. Kralj-Iglič and A. Iglič, J. Phys. II (France) 6 (1996) 477.
[6] C. Zheng and G. Vanderkooi. Biophys. J. 63 (1992) 935.
[7] S. McLaughlin, Curr. Top. Membr. Transp. 9 (1977) 71.
[8] K. Gawrisch, D. Ruston, J. Zimmerberg, V.A. Parsegian, R.P. Rand and N. Fuller, Biophys. J. 61 (1992) 1213.
[9] J.C. Franklin and D.S. Cafiso, Biophys. J. 65 (1993) 289.
[10] Y.A. Liberman and V.P. Topaly, Biophysics 14 (1969) 477.
[11] E. Gabev, J. Kasianowicz, T. Abbott and S. McLaughlin, Biochim. Biophys. Acta 979 (1989) 105.
[12] H. Rottenberg, Meth. Enzymol. 171 (1989) 364.
[13] E. Donath and V. Pastushenko, J. Electroanal. Chem. 116 (1979) 543.
[14] D. Cafiso, A. McLaughlin, S. McLaughlin and S. Winiski, Meth. Enzymol. 171 (1989) 342.
[15] D. Cafiso and W.L. Hubbell, Annu. Rev. Biophys. Bioeng. 10 (1981) 217.
[16] G.S.B. Lin, R.I. Macey and R.J. Mehlhorn, Biochim. Biophys. Acta 732 (1983) 683.
[17] D.H. Haynes, J. Membrane Biol. 17 (1974) 341.
[18] G.S.B. Lin and R.I. Macey, Biochim. Biophys. Acta 512 (1978) 270.
[19] I. Bernhardt, E. Donath and R. Glaser, J. Membrane Biol. 78 (1984) 249.
[20] S.A. Sundberg and W.L. Hubbell, Biophys. J. 49 (1986) 553.
[21] A. Iglič, M. Brumen and S. Svetina, Period. Biol. 89 (1987) 363.
[22] L.D. Bergelson and L.I. Barsukov, Science 197 (1977) 224.
[23] A. Elgsaeter, D.M. Shotton and D.M. Branton, Biochim. Biophys. Acta. 18 (1976) 101.
[24] S. Yoshida, Chem. Pharm. Bull. (Tokyo) 24 (1976) 3039.
[25] J. Slavik, Biochim. Biophys. Acta 694 (1982) 1.
[26] T. Horie, Y. Sugiyama, S. Awazu and M. Hanano, J. Pharm. Dyn. 1 (1978) 203.
[27] M. Brumen, R. Glaser and S. Svetina, Bioelectrochem. Bioenerg. 6 (1979) 227.
[28] G.J. Nelson, Biochim. Biophys. Acta 144 (1967) 221.
[29] R.F.A. Zwaal, B. Roelofsen and C.M. Colley, Biochim. Biophys. Acta 300 (1973) 159.
[30] D.H. Haynes and H. Staerk, J. Membrane Biol. 17 (1974) 313.
[31] T.L. Hill, An Introduction to Statistical Thermodynamics, (Addison-Wesley, Reading, 1962).
[32] M. Eigen and E. Wicke, J. Phys. Chem. 58 (1954) 702.
[33] W. Gruber and B. Deuticke, J. Membrane Biol. 13 (1973) 19.
[34] M.B. Feinstein, L. Spero and H. Felsenfeld, FEBS Lett. 6 (1970) 245.
[35] A. Iglič, Electrotechn. Rev. (Slovenia) 59 (1992) 223.
[36] A.J. Verkleij, R.F.A. Zwaal, B. Roelofsen, P Comfurius, D. Kastelijn and L.L.M. van Deenen, Biochim. Biophys. Acta 323 (1973) 178.
[37] R.B. Gennis, Biomembranes — Molecular Structure and Function (Springer-Verlag, New York, 1989).
[38] E.A. Eylar, M.A. Madoff, O.V. Brody and J.L. Oncley, J. Biol. Chem. 237 (1962) 1992.
[39] E. Donath and D. Lerche, Bioelectrochem. Bioenerg. 7 (1980) 41.
[40] M. Blank, R.G. King, L. Soo, R.E. Abbott and S. Chien, J. Coll. Interface Sci. 69 (1979) 67.
[41] E.J.W. Verwey and J.Th.G. Overbeek, Theory of the Stability of Lyophobic Colloids (Elsevier, New York, 1948).
[42] S. McLaughlin and H. Harary, Biochemistry 15 (1976) 1941.