ELSEVIER

Biochimica et Biophysica Acta 1370 (1998) 107–118

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Bromothymol blue as a probe for structural changes of model membranes induced by hemoglobin

Galina P. Gorbenko *

Department of Physics and Technology, Kharkov State University, Kharkov, 310077, Ukraine

Received 15 May 1997; revised 30 September 1997; accepted 21 October 1997

Abstract

The effect of methemoglobin on the structure of model membranes composed of phosphatidylcholine and diphosphatidylglycerol (18 : 1, mol : mol) was studied with the help of pH-indicator dye bromothymol blue. The partition coefficients characterizing the dye binding to methemoglobin or model membranes were derived from the pK_α^a dependences on the protein or phospholipid concentration. The observed character of the dye partitioning in the lipid or lipid–protein systems is interpreted in terms of the traditional electrostatic approach and some modern theories of membrane electrostatics. It is assumed that methemoglobin affects the structural and physicochemical parameters of lipid–water interface. © 1998 Elsevier Science B.V.

Keywords: Methemoglobin; Phosphatidylcholine–diphosphatidylglycerol liposome; Bromothymol blue; Protein–lipid interaction

1. Introduction

Acid–base indicator bromothymol blue is known as a probe highly sensitive to the alterations in the structure and physical properties of the proteins [1], phospholipid vesicles [19,27,29] and biomembranes [15,24,32]. The response of bromothymol blue to the changes of environmental conditions involves the shift of its protolytic equilibrium. In particular, the dye binding to phospholipid vesicles was found to be followed by the increase of the apparent ionization constant (pK_α^a) [19,27,29]. By studying the effect of various factors, including anesthetics [29], proteins [19] and temperature [27] on the structure of model phospholipid membranes, it has been demonstrated that pK_α^a shift of the bound dye reflects the changes in the molecular organization and physicochemical characteristics of the bilayer interfacial region.

In the present study, an attempt has been made to apply bromothymol blue for the investigation of lipid–protein interactions. The model system chosen includes hemoglobin as a protein component and liposomes composed of phosphatidylcholine (PC) and diphosphatidylglycerol (DPG) (18 : 1, mol : mol) as a lipid one. PC is a zwitterionic phospholipid, being the main component of most biological membranes. To obtain model membranes having a net negative charge, PC was mixed with DPG containing two charged phosphate groups. Although hemoglobin is not a naturally occurring membrane protein, it can effectively bind to phospholipids by means of electrostatic as well as hydrophobic interactions [25,35,47]. For this reason, the hemoglobin–lipo-

* Corresponding author. 52-52 Tobolskaya Str. 310072, Kharkov, Ukraine.

PII S0005-2736(97)00252-6

somes system appears to be suitable in elucidating the general features and mechanisms of protein–lipid interactions. The present study was focused on one of the protein forms, namely methemoglobin (metHb). This form is characterized by its relative stability in liposomal suspension since it is known that phospholipids can induce conversion of oxyhemoglobin to methemoglobin [22,28,44]. The main purpose of this work was to obtain information regarding the influence of protein on the properties of phospholipid bilayer.

2. Materials and methods

Egg-yolk phosphatidylcholine and beef-heart diphosphatidylglycerol were purchased from Bakpreparat (Kharkov, Ukraine). Both phospholipids gave single spots by thin-layer chromatography in the chloroform : methanol : acetic acid : water 25 : 15 : 4:2, v/v) solvent system. Indicator bromothymol blue was obtained from Reakhim (Russia) and used without further purification. Horse methemoglobin and thiourea were purchased from Reanal (Hungary).

A stock suspension of unilamellar phospholipid vesicles was prepared by the method of Batzri and Korn [3]. A 1 ml aliquot of the ethanol lipid solution containing 45 mM phosphatidylcholine and 2.5 mM diphosphatidylglycerol was injected into 13 ml of the 5 mM sodium-phosphate buffer under continuous stirring. Ethanol was then removed by dialysis. The average diameter of the vesicles, evaluated from sample turbidity measurements [26] was ca. 100 nm. Phospholipid concentration was determined according to the procedure of Bartlett [2]. Concentrations of metHb and bromothymol blue were found using extinction coefficients $E_{407} = 5.66 \times 10^5\ M^{-1}\ cm^{-1}$ for the protein [6] and $E_{617} = 2.25 \times 10^4\ M^{-1}\ cm^{-1}$ for the dye [1].

The pK_α were derived from the fitting of pH titration data to the equation [7]:

$$A = \frac{A_A 10^{-pH} + A_B^{-pK_\alpha}}{10^{-pK_\alpha} + 10^{-pH}} \quad (1)$$

where A is the dye absorbance at a given pH, A_A and A_B the absorbances at pH 5.0 and 11.0, respectively. The measurements were performed with SF-46 spectrophotometer at the wavelength 617 nm corresponding to the absorbance maximum of the indicator basic form.

The dye concentration employed for pK_α determination was 2×10^{-5} M. Methemoglobin was added to liposomes–dye mixture to a final concentration varying from 2×10^{-7} to 1.3×10^{-6} M. To avoid lipid peroxidation initiated by the protein, 170 mM of free-radical scavenger thiourea was introduced to the liposomal suspension. In the presence of 2 M KCl, no appreciable changes in the turbidity of the samples were observed, thereby indicating that no marked aggregation of liposomes occur. All experiments were performed at 20°C.

3. Theory

The acid–base equilibrium of bromothymol blue can be represented by:

$$HIn^- \Leftrightarrow In^{2-} + H^+$$

where HIn^- and In^{2-} are the protonated and deprotonated dye forms, respectively. According to the generally accepted electrostatic approach [18,33,34,37], the apparent ionization constant of the dye, distributing between membrane and aqueous phases, is given by:

$$pK_\alpha^a = pK_\alpha^w + \log\frac{\gamma_{In}}{\gamma_{HIn}} - \frac{\Psi F}{2.303RT} + \log\left(\frac{1 + \left(P_{HIn}(v_m/v_w)\right)^{-1}}{1 + \left(P_{In}(v_m/v_w)\right)^{-1}}\right) \quad (2)$$

where γ_{HIn}, γ_{In} and P_{HIn}, P_{In} represent transfer activity coefficients and partition coefficients for the protonated and deprotonated dye forms, respectively, v_m, v_w the volumes of membrane and aqueous phases, Ψ the electrostatic surface potential, F, R, T the Faraday constant, the gas constant and absolute temperature, respectively. The partition coefficients are defined as:

$$P_{HIn} = \frac{n_{HIn}^m v_w}{n_{HIn}^w v_m} \quad (3)$$

$$P_{\mathrm{In}} = \frac{n_{\mathrm{In}}^{\mathrm{m}} v_{\mathrm{w}}}{n_{\mathrm{In}}^{\mathrm{w}} v_{\mathrm{m}}} \quad (4)$$

where n is the number of moles of the different dye species in the membrane phase (m) and the bulk solution (w). On the other hand, partition coefficients can be considered as consisting of electrostatic and nonelectrostatic (P^0) terms:

$$P_{\mathrm{HIn}} \equiv P_{\mathrm{HIn}}^0 \exp(-z_{\mathrm{HIn}} \Psi F/RT) = \gamma_{\mathrm{HIn}}^{-1} \exp(-z_{\mathrm{HIn}} \Psi F/RT) \quad (5)$$

$$P_{\mathrm{In}} \equiv = P_{\mathrm{In}}^0 \exp(-z_{\mathrm{In}} \Psi F/RT) = \gamma_{\mathrm{In}}^{-1} \exp(-z_{\mathrm{In}} \Psi F/RT) \quad (6)$$

where z denotes the charge of the corresponding dye species. Taking into account the Eqs. (3)–(6), Eq. (2) can be transformed to:

$$\mathrm{p}K_\alpha^{\mathrm{a}} = \mathrm{p}K_\alpha^{\mathrm{w}} + \log\left(\frac{1 + P_{\mathrm{HIn}}(v_{\mathrm{m}}/v_{\mathrm{w}})}{1 + P_{\mathrm{In}}(v_{\mathrm{m}}/v_{\mathrm{w}})}\right) \quad (7)$$

Nonelectrostatic term in the Eq. (2) corresponds to the so-called intrinsic ionization constant (K_α^i):

$$\mathrm{p}K_\alpha^i = \mathrm{p}K_\alpha^{\mathrm{w}} + \log\frac{\gamma_{\mathrm{In}}}{\gamma_{\mathrm{HIn}}} \quad (8)$$

An approximate estimation of the $\mathrm{p}K_\alpha^i$ can be derived from the $\mathrm{p}K_\alpha^{\mathrm{a}}$ value determined at high bulk-phase ionic strength when electrostatic surface potential is thought to be negligibly small [29].

Traditional electrostatic approach, based on the Eqs. (2)–(8), being rather convenient for practical use, nevertheless, does not consider in details the actual factors governing the dye distribution between aqueous and membrane phases. It seems unreal to identify all these factors, but the main among them can be derived from the modern theories of membrane electrostatics. There are a number of excellent reviews, regarding the electrostatic properties of lipid–water interface [13,20,30,31,45,46], which prove to be of utmost importance in analyzing the experimental data. In particular, as concerns the solute partitioning, the following distribution function is introduced [13]:

$$g_i(x) = \exp\left[-w_i(x)/k_{\mathrm{B}}T\right] \quad (9)$$

where $w_i(x)$ is the mean force potential that corresponds to the integral potential, associated with the force between the particle i and the membrane vesicle and k_{B} the Boltzmann constant. The form of the mean force potential largely depends on the electrostatic model used and the membrane system under study. For example, in the case of hydrophobic ion distribution $w_i(x)$ can be approximately described as a sum of several constituents [13,23]:

$$w_i(x) = w_{\mathrm{el},i}(x) + w_{\mathrm{Born},i}(x) + w_{\mathrm{hydrat},i}(x) + w_{\mathrm{d},i}(x) + w_{\mathrm{n},i} + w_{\mathrm{others},i}(x) \quad (10)$$

The first term $w_{\mathrm{el},i}(x)$ characterizes the coulombic ion–membrane interactions:

$$w_{\mathrm{el},i}(x) = z_i e \Psi_{\mathrm{el}}(x) \quad (11)$$

By including only this term in Eq. (9), one obtains the commonly used Boltzmann distribution function. The contribution $w_{\mathrm{Born},i}(x)$ corresponds to the free energy of charge transfer between the media differing in dielectric constants [11]. The neutral energy term $w_{\mathrm{n},i}(x)$ includes hydrophobic, van der Waals and steric factors [23]. Of special interest is the hydration contribution $w_{\mathrm{hydrat},i}(x)$ which reflects a number of phenomena concerned with the capability of membrane components to bind water molecules [4,5,13]. Interfacial hydration can substantially affect the electrostatic membrane properties. In general, membrane surface potential must be considered as a sum of the common Coulomb potential (Ψ_{el}) and non-Coulomb dipole (or polarization) potential (Ψ_{d}) stemming from the molecular dipoles of phospholipids and interfacial water [46]. In turn, dipole potential of phospholipids (Ψ_{p}) is determined by ester carbonyl dipoles of glycerol backbone of the lipid molecules and phosphocholine dipoles of headgroups. The hydrational part of Ψ_{d} (Ψ_{h}) originates from the water dipoles oriented at the lipid–water interface. According to some estimates, Ψ_{d} of model membranes has a magnitude of several hundreds of mV, being positive inside [13,23]. The value of Ψ_{h} may vary from -300 to -800 mV [13,46]. Hydrational potential is appreciable only in the membrane vicinity since it decays on a linear scale of 0.07–0.4 nm [13]. Dipole potential Ψ_{d} is introduced in the Eq. (10) through the term $w_{\mathrm{d},i}(x)$. The contribution $w_{\mathrm{others},i}(x)$ accouts for a variety of residual solute–surface interactions which are thought to be negligibly small. Thus, by considering certain

average ion position x at the lipid–water interface, partition coefficient can be represented by:

$$P_i = \exp(-w_i/k_BT) \approx \exp\left[-\left(z_i e\Psi_{el} + w_{Born,i} + w_{hydrat,i} + w_{n,i} + w_{d,i}\right)/k_BT\right] \quad (12)$$

4. Results

The above-mentioned approaches provide the theoretical background for the analysis of bromothymol blue pK_α^a shift arising from the dye partitioning between aqueous and membrane phases. Interpretation of the pK_α^a changes observed for the lipid–protein system is complicated by the possible contribution of both lipid- and protein-associated dye species to the measured pK_α^a value. It therefore seemed important to examine the acid–base behavior of bromothymol blue in the solutions containing either liposomes or hemoglobin. The present study included the following main steps:

(a) quantitative analysis of the pK_α^a and pK_α^i dependences on the lipid or protein concentration aimed at the evaluation of the partition coefficients for different indicator forms;

(b) investigation of the protein effect on the pK_α^a and pK_α^i values of bromothymol blue bound to liposomal membranes; and

(c) estimation of the surface electrostatic potential of liposomal membranes from the pK_α^a measurements.

Figs. 1 and 2 show typical pH titration curves used for the pK_α^a determination. For bromothymol blue in aqueous bulk solution the pK_α^w was estimated to be ca. 7.18, the value being in good agreement with that reported elsewhere [50]. The dye binding to phospholipid vesicles resulted in the substantial increase of the pK_α^a as compared to pK_α^w. Similar changes, but much smaller in magnitude were observed for metHb. In order to approximately evaluate the intrinsic ionization constants, pH titrations have been performed in the presence of 2 M KCl. The salt concentration of 2 M was chosen for the screening of the membrane charge taking into account some theoretical estimates of electrostatic surface potential (see below) and the finding that, upon addition of KCl, the increase of pK_α^a reached plateau at KCl concentration of ca. 1.6 M. In Figs. 3 and 4, the pK_α^a and pK_α^i values determined for liposomes, metHb and their mixture are plotted against phospholipid or protein concentration. The fitting of these data to Eq. (7) allowed to estimate the partition coefficients for protonated and deprotonated forms of bromthymol blue (Table 1). The volumes of the membrane and the protein phases were calculated as follows:

$$v_m = N_A C_L V_L \quad (13)$$

$$v_p = N_A C_P V_P \quad (14)$$

where C_L, C_P are the molar phospholipid and protein concentrations, V_L, V_P are the volumes of phospholipid or protein molecules, respectively. Taking V_L to be 1265 A^3 [21], V_P to be $1.2 \times 10^5 A^3$ [12], it follows that $v_m \cong 0.762 C_L$ (dm^3), $v_P \cong 73.5 C_P$ (dm^3). Under the experimental conditions employed ($C_L \leq 1 \times 10^{-3}$ M, $C_P \cong 1.3 \times 10^{-6}$ M), the values of v_m and v_P appeared to be much less than the total volume of the system ($v_t = 1\,dm^3$), so that $v_w \approx v_t$.

The pK_α^a dependences on metHb concentration (Fig. 4) were fitted to Eq. (7) with C_L substituted for C_P. The measurements of pK_α^a in the lipid–protein system were performed at variable C_L and constant C_P values. In analyzing these results, two limiting cases have been considered. In the first case, the possibility of the dye binding to protein was ignored so that the experimental curves were fitted to Eq. (7). In the second case, the dye was assumed to distribute between independent lipid and protein binding sites and the data have been analyzed with the use of the following equation:

$$pK_\alpha^a = pK_\alpha^w + \log\left(\frac{1 + P_{HIn}^L(v_m/v_w) + P_{HIn}^P(v_p/v_w)}{1 + P_{In}^L(v_m/v_w) + P_{In}^P(v_P/v_w)}\right) \quad (15)$$

where superscripts L and P correspond to the lipid and protein, respectively. The values of P_{HIn}^P and P_{In}^P introduced in Eq. (15) were derived from the pK_α^a measurements performed with metHb in the absence of liposomes (Table 1). Partition coefficients estimated for the lipid–protein system by using Eqs. (7) and (15) could be considered as rough limits of the P_{HIn} and P_{In} characterizing the dye distribution between liposomal membranes modified by metHb and free protein in solution.

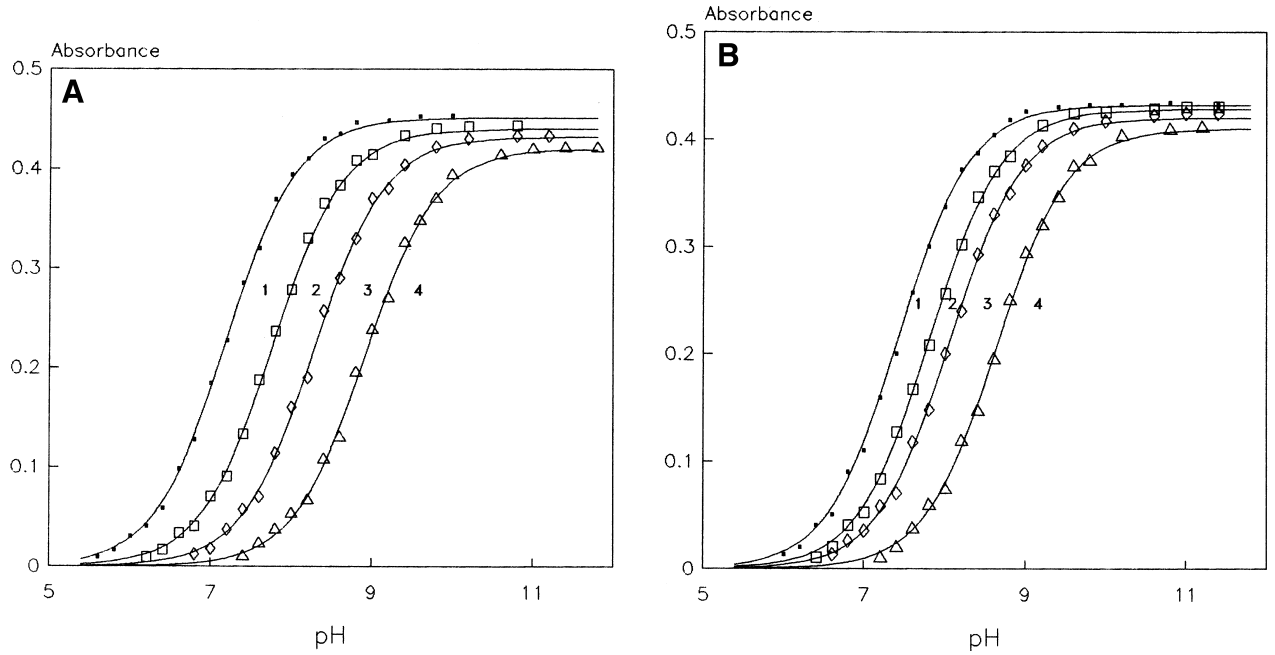

Fig. 1. Absorbance of bromothymol blue at 617 nm as a function of pH: (A) – liposomes PC : DPG (9 : 1) and (B) – liposomes + metHb, protein concentration $C_P = 1.3 \times 10^{-6}$ M: (1) 5 mM Na-phosphate buffer, lipid concentration (C_L), mM: (2) 0.11; (3) 0.29 (A), 0.22 (B); and (4) 0.95.

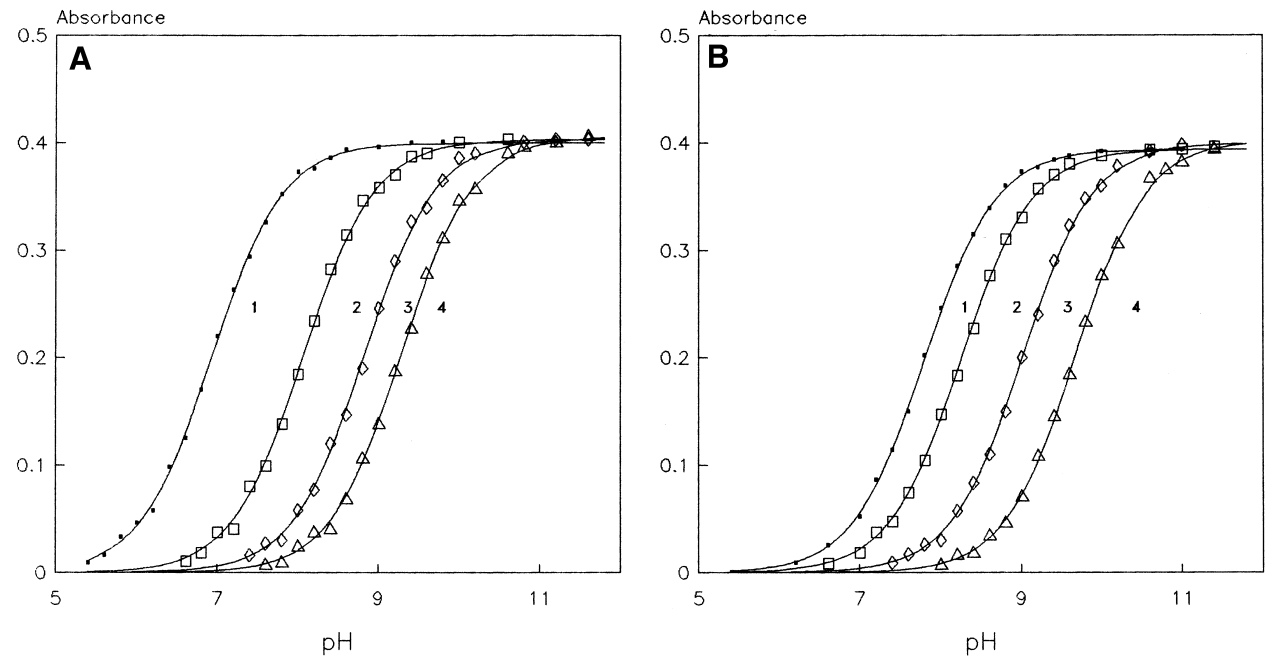

Fig. 2. Absorbance of bromothymol blue at 617 nm vs. pH: (A) – liposomes PC : DPG (9 : 1), (B) – liposomes + metHb, $C_P = 1.3 \times 10^{-5}$ M: (1) 5 mM Na-phosphate buffer, 2 M KCl; C_L: (2) 0.04; (3) 0.22; and (4) 0.95.

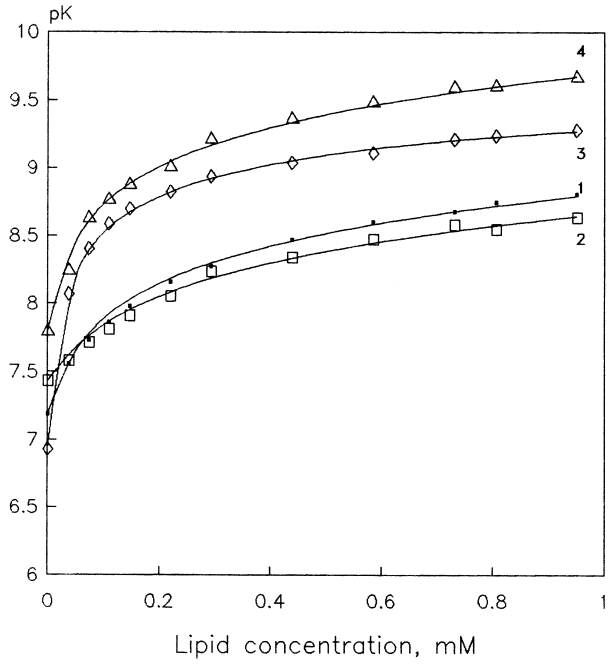

Fig. 3. Dependences of pK_α^a (1,2) and pK_α^i (3,4) on lipid concentration: (1,3) liposomes, (2,4) liposomes + metHb, $C_P = 1.3\times10^{-6}$ M.

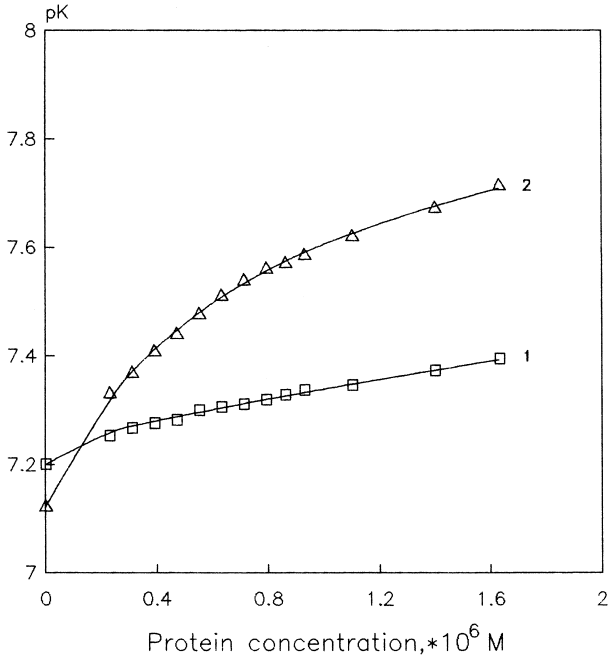

Fig. 4. Dependences of (1) pK_α^a and (2) pK_α^i on the concentration of metHb in the absence of liposomes.

As can be seen in Table 1, there is a substantial difference in the affinity of the various forms of bromothymol blue to the lipid bilayer or protein, the distinction being especially pronounced for liposomes. The protonated form of the dye was found to associate much more effectively with phospholipid vesicles than deprotonated one. By analyzing the pK_α^a dependences of bromothymol blue on phospholipid concentration in some cases (marked by superscript 'a' in Table 1), we failed to determine the P_{In} value with appropriate accuracy. This allows one to assume that, in liposomal suspension, a major part of deprotonated dye species remains in the aqueous phase. Additional arguments in favor of the very weak interaction of the form In^{2-} with phospholipids, or even lack of any binding, were provided by the measurements of the dye absorbance spectra and dialysis experiments. As illustrated in Fig. 5, the binding of the protonated form of bromothymol blue to liposomes was followed by the noticeable blue shift of the absorbance spectrum and increase in its intensity. By contrast, for deprotonated form no marked spectral changes were observed. These findings agree with the results of the dye release measurements presented in Fig. 6. The leakage of the protonated dye species from the dialysis tube containing liposomes was negligible (Fig. 6(A)), while the release of deprotonated ones was almost the same for liposomal suspension and aqueous solution (Fig.

Table 1
Partition coefficients of different forms of bromothymol blue calculated according to Eq. (7) and Eq. (15)

System	P_{HIn}	P_{In}	r
5 mM Na-phosphate buffer			
MetHb	1.6×10^4	6.4×10^3	0.995
Liposomes	5.1×10^4	—[a]	0.978
Liposomes + metHb	4.4×10^4	1.6×10^2	0.989
Liposomes + metHb[a]	6.1×10^4	—[a]	0.987
5 mM Na-phosphate buffer + 2 M KCl			
MetHb	2.8×10^4	2.3×10^3	0.996
Liposomes	3.1×10^5	1.1×10^3	0.990
Liposomes + metHb	4.4×10^5	—[a]	0.987
Liposomes + metHb[a]	5.1×10^5	—[a]	0.986

[a] P_{In} tends to zero upon fitting of experimental data to Eq. (7) or Eq. (15). The accuracy of the partition coefficients determination was ca. 12–16%, r being the correlation coefficient.

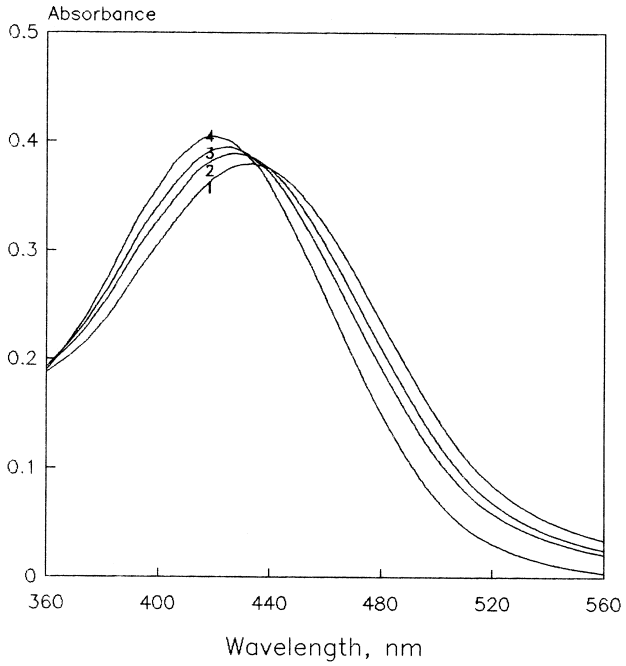

Fig. 5. Absorbance spectra of bromthymol blue: (1) pH 4.4, 5 mM Na-phosphate buffer; (2) $C_L = 0.1$ mM, (3) 0.25 mM; and (4) 1.3 mM.

6(B)). All these data suggest that substantial pK_α^a shift of bromothymol blue observed for liposomes could be explained mainly by the association of protonated dye species with the lipid bilayer. As a whole, the results of the binding experiments presented in Table 1 can be summarized as follows. MetHb molecules contain binding sites for both forms of bromothymol blue, but the protonated form binds more effectively to the protein as compared to deprotonated one. In the case of liposomes, partition coefficient of the indicator form HIn^- is several orders of magnitude greater than that of the form In^{2-}. Screening of the surface negative charge upon addition of 2 M KCl is followed by enhancing the association of the both dye species with phospholipid vesicles. Taking into account the accuracy of the P_{In} and P_{HIn} determination (being ca. 12–16%), it can be assumed that partitioning of the dye between the bulk solution (5 mM phosphate buffer) and lipid phase is not affected appreciably by metHb. Meanwhile, in the presence of 2 M KCl, the protein caused the opposite changes of the partition constants – a slight increase of P_{HIn} and substantial decrease of P_{In}.

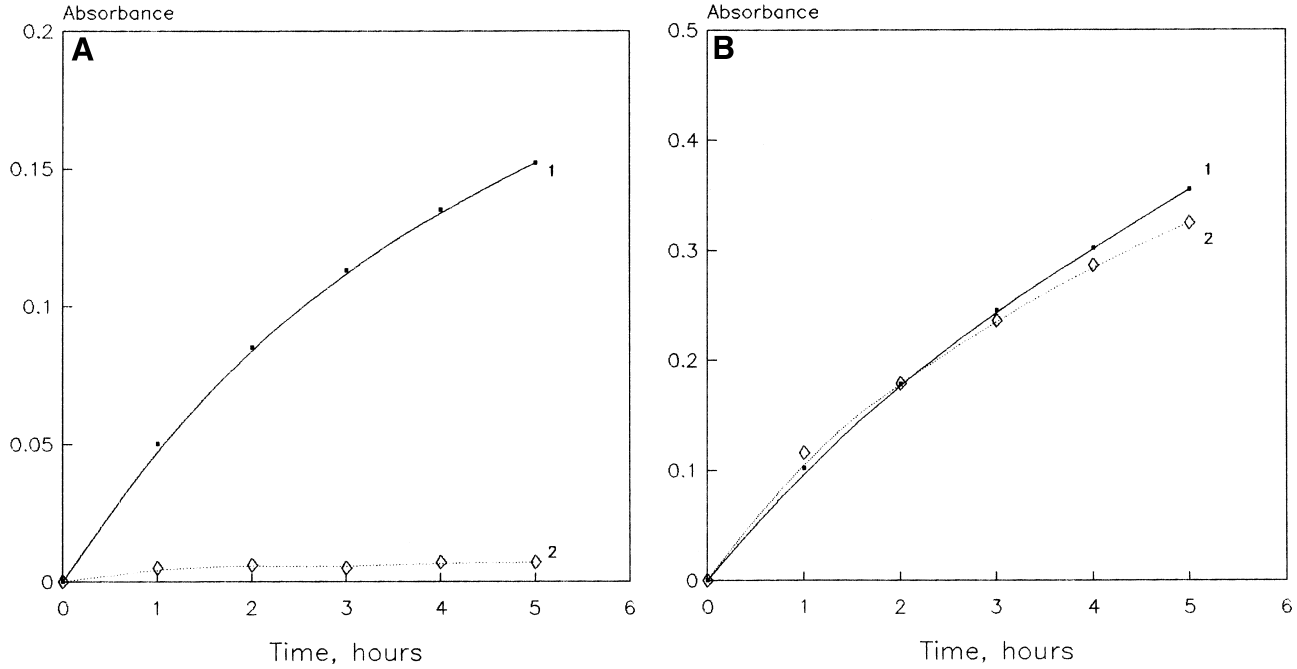

Fig. 6. Leakage of bromthymol blue from the dialysis tube: (A) – pH 4.4, 430 nm; and (B) – pH 12.2, 617 nm: (1) dye in 5 mM phosphate buffer; and (2) dye in the liposomal suspension $C_L = 1.8$ mM.

5. Discussion

Table 2 contains the pK_α^a values of bromothymol blue and their shifts with respect to pK_α^w observed for lipid and lipid-protein systems. These data correspond to phospholipid concentration of ca. 1 mM taken from the range where pK_α^a exhibits no strong dependence on C_L (Fig. 3). As can be seen in Table 2, upon addition of metHb pK_α^a and pK_α^i undergo the opposite changes. Both pK_α^a and pK_α^i reached constant values at C_P of ca. 8×10^{-7} and 5×10^{-7} M, respectively, so that the protein concentration of 1.3×10^{-6} M corresponds to the plateau region of the pK_α^a and pK_α^i dependences on C_P. The observed decrease of pK_α^a could hardly be ascribed to the redistribution of the dye between the lipid bilayer and protein molecules since phospholipid concentrations employed are substantially larger than the protein concentration so that the major part of the dye seems to be localized in the lipid phase.

According to our estimates, and those reported in the literature [40,47,48], hemoglobin interaction with negatively charged liposomes is characterized by the binding constant of ca. 10^6 M^{-1} and number of lipids constituting the protein binding site ca. 100. Using these values, we found that, for $C_P = 1.3 \times 10^{-6}$ M and $C_L = 10^{-3}$ M, the amount of the bound protein would be ca. 1.2×10^{-6} M, i.e. the concentration of the free protein appeared to be rather small.

In the lipid bilayer, bromothymol blue is thought to be located at the interfacial region presumably between hydrophilic headgroups and non-polar acyl chains [15,29]. The shift of the acid–base equilibrium, resulting from the dye binding to liposomes, is controlled by various factors including electrostatic surface potential, interfacial physicochemical properties and dye orientation within the membrane. The last two factors are believed to determine the intrinsic ionization constant. The contribution of the surface potential to the pK_α^a changes can be estimated in terms of the aforementioned electrostatic approach. Since, at low ionic strength (5 mM), partition coefficients of the deprotonated dye form appeared to be very small, the term $P_{In}(v_m/v_w)$ in Eq. (7) introduces a negligible correction so as to give the following:

$$pK_\alpha^a = pK_\alpha^w + \log\left(1 + P^0_{HIn}\frac{v_m}{v_w}\exp\left(-z_{HIn}\Psi F/RT\right)\right) \quad (16)$$

In the presence of 2 M KCl, when the surface potential of the liposomal membranes is supposed to tend to zero, the measured pK_α^a (pK_α^i) is given by:

$$pK_\alpha^i = pK_\alpha^w + \log\left(\frac{1 + P^0_{HIn}(v_m/v_w)}{1 + P^0_{In}(v_m/v_w)}\right) \quad (17)$$

Taking into account that for the high lipid concentration (≥ 1 mM) the terms containing P_{HIn} and P^0_{HIn} are substantially > 1, from the Eqs. (16) and (17), we have:

$$\Delta pK_\alpha^i = pK_\alpha^i - pK_\alpha^a \cong \frac{z_{HIn}\Psi F}{2.303RT} - \log\left(1 + P^0_{In}\frac{v_m}{v_w}\right) \quad (18)$$

The relationship (18) was employed to evaluate the electrostatic surface potential of the model membranes being studied. Using pK_α^a and pK_α^i values presented in Table 2, Ψ was estimated to be ca. -38 mV for liposomes and -61 mV for liposomes modified by metHb. It should be noted that an estimate of Ψ derived from the pK_α^a measurements is thought to represent the mean potential at the location of the indicator prototropic moiety in the interfacial region [29]. Another way to evaluate the electrostatic surface potential is based on the direct use of Eq. (5) with partition coefficients measured at low (5 mM) and high (2 M) ionic strengths instead of P_{HIn} and

Table 2
The effect of metHb on the pK_α^a and pK_α^i values of bromothymol blue at the lipid concentration of 9.5×10^{-4} M and protein concentration of 1.3×10^{-6} M

System	pK_α^a	pK_α^i	$pK_\alpha^a - pK_\alpha^w$	$pK_\alpha^i - pK_\alpha^w$
Liposomes	8.91 ± 0.04	9.28 ± 0.05	1.73	2.10
Liposomes + metHb	8.63 ± 0.06	9.67 ± 0.09	1.45	2.49

P^0_{HIn}. In this case, Ψ was found to be ca. -46 and -59 mV for lipid and lipid–protein model membranes, respectively. On the other hand, Ψ value can be estimated theoretically, e.g. from the following solution of non-linearized Poisson–Boltzmann equation, obtained for spherical vesicles [36]:

$$\sigma - \frac{2\varepsilon_r\varepsilon_0 kRT}{F}\sinh(Y/2)\left(1+\frac{2}{kr\cosh^2(Y/4)} + \frac{8\ln[\cosh(Y/4)]}{(kr)^2\sinh^2(Y/2)}\right)^{1/2} = 0 \quad (19)$$

where σ is the surface charge density, $Y = \Psi F/RT$, k^{-1} the Debye length and r the vesicle radius. The surface charge density of the mixed PC : DPG liposomal membranes was evaluated according to the following relationship:

$$\sigma = \frac{2e}{fA_{\mathrm{PC}} + A_{\mathrm{DPG}}} \quad (20)$$

where A_{PC}, A_{DPG} are the mean areas of PC and DPG headgroups, f the ratio of PC to DPG molar concentrations. Taking A_{PC} and A_{DPG} to be 0.7 nm^2 and 1.2 nm^2, respectively, [21] and f to be 18, it follows that $\sigma = 2.3\times10^{-2}$ C m^{-2}. Meanwhile, if the cation binding is taken into account, the effective surface charge density must be considered:

$$\sigma_{\mathrm{eff}} = \frac{\sigma}{1 + K_{\mathrm{B}}C_{\mathrm{B}}\exp(-z\Psi F/RT)} \quad (21)$$

where K_{B} is the binding constant, C_{B} the bulk concentration of a given ion (in our case, Na^+ or K^+). The typical K_{B} values characterizing the binding of monovalent cations to negatively charged phospholipids are reported to range from 0.4 to 1.0 M^{-1} [13,46]. If $K_{\mathrm{B}} = 1.0$ M^{-1} and $C_{\mathrm{B}} = 5$ mM, then σ_{eff} is equal to 2×10^{-2} C m^{-2}. By introducing σ or σ_{eff} in Eq. (19), the following estimates were derived: $\Psi \approx -87$ mV, $\Psi_{\mathrm{eff}} \approx -80$ mV, so that theoretical Ψ values appear to be greater than experimental ones. Besides, from the Eq. (19) it follows that, under the existing conditions of charge screening (2 M KCl), $\Psi \approx -7$ mV, and $\Psi_{\mathrm{eff}} \approx -2$ mV, i.e. the error associated with the assumption of zero Ψ value proves to be rather small. Notwithstanding this, it should be pointed out that all aforementioned estimates of surface potential were obtained in terms of the simplest approach, ignoring the complex nature of membrane electrostatic phenomena. In order to interpret the experimental data more adequately, we attempted to use Eq. (12) in analyzing the partition coefficients of bromothymol blue. As follows from the comparison of Eqs. (5), (6) and (12), the so-called non-electrostatic term of partition coefficient (P^0) can be represented by:

$$P^0_i = \exp\left[-(w_{\mathrm{Born},i} + w_{\mathrm{hydrat},i} + w_{\mathrm{n},i} + w_{\mathrm{d},i})/k_{\mathrm{B}}T\right] \quad (22)$$

The above-described estimation of surface potential is based on the assumption that the only consequence of increasing ionic strength is the substantial diminution of coulombic electrostatic term. However, the salt effects are not restricted to the common charge screening, but may include the alterations in dielectric properties of the interfacial region, extent of membrane hydration, conformation and mobility of phospholipids, etc. [13,30]. Hence, all possible contributions to P^0 (Eq. (22)) do not probably remain unchanged with the growth of ionic strength. Given all these complications, along with the observed predominant hemoglobin influence on the non-electrostatic constituents of partition coefficients (Table 1), it follows that – coming from the simplified model used – the conclusion about the protein-induced increase of surface potential may be wrong. Therefore, in considering hemoglobin effect, it seems more reasonable to analyse the changes in the partition coefficients for various dye species.

As already indicated, metHb does not significantly alter the indicator partition coefficients at low ionic strength. Meanwhile, at high ionic strength the P_{In} value was markedly lowered, resulting in the increase of $\mathrm{p}K^i_\alpha$. This effect cannot be explained by the dye partitioning between independent ''protein'' and ''lipid'' binding sites. As follows from Fig. 4 at the protein concentration considered (1.3×10^{-6} M), the measured $\mathrm{p}K^i_\alpha$ does not exceed 7.6. Hence the shift of $\mathrm{p}K^i_\alpha$ being ca. 2.5 units for the lipid–protein system could be attributed mainly to bromothymol blue interaction with the lipid bilayer. Another possible explanation involving the formation of new dye binding sites, due to phospholipid-induced conformational changes of metHb, seems unlikely because in this case the $\mathrm{p}K^i_\alpha$ measured for protein should be

appreciably greater than that for the lipid phase. Thus, an assumption can be made that the increase of pK_{α}^{i} caused by metHb reflects modification of the dye region in the liposomal membrane under the influence of the protein. A lot of evidence indicates that hemoglobin binding to the lipid bilayer involves electrostatic as well as hydrophobic interactions [9,25,35,47]. The results of the present work were obtained at the pH varying from 7.0 to 10.0. In this pH range, hemoglobin has for the most part negative net charge since its isoelectric point is known to be ca. 7.4. As far as the ionization state of phospholipids is concerned, it should be noted that PC is a zwitterion over the 3–12 pH range, while phosphate groups of DPG are fully ionized over pH 5.0 [46], therefore no significant changes of phospholipid ionization occur in the pH range studied.

In view of these facts, no appreciable electrostatic interaction between the protein and liposomes, being negatively charged, could be expected. Meanwhile, numerous studies provide evidence for a strong hydrophobic interaction of hemoglobin with the lipid bilayer. Such findings as hemoglobin-induced increase of liposome ion permeability [10,25], effective binding of the protein to lipid vesicles at high ionic strength [10], the character of its effect on the lipid thermotropic behavior similar to that observed for integral membrane proteins [35] are considered as arguments in favor of preferentially non-polar nature of hemoglobin complexes with phospholipids. The explanation for this phenomenon involves two possibilities:

(a) formation of the hydrophobic contact region upon the dissociation of hemoglobin molecule into dimers [10]; and

(b) lipid-induced conformational changes of hemoglobin leading to the enhanced exposure of the apolar residues at the protein surface [25].

However, there exist some evidence suggesting the incorporation of the heme moiety rather than globin part into the lipid bilayer [41,43].

Thus, it seems likely that metHb effect on the protolytic behaviour of bromothymol blue bound to liposomes is determined by the alterations in the dye microenvironment resulting from the hydrophobic protein interaction with phospholipids. This is also supported by the observed enhancement of the protein influence on the dye partitioning between aqueous and lipid phases upon addition of KCl, when electrostatic effects are expected to be substantially suppressed. On the other hand, taking into accout the possible contributions to P^0 (Eq. (22)), it becomes clear that decrease of the partition coefficient for In^{2-} dye form under the influence of the protein can be caused by various reasons including, in particular, the changes of interfacial hydration, conformational alterations of phospholipids, formation of structural defects, where the binding of ions In^{2-} is energetically unfavorable and so on. The opposite effects of hemoglobin observed for HIn^{-} and In^{2-} dye species suggest the involvement of non-coulombic electrostatic factors in the protein-induced phenomena. As already mentioned, the contributions of phospholipid and water dipoles to the net surface potential have the opposite sign. If under certain circumstances Ψ_h proves to be $> \Psi_p$, the dipole potential Ψ_d would be negative, thus preventing the penetration of two-charged hydrophobic ion In^{2-} in the interfacial region.

Unfortunately, by analyzing the data available in the literature we could not find information concerning the detailed mechanisms of hemoglobin effect on the molecular organization of the model membranes. Some arguments in favor of the lipid bilayer perturbation caused by hemoglobin were provided by ^{31}P-NMR and IR-spectroscopy [14,39]. However, it appeared difficult to compare our observations with the findings of the other authors. Nevertheless, it should be noted that the changes of phospholipid headgroup conformation and mobility have been reported for the cases of both electrostatic (basic myelin protein [42], melittin [8,17]) and hydrophobic (glycophorin [49], cytochrome oxidase [38], Ca-ATPase [16]) protein–lipid interactions. For example, cationic amphiphilic peptide melittin was found to affect choline dipole orientation in the negatively charged model membranes by rotating the N^{+} end of the dipole to the aqueous phase [8]. It is suggested that the extent of headgroup reorientation is controlled by both, the melittin influence on the total surface charge density and spatial positioning of the peptide with respect to the choline dipoles. As indicated above, under the experimental conditions employed in the present work, one might expect preferential hydrophobic interaction of hemoglobin with liposomal membranes. The protein incorporation into the lipid bilayer could

affect the conformation of phospholipid molecules and alter in such a way the orientation of ester carbonyl and phosphocholine dipoles. The spatial distribution of the protein charged groups seems to be of significance in determining the position of phospholipid dipoles. In addition, one cannot exclude the possibility of the protein influence on interfacial hydration or formation of structural defects in the bilayer regions in contact with the protein molecules.

In conclusion, the results of the present study can be summarized as follows. The apparent ionization constant of bromothymol blue was found to be sensitive to the structural changes of the model membranes caused by metHb. Using the pK_{α}^{a} dependences on phospholipid concentration, the dye partition coefficients for lipid and lipid–protein systems have been determined. The character of the protein effect on the distribution of dye species between aqueous and lipid phases suggests modification of the structure and physicochemical characteristics of the membrane interfacial region upon the formation of protein–lipid complexes.

Acknowledgements

I thank Prof. N.O. Mchedlov-Petrossyan (Department of Chemistry, Kharkov State University) for helpful discussions of the problem under study and the referee for valuable comments on the principal aspects of data analysis.

References

[1] E. Antonini, J. Wyman, R. Moretti, A. Rossi-Fanelli, Biochim. Biophys. Acta 71 (1963) 124–138.
[2] G. Bartlett, J. Biol. Chem. 234 (1959) 466–472.
[3] S. Batzri, E. Korn, Biochim. Biophys. Acta 298 (1973) 1015–1019.
[4] M.L. Belaya, Langmuir 3 (1987) 648–654.
[5] M.L. Belaya, V.G. Levadny, in: Water in Disperse Systems, Moscow, Khimiya, 1989, pp. 147–168.
[6] R. Benesch, E. Benesch, S. Yung, Anal. Biochem. 55 (1973) 245–248.
[7] I.Ya. Bershtein, Yu.L. Kaminsky, Spectrophotometrical Analysis in Organic Chemistry, Khimiya, Leningrad, 1986.
[8] G. Beschiaschvili, J. Seelig, Biochem. 29 (1990) 52–58.
[9] S.V. Bondarenko, I.P. Ushakova, A.F. Levit, G.A. Serebrennikova, R.P. Evstigneeva, Bioorg. Khimiya 11 (1985) 1385–1390.
[10] L. Bossi, S. Alema, P. Calissano, E. Marra, Biochim. Biophys. Acta 375 (1975) 477–482.
[11] M. Bucher, T. Poster, J. Phys. Chem. 90 (1986) 3406–3411.
[12] C.R. Cantor, P.R. Schimmel, Biophysical Chemistry, Part 2, W.H.Freeman and Company, San Francisco, 1980.
[13] G. Cevc, Biochim. Biophys. Acta 1031 (1990) 311–382.
[14] V.V. Chupin, I.P. Ushakova, S.V. Bondarenko, I.A. Vasilenko, G.A. Serebrenni-kova, R.P. Evstigneeva, G.Ya. Rosenberg, G.N. Koltsova, Bioorg. Khimiya 8 (1982) 1275–1280.
[15] R. Colonna, P. Dell'Antone, G. Azzone, Arch. Biochem. Biophys. 151 (1972) 295–303.
[16] A. Deese, E. Dratz, Progr. Prot.-Lipid Interact., Amsterdam e.a., 1986, pp. 45–82.
[17] C. Dempsey, M. Bitbol, A. Watts, Biochem. 28 (1989) 6590–6595.
[18] N. Funasaki, J. Phys. Chem. 83 (1979) 1998–2003.
[19] G.P. Gorbenko, Ukrain. Biokhim. Zh. 63 (1991) 94–98.
[20] P. Henderson, Progr. Surf. Sci. 13 (1983) 197–224.
[21] V.G. Ivkov, G.N. Berestovsky, Dynamic Structure of Lipid Bilayer, Nauka, Moscow, 1981.
[22] H. Itabe, T. Kobayashi, K. Inoue, Biochim. Biophys. Acta 961 (1988) 13–21.
[23] R. Flewelling, W. Hubbel, Biophys. J. 49 (1986) 541–552.
[24] J. Jackson, A. Crofts, Eur. J. Biochem. 10 (1969) 226–237.
[25] H. Kimelberg, Mol. and Cell. Biochem. 10 (1976) 171–190.
[26] V.I. Klenin, S.Yu. Shchegolev, S.Yu. Lavrushin, Characteristic Functions of the Light Scattering in Disperse Systems, Saratov, 1977.
[27] T.B. Makarova, N.P. Korolev, B.D. Milgram, I.I. Ivanov, Nauch. Dokl. Vish. Shkol. 1 (1983) 34–40.
[28] E. Marva, R. Hubbel, Blood 83 (1994) 242–249.
[29] T. Mashimo, I. Ueda, D. Shien, H. Kamaya, H. Eyring, Proc. Natl. Acad. Sci. USA 76 (1979) 5114–5118.
[30] S. McLaughlin, Annu. Rev. Biophys. Biophys. Chem. 18 (1989) 113–136.
[31] M. Mille, G. Vanderkooi, J. Coll. Interface Sci. 61 (1977) 455–474.
[32] P. Mitchell, J. Moyle, L. Smith, Eur. J. Biochem. 4 (1968) 9–19.
[33] J. Moller, U. Kragh-Hansen, Biochem. 14 (1975) 2317–2323.
[34] P. Mukerjee, K. Banerjee, J. Phys. Chem. 68 (1964) 3567–3574.
[35] A. Nelson, D. McQuarrie, J. Theor. Biol. 55 (1975) 13–27.
[36] H. Oshima, T. Healy, L. White, J. Coll. Interface Sci. 90 (1982) 17–26.
[37] F. Quina, H. Chaimovich, J. Phys. Chem. 83 (1979) 1844–1850.
[38] A. Seelig, J. Seelig, Biochim. Biophys. Acta 815 (1985) 153–158.
[39] S. Seleznev, N. Gromov, Biofizika 28 (1983) 521.
[40] N. Shaklai, J. Yguerabide, H. Ranney, Biochem. 16 (1977) 5593–5597.
[41] Y. Shviro, I. Zilber, N. Shaklai, Biochim. Biophys. Acta 687 (1982) 63–70.

[42] F. Sixl, G. Brophy, A. Watts, Biochem. 23 (1984) 2032–2039.
[43] J. Szebeni, E. Lorio, H. Hauser, H. Winterhalter, Biochem. 24 (1985) 2827–2832.
[44] J. Szebeni, C. Winterbourn, R. Carrell, Biochem. J. 220 (1984) 685–692.
[45] G. Torrie, J. Valleau, J. Phys. Chem. 86 (1982) 3251–3257.
[46] J. Tocanne, J. Teissie, Biochim. Biophys. Acta 1031 (1990) 111–142.
[47] I.P. Ushakova, I.A. Vasilenko, G.A. Serebrennikova, R.P. Evstigneeva, Bioorg. Khimiya 7 (1981) 613–620.
[48] I.P. Ushakova, I.A. Vasilenko, G.A. Serebrennikova, R.P. Evstigneeva, Bioorg. Khimiya 6 (1980) 1062–1067.
[49] P. Yeagle, D. Kelsey, Biochem. 28 (1989) 2210–2215.
[50] B. Zaslavsky, L. Miheeva, N. Gulaeva, A. Borovskaya, M. Rubtsov, L. Lukatskaya, N. Mchedlov-Petrossyan, J. Chem. Soc. Faraday Trans. 87 (1991) 931–938.