Dye Sorption as an Indicator of Erythrocyte Membrane Damage and Prehemolytic State of Erythrocytes

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High erythrocyte membrane permeability caused by detergents was associated with increased methylene blue sorption, which correlated with erythrocyte damage and preceded hemolysis. The content of sorbed dye in erythrocytes preincubated with 200 μM sodium dodecyl sulfate or cetyltrimethylammonium bromide increased by 58-74% and 2.4-3.8-fold surpassed that in the medium. New precise and convenient method for estimating erythrocyte binding capacity is proposed.

Key Words: *erythrocyte; dye sorption; damage; detergent*

Erythrocyte damage is as a general sign of toxicity of exo- and endotoxins accumulated in the blood [4]. It has long been known that damages to animal cells are accompanied by enhanced dye sorption [2,7]. The method for estimating binding capacity of erythrocytes (BCE) from the intensity of methylene blue sorption was recently proposed [6]. It was shown that BCE increases during purulent and septic processes [1,5,6].

Further studies revealed opposite changes in BCE and erythrocyte membrane permeability (EMP) measured by hemolysis in urea solutions [1,5]. Experimental data show that EMP increases and BCE decreases under conditions of compensated endogenous intoxication during peritonitis, while at the late stages MP decreases and BCE increases. It was assumed that the rise of BCE reflects abnormal density and reduced permeability of membranes, while the decrease in this parameter indicates the increase in membrane permeability [1,5]. This conclusion is in controversy with basic notions that dyes enter the cells due to impairment of the membrane barriers [2,3].

Here we estimated the interrelation between dye sorption and EMP under conditions of high membrane permeability, and optimized the method for analyzing BCE.

MATERIALS AND METHODS

Experiments were performed on erythrocytes obtained by centrifugation of donor blood. Methylene blue (Reakhim) was dissolved in isotonic solution containing 100 mM NaCl, 20 mM NaOH, and 30 mM sodium-phosphate buffer; pH was adjusted to 7.4. Basic detergent cetyltrimethylammonium bromide (CTAB) and acid detergent sodium dodecyl sulfate (Sigma) were dissolved in 50 mM sodium-phosphate buffer containing 100 mM NaCl (pH 7.4). Erythrocytes were treated with these detergents: 1 ml detergent solution was added to 300 μl packed red blood cells, incubated at room temperature for 10 min, and centrifuged at 3000 rpm for 10 min; the supernatant was then discarded.

Hemolysis was estimated by measuring optical (D_h) of the sample (220 μl supernatant and 1.5 ml distilled water) in a 0.5-cm cuvette at 540 nm on a KFK-2MP photometer. The optical density (D_{h0}) of lysed cells in the same dilution obtained by addition of 60 μl packed red blood cells to 1.5 ml water corresponded to 100% hemolysis. The degree of hemolysis (H) was calculated.

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TABLE 1. Hemolysis and Methylene Blue Sorption (%) by Erythrocytes before (Control) and after Treatment with Detergents ($M\pm m$, n=3)

Parameter	Equation	Control	CTAB, μM		Sodium dodecyl sulfate, μM	
			100	200	100	200
Degree of hemolysis (H)	$100D_h/D_{h0}$	0	1.5±0.2	5.0±0.9	0.7±0.1	2.7±0.7
$\Delta C_f/C_0$	$100(1-D_k/D_0)$	31.5±3.0	39.7±2.6 (126)	52.0±5.0 (165)	37.2±3.8 (118)	48.5±5.2 (154)
C_b/C_0	$100(V_S/V_E)(1-D_k/D_0)$	104.9±10.0	132±20 (126)	173±29 (165)	124±9 (118)	162±20 (154)
C_{bs}/C_0	$100(V_S/V_E)(1-D_k/D_0)/(1-0.01H)$	104.9±10.0	134±21 (128)	183±52 (174)	126±9 (120)	166±22 (158)
Q	$(V_S/V_E)(D_0-D_k)/D_k$	1.53±0.3	2.9±0.6 (190)	5.8±2.3 (380)	2.3±0.4 (148)	3.6±0.5 (237)

Note. % of control is shown in brackets. V_E and V_S are volumes of packed red blood cells and dye, respectively.

Methylene blue sorption on erythrocytes was determined by a modified method described elsewhere [6]: 300 μl native or detergent-treated packed red blood cells were incubated with 1 ml 600 μM methylene blue for 30 min and then centrifuged. The supernatant was collected and added (150 μl) to 2 ml water. Optical density (D_k) was measured at 670 nm. The initial optical density (D_0) was estimated by the same method: the initial solution of methylene blue (150 μl) was added to 2 ml water.

After incubation of erythrocytes with methylene blue, the reduction of dye concentration in the supernatant was calculated ($\Delta C_f/C_0$, where $\Delta C_f=C_0-C_f$ and $C_f=C_0D_k/D_0$) [6]. We adapted the method for 1.5-ml standard plastic tubes. Methylene blue was diluted before photometry to avoid underestimation at high dye concentration. The concentration of methylene blue sorbed on cells ($C_b=(V_S/V_E)\Delta C_f$) was measured and corrected for the number of nonhemolyzed cells: $C_{bs}=C_b/(1-0/01H)$. It should be emphasized that the parameter characterizing an equilibrium distribution of the dye between cells and medium was the most informative: $Q=C_b/C_f$ [2]. Parameters of sorption (ΔC_f, C_b, and C_{bs}) were calculated in a dimensionless form as the ratio of current to initial concentration of methylene blue (equations are shown in Table 1).

RESULTS

Detergents, hemolytic viruses, and many bacterial and animal toxins belong to channel-forming agents forming hydrophilic pores in cell membranes [9]. It was shown that EMP increases with increasing the content of detergents in the medium and culminates in cell lysis due to their colloid and osmotic swelling [8]. In our experiments detergents were used in concentrations corresponding to the prehemolytic level of EMP: the degree of hemolysis at maximum detergent concentration was 3-5% (Fig. 1). Irrespective of the charge of detergents, the increase in their concentrations led to rapid elevation of methylene blue content in nonhemolyzed cells (Table 1). It is interesting that the rise of $\Delta C_f/C_0$ practically did not differ from that observed during purulent and septic processes (182-188%) [4]. Therefore, the model used in our experiments approximates actual degree of toxin-induced erythrocyte

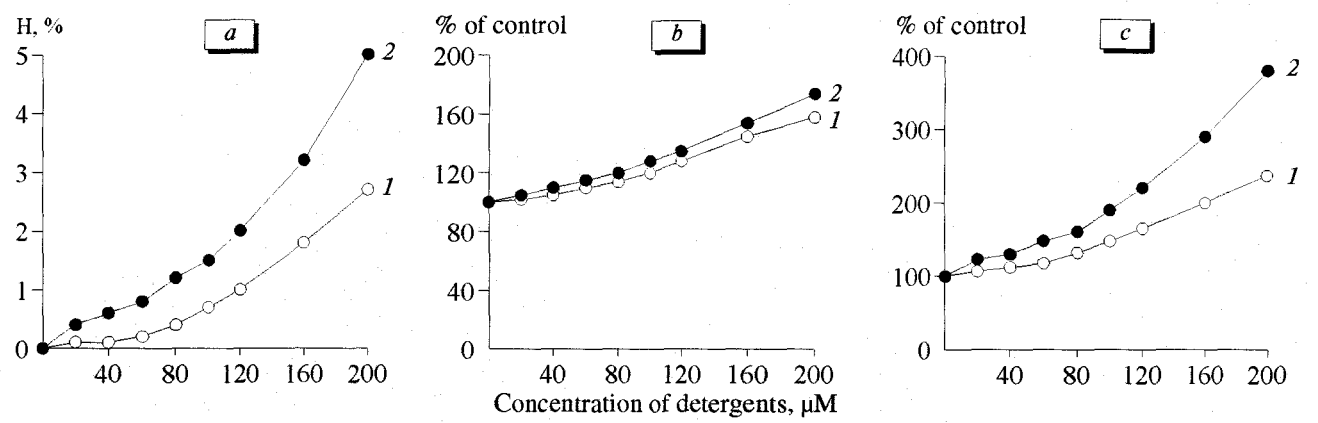

Fig. 1. State of erythrocytes incubated with sodium dodecyl sulfate (*1*) and cetyltrimethylammonium bromide (*2*). Ordinate: degree of hemolysis (*H*, *a*), sorption of methylene blue (*b*), and *Q* (*c*).

damage in patients with sepsis. Q in native erythrocytes was above 1, which agrees with published data on high membrane permeability for basic dyes and their intensive sorption by cell proteins [2,7]. In erythrocytes treated with detergents, Q 2.4-3.8-fold surpassed that in the control (Table 1). Thus, methylene blue sorption correlates with the rise of EMP and precedes hemolysis. Our results showed that Q is a more accurate measure of BCE (compared to other parameters).

Simultaneous increase in methylene blue sorption and EMP suggests that previous interpretation of changes in BCE and EMP observed during endogenous intoxication should be reconsidered. From the principle of EMP estimation, we assume that hemolysis (and, therefore, membrane resistance to osmotic swelling) is a rate-limiting step of the process. Therefore, hemolysis in the urea solution is a variant of osmotic resistance of erythrocytes, rather than the measure of EMP. Therefore, the greater is the degree of membrane damage, the greater BCE and the lower erythrocyte resistance. The apparent attenuation of the degree of erythrocyte damage [1,5] is probably related to variations in red blood cell population (intensive destruction of less resistant old cells and recruitment of more resistant young cells into the circulation).

Our experiments showed that measurements of methylene blue sorption can be used for quantitative assay of toxin-induced erythrocyte damage associated with elevated EMP. We proposed a new precise and convenient method for estimating BCE. This method adapted for small amounts of reagents is based on the analysis of dye distribution between cells and medium.

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