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The mechanism of Zn-phthalocyanine photosensitized lysis of human erythrocytes

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Abstract

The phthalocyanines have recently been suggested as one of most effective possible sensitizers for photodynamic therapy and the blood viral inactivation. The further characterisation of the mechanism of human red blood cell lysis and membrane alterations upon photodynamic treatment in the presence of Zn-phthalocyanine was the aim of this study. It was found that there were $(2.7\pm0.4)\cdot10^7$ dye binding sites per red blood cell with the association constant equal to $(1.4\pm0.3)\cdot10^4$ M^{-1}. Two types of the photosensitized haemolysis: haemolysis during irradiation (''light'' haemolysis) and post-irradiation haemolysis (''dark'' haemolysis) were studied. The erythrocyte membrane hyperpolarisation, membrane fluidisation and cell swelling preceded the ''light'' haemolysis. The modification of the erythrocyte membrane band 3 protein by DIDS (an inhibitor of anion exchange) increased the rate of the ''light'' haemolysis. The rate of ''dark'' haemolysis was higher and that of ''light'' haemolysis was lower in potassium media in comparison to sodium ones. The rates of photohaemolysis depended on the erythrocyte membrane potential: a decrease of membrane potential inhibited both types of haemolysis. The cell shrinkage in the presence of sucrose (up to 15 mM) inhibited the ''dark'' haemolysis but significantly increased the ''light'' haemolysis. Oxidation of intracellular oxyHb to metHb by nitrite, which drastically decreases intracellular oxygen concentration, as well as GSH concentration, inhibited the rate of the ''light'' haemolysis. The results allow for the conclusion that the mechanism of photochemical (''light'') haemolysis is not of a colloid-osmotical type, in contrast to the post-irradiation (''dark'') haemolysis. The photochemical oxidation or denaturation of band 3 protein plays a significant role in the formation of haemolytic holes. The membrane lipid peroxidation, as well as glutathione oxidation, does not participate in the process of photosensitized haemolysis. From the inhibition of ''dark'' haemolysis by sucrose the apparent pore radius was estimated to be about 1.1 nm. The pores appear to be transient short-lived ones, the average pore number per cell was 0.02.

Keywords: Photosensitized haemolysis; Erythrocytes; Zn-Phthalocyanine; Membrane

1. Introduction

Photodynamic therapy (PDT) is a cytotoxic treatment, which can induce cells to initiate a rescue response, or to undergo cell death, either apoptosis or necrosis [1]. Several second generation photosensitizers are currently under development for application to photodynamic therapy, both in oncology as well as in non-oncological diseases and cell blood product photosterilization [2,3]. Derivatives from the group of phthalocyanines (Pcs) have been the sensitizers of choice [2,3].

The subcellular localisation of the photosensitizer is of special importance, since it determines the localisation of the primary damage and a detailed understanding of the cellular photoinduced processes seems very important for the better application of the photodynamic therapy.

It was earlier shown that photodamage to red blood cells could be involved in haemostasis induced by photodynamic therapy [4]. The photoinduced ''light'' and ''dark'' haemolysis had been seen as the most pronounced results of photoinduced erythrocyte damage. Photohaemolysis is usually considered to be a colloidal-osmotic process, during which loss of selective permeability of the photodamaged membrane leads to indiscriminate entrance of solute molecules, swelling and eventual membrane rupture [4–7]. The steps leading from the primary photochemical events on the key elements of the cell membrane to cell damage and haemolysis are still obscure, as well as the key elements of membrane themselves. However, the phenomenon of photoinduced haemolysis has been known for many years [8].

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The aim of this report was to further characterize the molecular mechanisms underlying ZnPc-mediated cell photodamage using human red blood cells (RBCs), lacking protein-impairment-repair-machinery, as a human cell model.

In our experiments the mechanism of photosensitized haemolysis was revealed by evaluating red blood cell membrane structural transformations, parameters of haemolysis, rates of cellular glutathione oxidation and membrane lipid peroxidation, and the size and numbers of membrane pores formed. This was achieved by examining the effects of postirradiation treatments and irradiation conditions on the courses of haemolysis. Two types of cell processes: ''light'' processes under irradiation (light phase of photoreaction) and ''dark'' ones (post-irradiation events of the preliminarily irradiated cells during dark incubation) were separated.

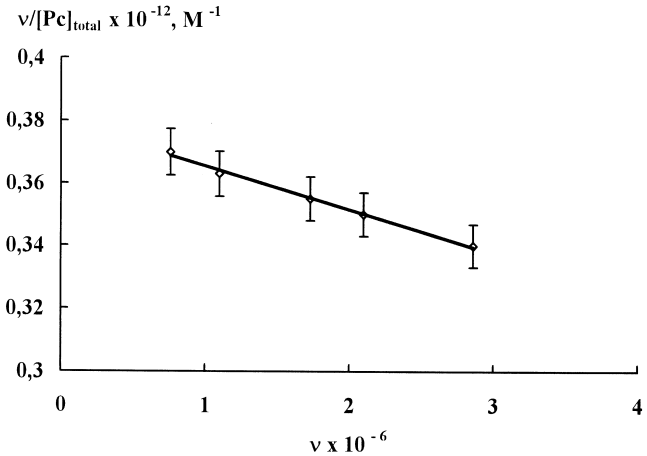

Fig. 1. The Scatchard plot of the Zn-phthalocyanine binding by human erythrocytes. $6.75 \cdot 10^8$ cell/ml, PBS, pH 7.4, 22 °C. The dye concentration was changed from 2 to 8 μM.

2. Materials and methods

2.1. Chemicals and cells

Reagents of analytical grade were from POCH (Gliwice, Poland); 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), 2-thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were from Sigma–Aldrich, Germany; the fluorescent probe 1-(trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH) was from Molecular Probes, Eugene, OR, USA; Zn-phthalocyanine (ZnPc) was from Acros Organics, NJ, USA; dimethyl sulfoxide was from Sigma, St. Louis, MO, USA.

Blood from healthy donors was purchased from the Central Blood Bank in Lodz, Poland. Blood was taken into 3% sodium citrate. After removing plasma and the leukocyte layer, erythrocytes were washed three times with cold (4 °C) phosphate-buffered saline (PBS: 0.15 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4). RBCs were used immediately after isolation.

2.2. ZnPc binding to RBCs

The parameters of the dye binding by RBCs were estimated by the Scatchard plot (Fig. 1), where ν is the ratio of the concentration of the bound dye ($[Pc]_{bound}$) to the concentration of the cells [(cell number)/(per litre)]. $[Pc]_{bound}$ was calculated from: $[Pc]_{bound}=[Pc]_{total}-[Pc]_{free.}$

The concentrations of the free dye were determined spectrophotometrically (using calibration curve) in the supernatant after centrifugation of cell suspensions (4000 *g*, 5 min), containing $6.75 \cdot 10^8$ cell/ml at different concentrations of added (total) ZnPc (2–8 μM) in PBS, pH 7.4, 22 °C. Before centrifugation, cells were incubated with ZnPc for 20 min.

The plot slope is equal to (K_a) (association constant) and the interception point with the *y*-axis is equal to (nK_a) (*n* is the number of the equal and independent binding sites per cell).

2.3. Photo oxidative damage of erythrocytes

2.3.1. Conditions of erythrocyte irradiation

The susceptibility of erythrocytes was measured in terms of the accumulation of TBA-reactive species (TBARS), oxidation of intracellular reduced glutathione (GSH), changes of red blood cell membrane fluidity and cell haemolysis. These parameters were measured immediately after irradiation (''light'' processes) or after dark storage of irradiated cells (''dark'' processes).

The erythrocyte suspensions in PBS (5% haematocrit) were dark incubated with ZnPc (3 μM) at 22 °C for 20 min [ZnPc was added as 0.5 mM stock solution in dimethyl sulfoxide (DMSO)]. After incubation RBC suspensions (2 ml) were irradiated with laser light of 670 nm in standard 1.5 cm glass tubes through uncovered surface, with intermittent gentle mixing to expose all cells to the same number of photons. The light source was a semiconductor laser (Toshiba TOLD 9215, Japan) with regulated light emission power (0 –7 mW) and width of a light beam. Usually cell suspensions were irradiated 30 min at light source power of 7 mW (light dose=12.6 J) and 22 °C. To remove the photosensitizer after irradiation, erythrocytes were washed before measuring the most of cell parameters.

2.3.2. TBARS assay

The amount of TBARS formed was measured using the method of Stocks and Dormandy [9]. Briefly, 0.2 ml of 25% TCA was added to 2 ml of final red blood cell suspension and centrifuged. To 1 ml of the supernatant 0.25 ml of 1% TBA in 0.05 M NaOH and 0.025 ml of butylated hydroxytoluene in methanol (88 mg/10 ml alcohol) were added. This was kept in a boiling water bath

for 15 min. The concentration of the lipid peroxidation products–TBA complex was assessed spectrophotometrically using the extinction coefficient 156 mM^{-1} cm^{-1} (532 nm).

2.3.3. Intracellular GSH assay

The GSH level was determined by the method of Ellman et al. [10]. Briefly, 0.2 ml of 25% TCA was added to 2 ml of final RBC suspension and centrifuged. To 1 ml of the supernatant, 1 ml of 1 M phosphate buffer (pH 7.4) and 0.1 ml Ellman's reagent (10^{-3} M) were added for GSH determination. Concentration of GSH was monitored spectrophotometrically at 412 nm using the extinction coefficient 13.6 mM^{-1} cm^{-1}.

2.3.4. RBC membrane fluidity evaluation

Fluorescence anisotropy of probe TMA-DPH, incorporated in cell membrane is inversely related to membrane fluidity [11]. Measurements of steady-state fluorescence anisotropy of TMA-DPH were performed at room temperature with a Perkin-Elmer LS-5B spectrofluorimeter. Prior to anisotropy measurements, samples of red blood cells were diluted to the haematocrit of 0.05% to avoid depolarization effects due to light scattering. The final concentration of the fluorescent probe in samples was 1 μM, $\lambda_{ex}=356$ nm and $\lambda_{em}=428$ nm.

The steady-state fluorescence anisotropy was calculated according to the equation [11]:

$$r = (I_{vv} - I_{vh}G)/(I_{vv} + I_{vh}G)$$

where I_{vv} and I_{vh} are the components of emitted light intensity which are parallel and perpendicular, respectively, with reference to the direction of polarisation of the excitation light, and G is the factor ($G=I_{hv}/I_{hh}$) used to correct for unequal transmission in the optical system. Under these conditions the effect of light scattering was negligible.

2.3.5. "Light" and "dark" haemolysis evaluation

Percentage of "light" haemolysis was calculated as the haemoglobin release after photosensitization: 0.05 ml of irradiated suspension was added to 2 ml of PBS and centrifuged immediately at 4 °C. The haemoglobin content in the supernatant was measured spectrophotometrically by absorbance at 414 nm.

For registration of the process of "dark" haemolysis, preliminarily irradiated (as described) and twice washed RBCs were incubated in the dark in PBS (haematocrit 0.5%) at 22 °C. Haemolysis were determined after suitable time intervals by measuring haemoglobin contents in the supernatant after cell centrifugation and in the total suspension (for estimation of 100% haemolysis).

2.3.6. Osmotic fragility evaluation

For the determination of the osmotic fragility, irradiated erythrocytes were washed immediately and diluted to the 0.25% haematocrit with the NaCl solutions of various concentrations (50–150 mM) buffered with 5 mM sodium phosphate (pH 7.4). After 20 min of incubation, cell suspensions were centrifuged and the percent of osmotically lysed cells was estimated.

2.3.7. Membrane pore parameter evaluations

The apparent area of the holes, formed under irradiation we calculated according to the equation of Lieber et al. [12]:

$$A = d\left[(V^*)^2 - (V_0)^2\right]/2DV_0t_{50}$$

where d is the membrane thickness ($d=6\cdot10^{-7}$ cm) [12], V^* is the average erythrocyte aqueous volume of the maximally swollen cell (critical haemolytic volume) (cm^3), V_0 is the average RBC aqueous volume before irradiation, D is the diffusion coefficient of the solute in the medium (cm^2/s), and t_{50} is the time required for 50% haemolysis (s).

The true mean radius (r) and number (n) of the pores can be calculated from:

$$r = (b\sqrt{A_a} - a\sqrt{A_b})/(\sqrt{A_a} - \sqrt{A_b})$$

$$n = A_a/\left[\pi(r-a)^2\right] = A_b/\left[\pi(r-b)^2\right]$$

We used 20 mM NaCl and 40 mM sucrose as solutes added to iso-osmotic PBS solution. For these pair of solutes, Stokes radii and diffusion coefficients are $a=0.13$ nm and $D_a=12.6\cdot10^{-6}$ cm^2/s (NaCl) and $b=0.53$ nm and $D_b=4.7\cdot10^{-6}$ cm^2/s (sucrose) [12–14].

2.4. Photo oxidative damage of modified RBCs

2.4.1. Spectrin heat-denaturation

The temperature of the heat denaturation of spectrin is 48–50 °C [15]. For membrane protein thermodenaturation experiments, the RBCs (5% haematocrit in PBS) were incubated in the dark at different temperatures for 20 min, then cooled on ice.

2.4.2. Band 3 protein modification

The RBCs (5% haematocrit in PBS) were incubated with 10^{-3} M 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (disodium salt) (DIDS) (Sigma) at 22 °C for 30 min, washed twice by the excess of PBS and ZnPc was added.

2.4.3. Intracellular oxyHb oxidation

The erythrocytes (10% haematocrit in PBS) were incubated in the presence of different concentrations of sodium nitrite at 22 °C for 30 min, washed twice by the excess of PBS, and suspended at a haematocrit of 5%.

Results were expressed as mean±S.D. and statistical significance of differences was evaluated using unpaired Student's t-test.

3. Results

Scatchard's plot analysis of the parameters of the ZnPc binding by the human RBCs showed that there are $(2.7\pm0.4)\cdot10^{7}$ dye binding sites per cell with the association constant equal to $(1.4\pm0.3)\cdot10^{4}$ M^{-1} (Fig. 1). At the concentration of the dye used approximately 10^{6} dye molecules per cell were bound. Irradiation of cell suspension in the presence of the dye resulted in the damage to the erythrocytes: accumulation of the products of membrane lipid peroxidation (TBARS formation), cellular reduced glutathione oxidation and haemolysis (Fig. 2a–c). Membrane soluble antioxidant butylated hydroxytoluene (BHT) (10^{-4} M), when added before irradiation, inhibited the process of membrane lipid peroxidation (Fig. 2b), but it neither influenced the percent of ''light'' haemolysis (Fig. 2a) nor protected intracellular reduced glutathione from oxidation (Fig. 2c). It must be stressed that no intracellular oxyHb oxidation was observed during the process of photosensitization.

Preliminarily irradiated RBCs undergo some processes which develop over time during dark incubation (''dark'' processes). We observed slow ''dark'' accumulation of lipid peroxidation products, some recovery of the intracellular level of GSH oxidized by irradiation (Fig. 3) and ''dark'' haemolysis (Fig. 4b). The process of ''dark'' haemolysis of photodamaged cells can be described by a simple equation: $N=N_0\cdot e^{-kt}$, where N_0 and N are the numbers of the nonlysed cells at the time zero and t, respectively, and k is the apparent rate constant of ''dark'' haemolysis (Fig. 4b). Presence of BHT during irradiation significantly inhibited ''dark'' accumulation of TBARS (Fig. 3) but did not decrease the rate of ''dark'' haemolysis.

Photooxidation of erythrocytes decreased the fluorescence anisotropy r of the fluorescence probe TMA-DPH and increased the fluorescence intensity of the probe, reflecting remarkable RBC membrane structural transformations (Fig. 5).

The vehicle alone (DMSO) in the concentration used (less then 0.5%) (data not shown) or irradiation without photosensitizer (Fig. 5) did not produce any measurable effect on RBCs.

Osmotic fragility curves of erythrocytes irradiated with various doses are shown in Fig. 6. Irradiation increased the RBC osmotic fragility. The concentration of NaCl corresponding to 50% osmotic haemolysis of native erythrocytes was 60.8±3.6 mM whereas that corresponding to 50% haemolysis of cells irradiated 30 min at 7 mW of laser power (12.6 J) was 75.2±3.6 mM. Taking into account the osmolarity of the buffer, these values correspond to the osmolarity of 134.1 and 162.9 mosmol/l, respectively.

The rate of haemolysis significantly depended on the medium of irradiation and on the RBC membrane state. Cell ''light'' haemolysis was higher in a buffered sodium-containing medium, in comparison to the potassium one

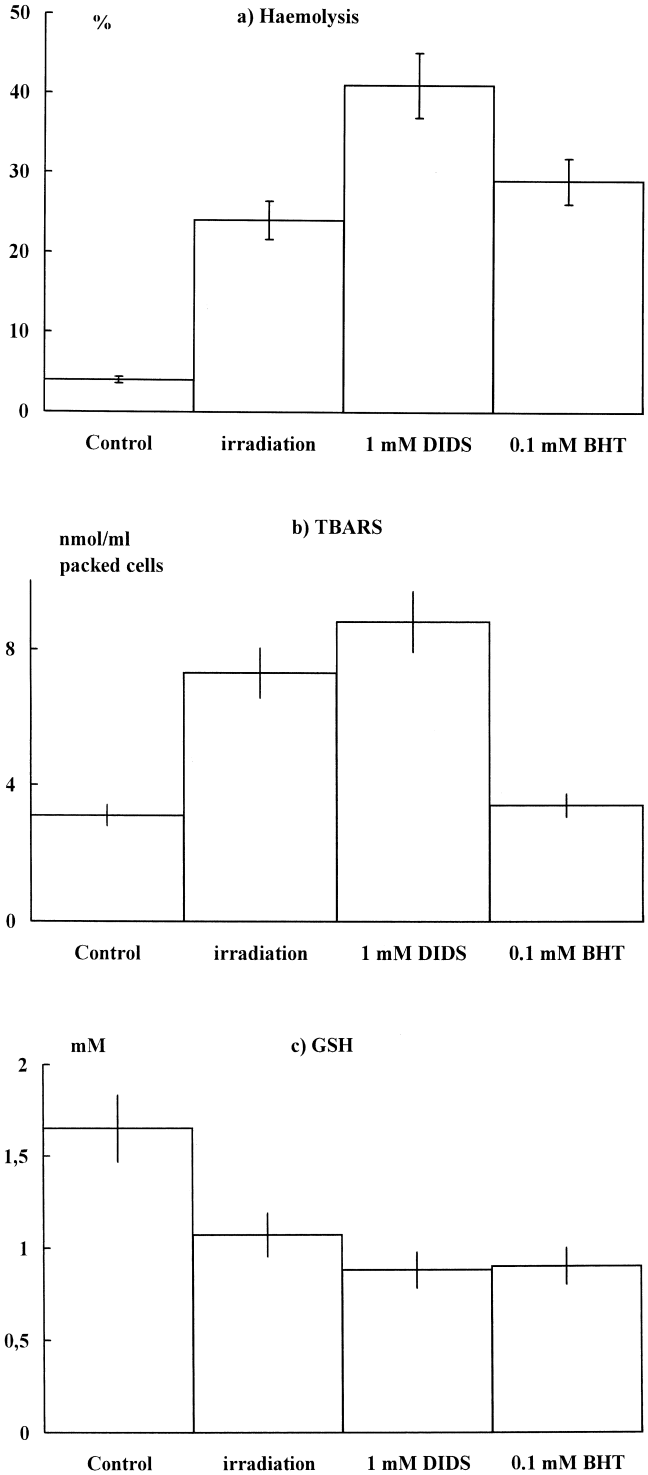

Fig. 2. Light-induced processes in RBCs before and after modification by DIDS (10^{-3} M) or in the presence of BHT (10^{-4} M): (a) percent of ''light'' haemolysis; (b) TBARS formation; (c) intracellular GSH oxidation. Cell suspensions were irradiated 30 min at light source power of 7 mW (light dose = 12.6 J).

(Fig. 4a). Cell membrane hyperpolarization by valinomycin addition in a non-potassium medium inhibited ''light'' haemolysis, whereas valinomycin did not change

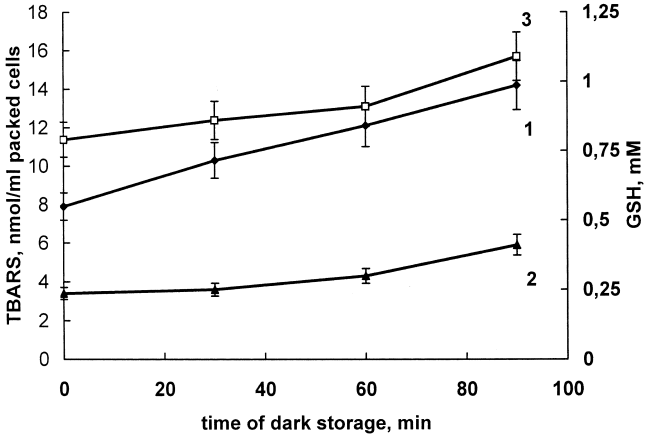

Fig. 3. ''Dark'' processes in the preliminarily irradiated RBCs; changes in TBARS (1, 2) and GSH (3) levels; cells were irradiated and incubated in dark in the absence (1, 3) or presence of 10^{-4} M BHT (2).

the rate of haemolysis in a potassium containing medium (in this case valinomycin did not change the potassium distribution) (Fig. 4a). Perhaps the dye binding to the membrane depends on cell membrane charge. In agreement with previous observations [5], ''dark'' haemolysis was accelerated in KCl solutions versus NaCl solutions (isotonic KCl or NaCl media buffered with 10 mM Tris–HCl, pH 7.4) (Fig. 4b). It also depended on membrane potential: the enhancement of membrane potassium permeability by valinomycin decreased the rate constant of haemolysis in isotonic NaCl solutions and increased this parameter in isotonic KCl solutions (Fig. 4b).

Hyperosmotic shrinkage of erythrocytes by sucrose (up to 15 mM of sucrose added to buffered isotonic NaCl solution) increased the percent of lysed cells under irradiation (Fig. 7). Only at higher sucrose concentrations we observed some inhibition of ''light'' haemolysis. However, a complete cell protection by hyperosmotic shrinkage in the presence of sucrose was not achieved. One can conclude that haemolysis during irradiation is not a colloid-osmotic process. The effect of sucrose, added to irradiated RBCs, on the rate constant of ''dark'' haemolysis, is shown in Fig. 7. Hyperosmotic shrinkage caused by high sucrose concentrations completely prevented ''dark'' haemolysis of photodamaged cells.

To estimate the role of different cell (or membrane) constituents on the RBC photostability, the photoinduced processes in the preliminarily modified cells were studied. RBC modification by DIDS, the inhibitor of anion transport mediated through the membrane band 3 protein, accelerated the ''light'' haemolysis about two times and slightly increased both the rate of photoinduced GSH oxidation and membrane lipid peroxidation (Fig. 2). Similarly, RBC modification by DIDS enhanced the rate of ''dark'' haemolysis (data not shown).

Preliminary heat-denaturation of spectrin resulted in an increase of the percent of ''light'' haemolysis and in a

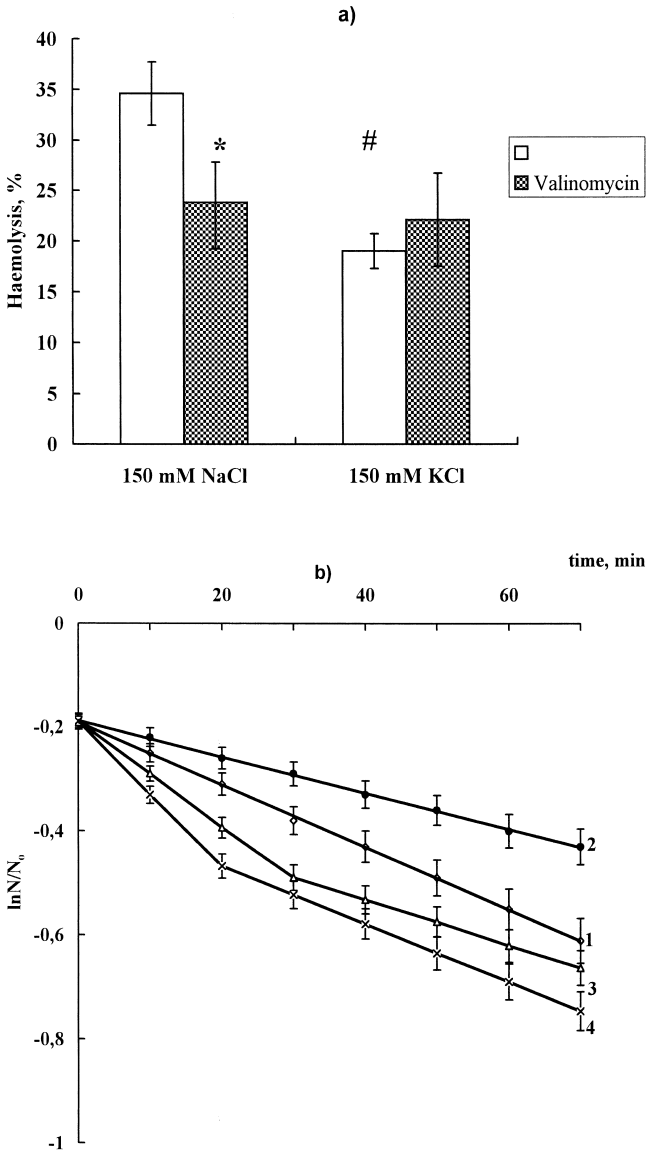

Fig. 4. (a) The percent of ''light'' haemolysis of RBCs in the Na^+- or K^+-containing media (150 mM NaCl or 150 mM KCl + 10 mM Tris–HCl, pH 7.4) in the presence or absence of valinomycin ($5 \cdot 10^{-6}$ M); * or # $p < 0.05$ in comparison to haemolysis in 150 mM NaCl in the absence of valinomycin. (b) ''Dark'' haemolysis of the preliminarily irradiated erythrocytes in different media: (1) 150 mM NaCl + 10 mM Tris–HCl, pH 7.4; (2) 150 mM NaCl + 10 mM Tris–HCl, pH $7.4 + 5 \cdot 10^{-6}$ M valinomycin; (3) 150 mM KCl + 10 mM Tris–HCl, pH 7.4; (4) 150 mM KCl + 10 mM Tris–HCl, pH $7.4 + 5 \cdot 10^{-6}$ M valinomycin.

slight increase of the efficiency of intracellular GSH photooxidation and did not change the process of membrane lipid photoperoxidation (Fig. 8). Heating alone did not produce cell haemolysis, GSH oxidation or TBARS formation (Fig. 8).

Next, the question was asked whether haemoglobin plays any role in cell photostability. Oxidation of intracellular oxyHb to metform significantly inhibited photosensitized ''light'' haemolysis, the level of intracellular metHb (produced by nitrite treatment) inversely correlated with

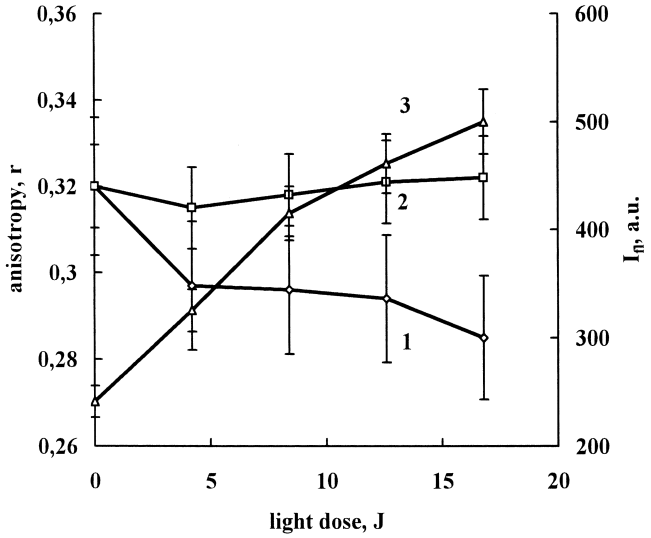

Fig. 5. The dependences of fluorescence anisotropy (1, 2) and intensity (3) of TMA-DPH incorporated in red blood cell membranes on light irradiation dose. Cells were irradiated in the presence (1, 3) or absence (2) of ZnPc for various times at a light source power of 7 mW.

the level of haemolysed cells (Fig. 9a). The increased level of products of lipid peroxidation in the preliminarily oxidized cells (Fig. 9b) can be due to a decreased level of intracellular GSH at high (above 3 mM) nitrite concentrations (Fig. 9c). (Nitrite treatment did not produce any haemolysis or TBARS formation, Fig. 9).

To study the role of metabolic cell state in photodestruction, RBCs were irradiated in the presence of high glucose concentrations (50–100 mM in PBS). Presence of glucose during irradiation slightly increased the rate of GSH

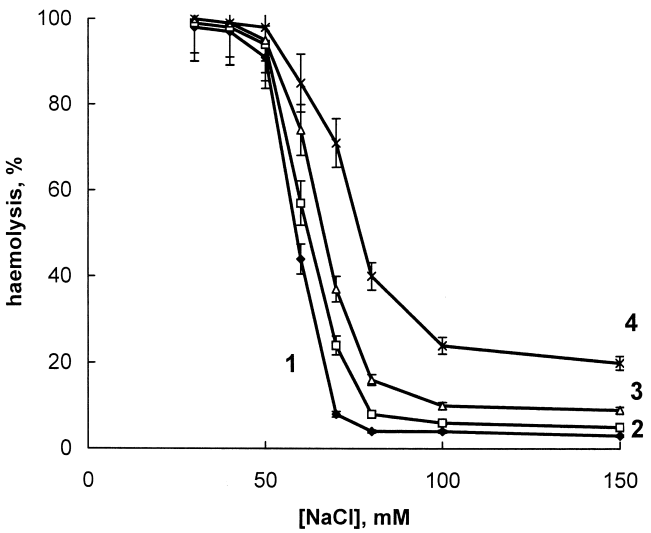

Fig. 6. Osmotic fragility of erythrocytes irradiated with various light powers for 30 min (various light doses) in the presence of ZnPc (3 μM): 1—control RBCs; 2—2 mW (3.6 J); 3—3 mW (5.4 J); 4—7 mW (12.6 J). After irradiation RBCs were immediately washed and incubated 20 min in different buffered NaCl solutions (haematocrit 0.25%). The percent of osmotically lysed cells was measured.

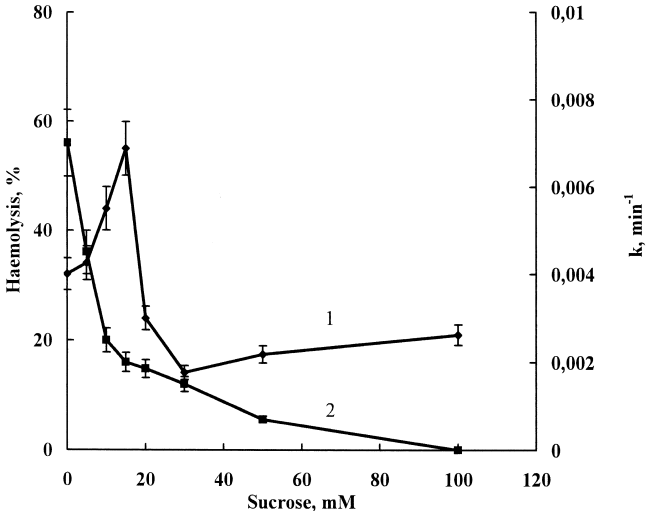

Fig. 7. The effect of sucrose on the percent of lysed RBCs under irradiation (''light'' haemolysis) (1) and on the rate constant of ''dark'' haemolysis (in this case RBCs after irradiation were washed twice and incubated in the correspondent sucrose solutions in PBS in dark) (2).

photooxidation and did not significantly change either the level of accumulated TBARS or the rate of ''light'' haemolysis (data not shown).

Fig. 10 shows the Arrhenius plot of the temperature dependence of the rate constant of ''dark'' haemolysis, which is not linear. One can see the inflection point around 27–30 °C between two parts of the curve. We calculated the apparent activation energy of the process of ''dark'' photohaemolysis in the temperature range 37–44 °C assuming that the temperature dependence of rate constant of haemolysis in narrow temperature range can be described by Arrhenius equation. A value of $\Delta E = (101 \pm 17)$ kJ/mol was obtained.

For calculation of apparent radii and numbers of pores

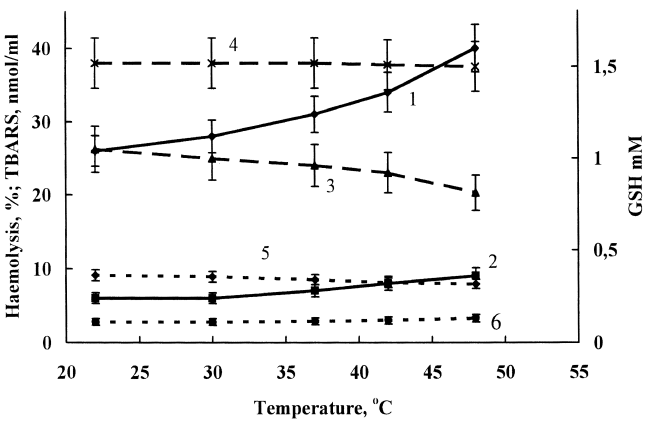

Fig. 8. The effect of RBCs preliminary heating on ''light'' haemolysis (1), intracellular GSH oxidation (3) and RBC membrane lipid peroxidation (TBARS formation) (5) for photosensitized erythrocytes. The percent of RBC haemolysis (2), level of GSH (4) and TBARS (6) in the preliminarily heated erythrocytes after 30 min incubation in the presence of 3 μM ZnPc without irradiation were measured for comparison.

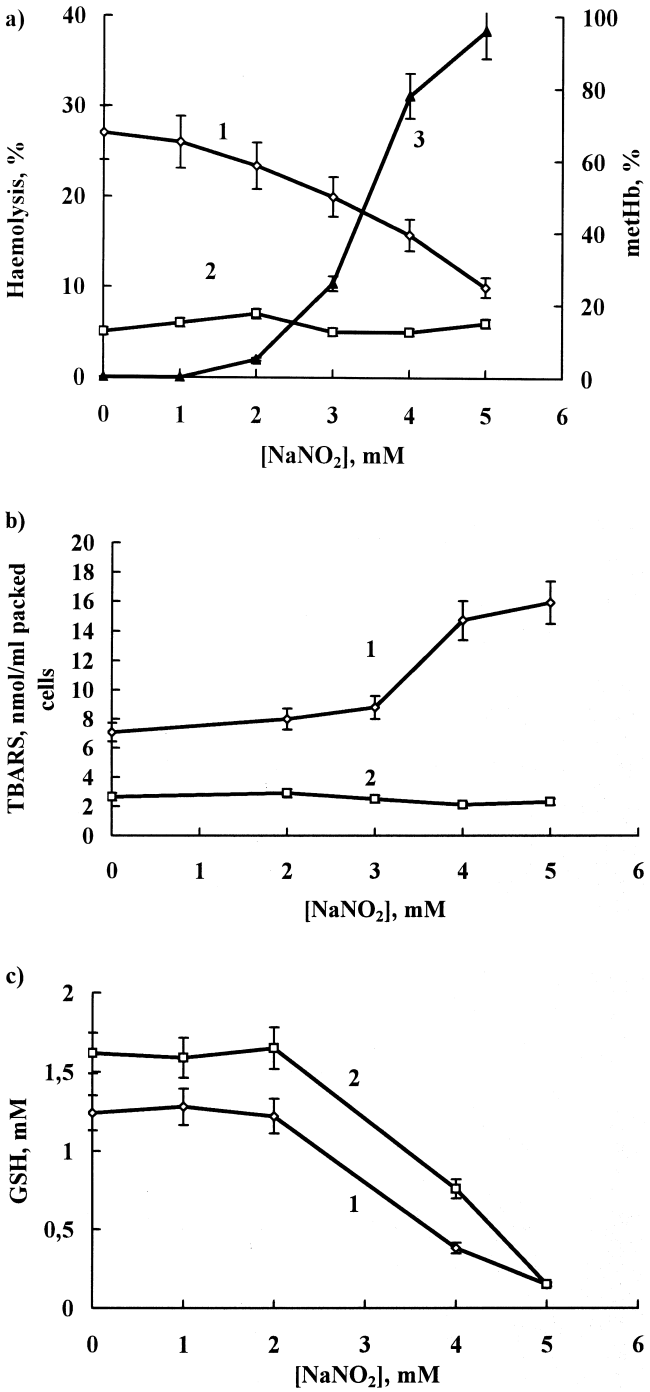

Fig. 9. Light-induced processes in metHb-containing erythrocytes. The dependences on nitrite concentration of: (a) the percent of lysed cells (''light'' haemolysis) (1, 2) and metHb content in erythrocytes (3); (b) the level of lipid peroxidation (1, 2) and (c) the reduced glutathione (GSH) content (1, 2). The parameters were measured immediately in erythrocytes irradiated (1) or incubated in dark (2, 3) for 30 min after modification by different concentrations of $NaNO_2$.

formed under photosensitization it can be assumed for human erythrocytes that the RBCs have an aqueous volume $V_0 = 9.8 \cdot 10^{-11}$ cm^3 and that the osmotically active volume of the cell is 0.7 of its total volume [16]. The

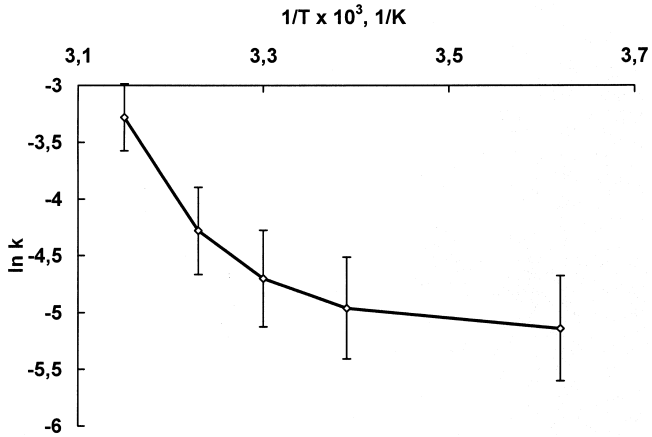

Fig. 10. Arrhenius plot of the temperature dependence of the rate constant of ''dark'' haemolysis. RBCs were irradiated at 22 °C, washed and dark stored at different temperatures.

aqueous volume of the maximally swollen RBC V^* can be determined from the osmotic fragility curve as the sum of the osmotically inactive volume (V_{ina}) and the osmotically active volume (V_a) corresponding to 50% osmotic haemolysis, assuming that the osmotically active volume of the RBC behaves as a perfect osmometer and the osmotically inactive volume does not change after photosensitization [13]. Critical haemolytic volume would be: $V^* = V_{ina} + V_{a\ iso} \cdot (C_{iso}/C_{50\%})$, where $V_{ina} = 2.94 \cdot 10^{-11}$ cm^3, $V_{a\ iso} = 6.86 \cdot 10^{-11}$ cm^3, C_{iso} is the osmolarity of isosmotic medium (300 mosm), and $C_{50\%}$ is the osmolarity of the medium of 50% osmotic haemolysis. V^* was equal to $18.3 \cdot 10^{-11}$ cm^3 for nonirradiated cells and to $15.6 \cdot 10^{-11}$ cm^3 for photodamaged cells at light dose of 12.6 J (Fig. 6). Cell photosensitization decreased the average aqueous haemolytic volume (decreased membrane stability and deformability) due to cell photoinduced impairments.

For two solutes, 20 mM NaCl (a) and 40 mM sucrose (b), t_{50} was measured to be 5910 s for NaCl solution and 41400 s for sucrose solution. Two different hole area values $A_a = 6.03 \cdot 10^{-2}$ nm^2 and $A_b = 2.31 \cdot 10^{-2}$ nm^2 were obtained.

According to our calculations irradiation of photosensitized RBCs induces membrane pores with apparent radius $r = 1.1$ nm and the average pore number per cell is $n = 0.02$.

4. Discussion

Cell photosensitization results in a complex cascade of cellular events, involving several signalling pathways [1]. For different types of cells and photosensitizers, a photostimulated expression of pro-/antiapoptotic proteins [17], stimulation of protein phosphorylation [1], immediate transient increase of cytoplasmic free calcium. This latter

effect was mediated by damage of critical cell targets via singlet oxygen or other reactive oxygen species [18]. In the case of RBCs, lysis produced by different photosensitizers originates from the oxidatively induced formation of aqueous membrane pores permeable to small ions, and also to nonelectrolytes and macromolecules up to a limiting size [5,6]. Photodynamic treatment of erythrocytes in the presence of aluminum chlorotetrasulphonate Pc enhanced leaks for K^+ as well as for choline or erythritol. This enhancement was accompanied by a marked increase of the transbilayer reorientation rate of the membrane lipids [6]. Additionally, Pc induced photodamage to RBCs resulted in cell swelling, spherocytosis [19], reduction in negative cell surface charge on RBCs immediately after treatment [20], and cell aggregation [21]. Photodynamic activity of Pcs correlated with a binding of the dye to the cell [19].

We have previously shown that the first step of RBC membrane photodamage is a considerable membrane hyperpolarization due to specific potassium ion leakage not compensated by sodium ion moving into the cell [22].

Photosensitized damage of RBCs produced a significant increase of membrane fluidity (Fig. 5), which is in agreement with previous observation of a marked increase of the transbilayer reorientation rate of lipid probe [6]. Assuming that fluorescence intensity of TMA-DPH incorporated into RBC membrane is proportional to the cell membrane surface area [23], a considerable increase of the RBC membrane area was observed (Fig. 5). This correlates with the observed photosensitized cell swelling [19]. Membrane fluidization may be due to the formation of RBC membrane defects. Such membrane defects and impairments of membrane integrity decreased RBC osmotic stability (Fig. 6), despite the fact that the observed K^+-leakage, as well as an increase of cell surface area, should result in an increase of osmotic stability.

For the photochemical phase of sensitized cell damage, we observed TBARS formation, intracellular GSH oxidation and haemolysis. We suggest that lipid peroxidation is not a reason for ''light'' haemolysis, as BHT did not prevent ''light'' haemolysis (Fig. 2). This is in accordance with our earlier observation [22].

Specific blockage of band 3 protein of RBC membranes by DIDS considerably increased the photosensitivity of the cells (increased the rate of photohaemolysis) (Fig. 2). DIDS is a known inhibitor of anion exchange mediated in RBCs by the band 3 protein [24]. It was shown that scavengers of singlet oxygen interacting with band 3 protein could selectively protect the red cells against damage induced by Pc photosensitization [3]. We therefore conclude that band 3 protein plays an important role in RBC photostability. Thermal denaturation of another membrane protein, spectrin, also enhanced the rate of ''light'' haemolysis (Fig. 8). Band 3 protein modification and spectrin denaturation only slightly increased or did not change either intracellular GSH photooxidation or membrane lipid peroxidation (Figs. 2 and 8).

Transformation of intracellular oxyHb into the met-form by nitrite treatment prevented ''light'' haemolysis of RBCs even after prior oxidation of intracellular GSH by nitrite (Fig. 9). Such cell treatment drastically decreased intracellular Hb bound oxygen concentration and this may be a reason for cell photostabilization. The process of ''light'' haemolysis did not depend either on the level of GSH or TBARS formed (Fig. 9). ''Light'' haemolysis did not accelerate when the NaCl medium was replaced by the KCl one (Fig. 4a), as one could expect if photosensitized haemolysis is due to disturbance in passive ion permeability.

Similarly, increased tonicity of the medium by sucrose addition did not inhibit ''light'' haemolysis (Fig. 7). Thus, photochemical ''light'' haemolysis is not a simple colloid-osmotic one.

It was shown earlier that Pc-sensitized photohaemolysis was preceded by a reduction in ATP level and that the glycolysis is important in preventing RBC photodamages [5]. However, addition of exogeneous glucose during irradiation did not protect RBCs from damage in our experiments (data not shown).

Previously, the ''dark'' haemolysis of irradiated RBCs has mainly been studied [2,4–6,19,20]. In our experiment different features of ''dark'' and ''light'' cell ruptures have been observed. Some slow ''dark'' processes in photosensitized cells (''dark'' haemolysis, TBARS formation, GSH recovery) are similar to the processes in γ-irradiated cells [13]. ''Dark'' haemolysis is inhibited by hyperosmotic shrinkage of RBCs in the presence of sucrose (Fig. 7) and accelerated in potassium-containing medium (Fig. 4b). Valinomycin, a K^+-ionophore, hyperpolarizes RBC membrane and inhibits ''dark'' haemolysis in NaCl medium. In KCl medium it depolarizes the membrane and accelerates haemolysis (Fig. 4b). ''Dark'' and ''light'' haemolysis (Fig. 4) are dependent on RBC membrane potential.

''Dark'' haemolysis is not due to the progressive development of lipid peroxidation during postirradiation storage or under irradiation. Alkoxyl-radicals do not participate in photodynamically-induced haemolysis (BHT did not inhibit both types of haemolysis, but prevented lipid peroxidation, Figs. 2 and 3).

Intracellular GSH utilized in the ''light'' processes, perhaps in reduction of the products of membrane lipid peroxidation, partly recovered it's concentration during ''dark'' storage (Fig. 3). It was shown that singlet-oxygen mediated photooxidation of membrane cholesterol resulted in generation of three different hydroperoxide products after erythrocyte ghost treatment, using chloroaluminum Pc tetrasulphonate as sensitizer. All hydroperoxide species underwent glutathione/glutathione peroxidase-dependent reduction [25].

ZnPc is nonspecifically bound to RBC membranes, as one can conclude from the value of binding constant. The number of ZnPc binding sites $(2.7\pm0.4)\cdot10^7$ per cell is higher than that determined for another dye merocyanine

540, i.e., $9.3 \cdot 10^6$ per cell [26]. Photoexcited ZnPc molecules, which bind to membrane sites, probably in the vicinity of membrane proteins, destroy RBCs.

Ben-Hur and Orenstein have shown that ''dark'' photohaemolysis induced by AlPc displays a curved Arrhenius plot and they suggested that for each temperature range a different reaction (with different activation energy) is rate-limiting for overall process [4]. For ZnPc induced ''dark'' haemolysis we observed similar non-linear temperature dependence (Fig. 10). ''Dark'' haemolysis is a complex multi-step process, with activation energy increasing as temperature increase. The apparent value of activation energy of ''dark'' haemolysis (101 ± 17 kJ/mol) corresponds to that of protein denaturation [27].

According to Deuticke et al., formation of short-lived fluctuating in time and space barrier defects in RBC membrane seems to be responsible for the membrane damage after photodynamic treatment in the presence of Pc [6]. Ben-Hur and Orenstein, on the basis of nonlinear Arrhenius plot of temperature dependence of Pc-photosensitized ''dark'' haemolysis rate, suggested photoinduced opening of the ion channels in the membrane [4].

Deuticke et al. determined a radius of photodynamically produced membrane pores as the Stokes radius of the smallest nonelectrolite providing full protection against lysis [6]. The apparent pore radius was about 0.75 nm and the apparent number of pores after 90 min of irradiation in the presence of Pc was 0.5 pores/cell [6]. We recalculated these parameters using our data on inhibition of ''dark'' haemolysis when irradiated cells were suspended in sucrose-containing-solutions instead of NaCl solutions (Fig. 7) and obtained the higher pore radius and the smaller pore numbers per cell. Like many different haemolytic agents, i.e., γ-radiation [13] and ethanol [14], photosensitization produces in cell membrane the limited number of pores through which molecules of various diameter permeate with different rate.

Thus, red light irradiation of RBCs in the presence of ZnPc induced some ''light'' and ''dark'' processes which, as end result, have the formation of transient dynamic short-living pores. The average number of pores per cell was much less than unity, similarly to the number of pores formed by γ-irradiation [13].

We suggest that the damage of the same membrane element(s) resulted in ''light'' and ''dark'' haemolysis. Haemolytic holes appeared as a result of membrane protein photodenaturation and aggregation. Band 3 protein participates in these processes and plays a significant role in RBC photostability.

The rates of photohaemolysis were potential dependent: a decrease of membrane potential inhibited both types of haemolysis. The results allow for the conclusion that photochemical (''light'') haemolysis is not a colloid-osmotical process, in contrast to the post-irradiation (''dark'') haemolysis. The membrane lipid peroxidation as well as glutathione oxidation does not participate in the processes of photosensitized haemolysis.

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