

## Comparison between the Mechanisms of Acid- and Alkali-Induced Hemolyses of Human Erythrocytes

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**Abstract**—The mechanisms of acid- and alkali-induced hemolysis of human erythrocytes were compared. The results obtained suggested that, at the initial step of alkali hemolysis, alkali is transferred into the cytosol. This induces oxidative stress in the membranes. Ellman's reagent does not affect the alkali hemolysis but completely inhibits the acid hemolysis. The inhibition of the acid hemolysis is accompanied by crosslinking of membrane proteins, probably, because of their acylation. In the presence of SH-reducing agents such as cysteine or dithiothreitol, and, to a lesser extent, albumin, the membrane proteins are not crosslinked, and the barrier functions of the membranes are impaired. It was found that the crosslinking of membrane proteins with Ellman's reagent could not protect them from oxidative damage but could probably prevent their aggregation. From the results obtained, an inference was drawn that the impairment of the barrier properties of the membranes during acid hemolysis is a consequence of the aggregation of membrane proteins that have undergone oxidative damage.

**Key words:** erythrocytes, acid and alkali hemolyses, mechanism

### INTRODUCTION

The main functions of erythrocytes are the oxygen and carbon dioxide transport, and also the binding of acids and alkalis formed during the metabolism in tissues. For this purpose, erythrocytes have a powerful and rapid system containing the anion-exchanger protein, which transfers acid and base equivalents through the erythrocyte membranes [1]. In an acidic medium, extracellular  $\text{Cl}^-$  is exchanged for intracellular  $\text{HCO}_3^-$  or  $\text{OH}^-$ , which results in the formation of  $\text{H}^+$  ions in the cytosol. The cytosolic acidification is weakened by the buffer capacity of hemoglobin and the release of alkali cations. In an alkaline medium, intracellular  $\text{Cl}^-$  or  $\text{HCO}_3^-$  is exchanged for extracellular  $\text{OH}^-$ . This cytosolic alkalization is inhibited by the  $\text{H}^+$  liberation from hemoglobin and the binding of alkali cations. As a consequence, erythrocytes swell in an acidic medium and shrink in an alkaline medium.

**Abbreviations:** DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonate; DTNA, 5,5'-dithiobis(2-nitrobenzoic acid).

Many types of cells, including the intestinal bacterial flora, *inter alia*, pathogenic bacteria, live well in an acidic medium. In such a medium, leukocytes are not lysed, whereas erythrocytes are lysed rapidly. Therefore, the effect of acidic and alkaline media on erythrocytes was studied in many works. However, little has hitherto been known about the sequence of processes that lead to hemoglobin release during acid- or alkali-induced hemolysis [2, 3]. It is considered that the cell membrane is a critical target in the pH-induced hemolysis mechanism. It is assumed that, in media with nonphysiological pH values, the outer membrane surface undergoes changes, e.g., pH-induced conformational changes in membrane proteins. These changes lead to impairment of the barrier functions of the membrane and to hemolysis. It is also assumed that the hemolysis is related to the generation of the transmembrane potential difference with changing the pH of a suspension medium, which causes electrical breakdown of the membranes. It is known that the alkali hemolysis occurs via a typical colloid-osmotic mechanism [4], whereas the mechanism

of acid hemolysis is different [5]. The spectrin network of peripheral proteins precipitates at the isoelectric pH 4.5 as intramembrane particles consisting of spectrin aggregates [6]. The acid hemolysis is inhibited by the membrane protein glycophorin [5] and lipophilic Ellman's reagent [7]. It was shown that treatment of erythrocytes with acridine orange, which brings about clustering of membrane proteins (for the most part, anion channel proteins) increases the acid resistance [8]. These results suggested that a certain step of acid hemolysis involves modified membrane proteins.

Previously, data were obtained on the role of oxidative stress in the prelysis membrane damage during acid hemolysis. It was found that the HCl-induced hemolysis is preceded by the acid transfer into the cytosol [9, 10], which is accompanied by the formation of free radicals damaging the cell membranes [9]. The passage of an equivalent acid amount through the erythrocyte anion exchanger is a limiting step in the acid hemolysis of animal erythrocytes and also in the specific inhibition of the anion transport [9]. The free radical generation is likely to be related to the change in the hemoglobin content, since the cytosolic acidification required for triggering the hemolysis of reconstituted erythrocytes with lower protein content was much weaker than that for intact erythrocytes ( $pH_{\text{cyt}} 3.7$  against  $pH_{\text{cyt}} 5.7$ , respectively), and so was the impairment of the barrier functions of the membranes [9].

In this work, the alkali hemolysis mechanism was studied on the basis of a previously developed [9] experimental approach. For this purpose, various specific reagents were applied: 4,4'-diisothiocyano-2,2'-stilbene disulfonate (DIDS), the enzymes superoxide dismutase and catalase, and Ellman's reagent. DIDS is a membrane-penetrating inhibitor, which covalently binds to the erythrocyte anion exchanger [11]. Ellman's reagent—5,5'-dithiobis(2-nitrobenzoic acid) (DTNA)—is known to be a bifunctional reagent capable of crosslinking the membrane proteins [12]. Superoxide dismutase and catalase decrease the concentration of the superoxide anion and hydrogen peroxide, respectively. Thereby they inhibit oxidative stress and diminish the oxidative cell damage [13].

## EXPERIMENTAL

The following reactants were used: DIDS, DTNA, dithiothreitol,  $\beta$ -mercaptoethanol, dimethyl sulfoxide,

superoxide dismutase, catalase, and bovine serum albumin (all from Sigma, USA); and also L-cysteine (Reanal, Hungary).

Erythrocytes were isolated from the fresh human blood and were washed by triple centrifugation with physiological salt solution (150 mM NaCl). Before using, the erythrocytes were suspended (the hematocrit 0.05) in the same solution supplemented with 5 mM glucose. A 40 mM DTNA solution in dimethyl sulfoxide was added to the suspension medium in a volume ratio corresponding to a sought concentration. In all samples, including the reference sample, the dimethyl sulfoxide concentration was 1 vol %.

The erythrocyte anion exchanger was inhibited by incubating the cells (the hematocrit 0.15) for 15 min at 25°C in the dark in physiological salt solution containing 10 mM phosphate or borate buffer and 10  $\mu$ M DIDS [11]. For attaining different degrees of inhibition, the pH of the buffer was varied over a range of 7.0 to 8.0. Before using, the treated cells were washed thrice with physiological salt solution. The degree of inhibition of the ion transport was determined according to a published procedure [14] by monitoring the changes in the pH of an isotonic sucrose solution containing 20 NaCl after the intact or DIDS-treated cells had been suspended (the hematocrit 0.05) in it.

To 1.8 ml of nonbuffered physiological salt solution during continuous agitation, 20  $\mu$ l of a suspension of intact or DIDS-treated erythrocytes was added. The degree of hemolysis was determined by measuring the optical density at 700 nm with a Specol 20 spectrophotometer (Carl Zeiss, Germany) after mixing of 20  $\mu$ l of diluted HCl or NaOH with the erythrocyte suspension [2] at 25°C. The final pH was measured with a Radelkis pH-meter (Hungary) in a special experiment after completion of the hemolysis.

The osmotic properties and the deformability of the membranes of the cells fixed by DTNA in an acidic medium were compared with the respective properties of the membranes of the intact cells by examining the dependence of the equilibrium cell volume on the osmotic pressure of the suspension medium. The equilibrium erythrocyte volume is inversely proportional to the osmotic pressure of the medium. Twenty microliters of the suspension of the intact or fixed cells was added to 1.8 ml of a NaCl solution of the given concentration. The cell volume was estimated in 3 min after equilibration as shown

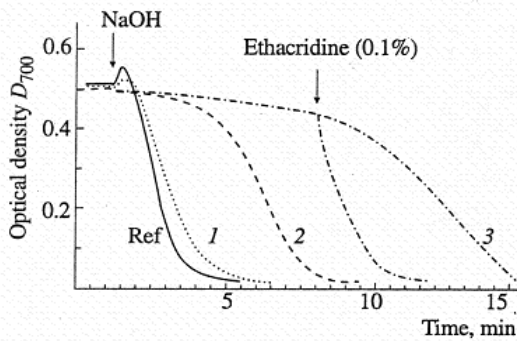


Fig. 1. The kinetic curves of alkali hemolysis of human erythrocytes at different degrees of inhibition of the erythrocyte anion exchanger for (Ref) the intact cells and the cells treated by DIDS at (1) pH 7.0, (2) pH 7.6, and (3) pH 8.0. The degree of hemolysis was determined from the optical density  $D_{700}$  of suspensions. The arrow-head points to the moment of addition of NaOH (the final pH 12) and ethacridine (the final concentration 0.1%).

by the optical density  $D_{700}$  at a wavelength of 700 nm [15]. Under these conditions,  $D_{700}$  is inversely proportional to the cell volume [15].

The fractionation of the erythrocyte membrane was performed as described in the literature [16]. The cells fixed by DTNA in an acidic medium were lysed with a detergent solution containing 4% pentanol and 0.4% Triton X-100. The membranes were sedimented by centrifugation for 10 min at 10,000  $g$  and were washed with 5 mM phosphate buffer (pH 7.9) to remove hemoglobin. The sedimented membranes were placed into a 1:2 methanol-chloroform mixture. In 5 min, distilled water was added to a ratio of 1:1 during intense agitation. The solution was centrifuged for 5 min at 10,000  $g$ , which resulted in its separation into three layers. The upper, water-methanol layer contained glycophorin and other hydrophilic membrane components, whereas the lower layer contained chloroform and lipids. Most of membrane proteins, including spectrin and SH-containing integral proteins were located in the middle layer.

The DTNA concentration in the media was determined from the optical density at a wavelength of 400 nm after alkalization by adding a NaOH solution to pH 12.0.

## RESULTS AND DISCUSSION

As in acid hemolysis [9], the specific inhibition of the erythrocyte anion exchanger strongly affected

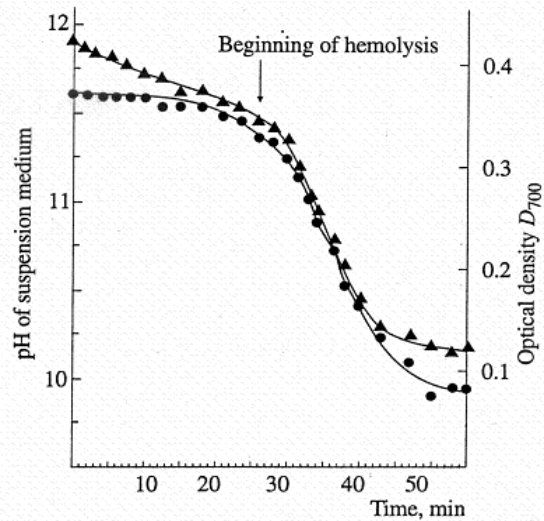


Fig. 2. The synchronously measured curve of change in the pH of the hemolytic medium (triangles) and curve of alkali hemolysis of DIDS-treated erythrocytes (circles). The cells treated with DIDS (10  $\mu$ M, pH 8) were suspended (the hematocrit 0.03) in physiological salt solution. NaOH was added at the initial moment of time. The pH of the suspension medium,  $pH_{med}$ , was measured with a pH-meter and recorded with a recorder. The degree of hemolysis was determined from the change in the optical density  $D_{700}$  of aliquots, which were periodically taken and diluted with physiological salt solution. The arrow-head points to the beginning of the hemolysis.

the run of the alkali hemolysis (Fig. 1). The NaOH-induced hemolysis of both intact and DIDS-treated cells began after a prelysis delay time, but this time for the DIDS-treated cells was much longer. The higher the degree of inhibition of the anion channel (Fig. 1, curves 1, 2, 3), the longer the prelysis step of hemolysis and the half-hemolysis time  $t_{1/2}$ .

In a special experiment, to a suspension of the DIDS-treated cells (the hematocrit 0.03), NaOH was added, and the change in the pH of the suspension medium ( $pH_{med}$ ) was measured with an immersed pH-meter (Fig. 2). The degree of hemolysis was determined from the change in the optical density  $D_{700}$  of aliquots, which were periodically taken and diluted with equal volumes of physiological salt solution containing 20 mM phosphate buffer (pH 7.4). The end of the prelysis step and the beginning of lysis were determined with a microscope by emergence of individual hemolyzed cells. At the prelysis step, the  $pH_{med}$  linearly decreased, which suggested that alkali entered the cytosol and was bound owing to the buffer

capacity of hemoglobin. The molar concentration of hydroxyl anions  $\text{OH}^-$  in the hemolytic medium, i.e.,  $10^{-(14-\text{pH}_{\text{med}})}$ , was found at the beginning ( $c_{\text{in}}$ ) and the end ( $c_{\text{fin}}$ ) of the prelysis step. As Fig. 2 shows, about 60% of the initial alkali amount entered the cell cytosol prior to hemolysis. That is, the impairment of the barrier functions of the cell membranes and the hemoglobin release were preceded by the ingress of a considerable alkali amount into the cell cytosol and its associated deviation of the pH of the cytosol from the physiological value. It is clear that the alkali hemolysis mechanism should take into account not only the changes in the outer cell surface but also the changes in the cytosol after alkalization of the suspension medium.

This inference is consistent with the results shown in Fig. 1. The delay in the beginning of hemolysis, which was caused by the inhibition of the erythrocyte anion exchanger and its associated decrease in the ingress of alkali into the cytosol, was the longer, the stronger was the inhibition of the erythrocyte anion exchanger. Moreover, addition of ethacridine (to a concentration of 0.1%) to a suspension of the DIDS-treated cells at any moment of the prelysis step brought about rapid hemolysis (Fig. 1). In the extracellular medium (at pH  $\sim 11.5$ ), ethacridine molecules ( $\text{p}K_a \sim 10.2$ ) are electrically neutral and, owing to their lipophilicity, readily penetrate the cell membrane. On the other membrane side, ethacridine dissociates and, thereby, alkalizes the cytosol, thus causing hemolysis. In addition, the increase in the optical density at the prelysis step after introduction of alkali (Figs. 1, 3) is explained by the cell shrinkage in response to the replacement of osmotically active intracellular  $\text{Cl}^-$  by extracellular  $\text{OH}^-$  [17]. The DIDS-treated cells experienced no prelysis shrinkage (Fig. 1), which suggested the inhibition of this fast way of penetration of alkali into the cell cytosol.

A deviation of the pH of a hemoglobin solution from its physiological value triggers a number of well-studied processes. The globin conformation changes, and tetramers begin to dissociate. The autooxidation of hemoglobin into methemoglobin to form the superoxide anion radical  $\text{O}_2^-$  is accelerated [13]. At high concentrations, these radicals dismutate to give hydrogen peroxide  $\text{H}_2\text{O}_2$  capable of entering into the Fenton reaction. Hydrogen peroxide itself reacts with  $\text{O}_2^-$  to yield the hydroxyl radical  $\text{OH}^\cdot$  (the Haber-Weiss reaction). If the production of radicals

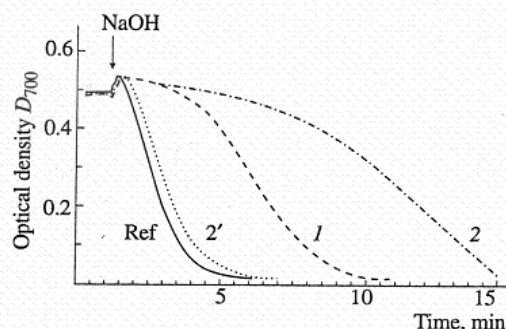


Fig. 3. The kinetic curves of alkali hemolysis of intact erythrocytes in 0.15 M NaCl (Ref) in the absence of antioxidant enzymes and in the presence of (1) 250 and (2) 400 units per ml of superoxide dismutase, and (3) 400 units per ml of thermally inactivated superoxide dismutase. The other notation is as in Fig. 1.

and oxidants exceeds the antioxidant protection capacity of the cells, these radicals and oxidants oxidize SH groups of hemoglobin and methemoglobin, which results in denaturation, detachment of globin from heme, and release of iron ions [18]. In the cytosol, these processes initiate oxidative stress in the membranes, and a part of denatured globin is adsorbed on the inner membrane surface [19], changing the conformations of the membrane components [20].

Figure 3 presents the kinetic curves characterizing the effect of the antioxidant enzyme superoxide dismutase on the NaOH-induced hemolysis of the intact cells. The enzyme at concentrations to 100 units per ml did not affect the run of the hemolysis. As in acid hemolysis [9], above this concentration, there was inhibition of the alkali hemolysis, which strengthened with increasing the enzyme concentration. For example, at a superoxide dismutase concentration of 400 units per ml, the alkali resistance of erythrocytes increased approximately fourfold. A similar result was obtained in the presence of catalase in concentrations 300–1500 units per ml (data are not presented). In the presence of both enzymes at their maximum activity, alkali hemolysis virtually did not take place (data are not presented). Before their addition, the enzymes were dissolved in an aqueous NaCl solution, but this did not change the pH of the medium. Thermal preinactivation of the enzymes (at 100°C for 10 min) virtually completely eliminated their protective role in alkali hemolysis (Fig. 3).

According to the existing concepts, the establishment of the protective role of the antioxidant

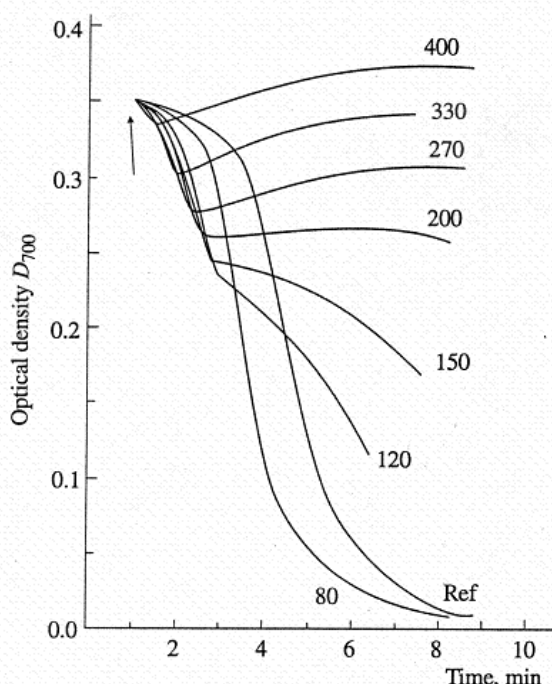


Fig. 4. The kinetic curves of acid hemolysis of intact erythrocytes in the presence of Ellman's reagent (DTNA). The arrowhead points to the moment of addition of the cells to the hemolytic medium containing 150 mM NaCl, HCl (pH 3.2), 1 vol % dimethyl sulfoxide, and DTNA at the concentrations ( $\mu\text{M}$ ) indicated at the curves. The degree of inhibition of hemolysis at a given DTNA concentration  $c$  was calculated as the ratio of the changes in the optical density  $D_{700}$  of the suspension:  $(D_{in} - D_{fin}) / (D_{in} - D_{fin})_0$ .

enzymes superoxide dismutase and catalase in a cell damage is regarded as a direct proof of the participation of free radicals in causing this damage [13]. After alkalization of an erythrocyte suspension, as well as in acid hemolysis, oxidative stress develops, which is likely to play a decisive part in the alkali hemolysis mechanism, as suggested by the inhibition of the hemolysis in the presence of the antioxidant enzymes. Apparently, the formed radicals are capable of disturbing the barrier functions of the membranes and causing hemolysis if their formation rate exceeds the antioxidant protection capacity of the enzymes in the suspension medium (about 400 units per ml of superoxide dismutase and 300–1500 units per ml of catalase).

The most probable source of this oxidative stress is the deviation of the pH of the cytosol from its physiological value. At the same time, the inhibition of the anion channel protein can in no way cancel the

contributions of other factors related to changes in the outer membrane surface, in particular, pH-induced conformational changes in membrane proteins or the generation of the transmembrane potential, which emerge after addition of alkali to a cell suspension. Therefore, under the alkalization conditions, these two lytic factors undoubtedly affect the membranes of the intact and DIDS-treated cells. However, the fact that the prelysis step for the DIDS-treated cells was three times longer shows that the contributions of these factors to the alkali hemolysis mechanism were not very large. Moreover, the hemolysis of the DIDS-treated cells began not at the moment of introduction of alkali, when the transmembrane potential is generated, but in the time when the hydroxyl anion concentration gradient and, hence, the transmembrane potential have already been largely equilibrated. The main function of the anion channel protein is the transfer of acids and alkalis from the extracellular environment into the cytosol. The inhibition of this channel decreases the rate of ingress of acid or alkali into the cytosol; correspondingly, there emerges a delay of the moment when the pH of the cytosol critically deviates from its physiological value, and oxidative stress begins to be generated. On the contrary, addition of ethacridine, which under these conditions acts as a hydroxylophore, increases the rate of ingress of alkali equivalents into the cytosol and initiates hemolysis. With regard for these considerations, on the basis of the results presented, one can state that alkali hemolysis, as well as acid hemolysis, involves the transfer of alkali through the erythrocyte anion exchanger and the generation of oxidative stress in the membranes.

The presence of Ellman's reagent (DTNA) in the hemolytic medium did not influence the NaOH-induced hemolysis but strongly affected the final degree of HCl-induced hemolysis (Fig. 4). In these experiments, the cells were placed into a medium that already contained DTNA and acid. The hemolysis was complete and rapid at DTNA concentrations below 100  $\mu\text{M}$ ; however, at concentrations above 100  $\mu\text{M}$ , DTNA acted as an inhibitor of hemolysis (Figs. 4, 5). At concentrations above 400  $\mu\text{M}$ , Ellman's reagent inhibited completely the hemolysis of the intact cells and, to a lesser degree, the hemolysis of the DIDS-treated cells (Fig. 5). However, of decisive importance for the inhibitory effect of DTNA was the sequence of mixing of DTNA, acid, and the cells. When acid was added in 1 min after mixing of the cells and

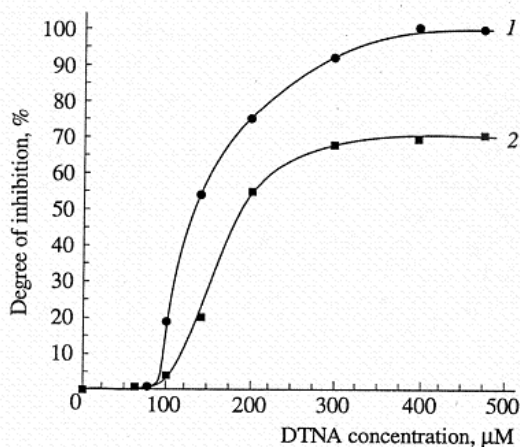


Fig. 5. The degrees of inhibition of acid hemolysis of (1) intact and (2) DIDS-treated erythrocytes as functions of the concentration of Ellman's reagent. The degree of inhibition was calculated as shown in Fig. 4.

a DTNA solution, there was rapid and complete hemoglobin release, which was rapid than the hemolysis in a DTNA-free medium (data are not presented). Even when the cells were introduced into a medium containing DTNA and acid (Fig. 4), a part of cells immediately began to be hemolyzed. This part was the smaller, the higher was the DTNA concentration. The effect of DTNA on the cells was likely to be dual. On the one hand, it sensitized the cells to the hemolytic action of acid; and on the other, it protected the cells from hemolysis, but it did it only in an acidic medium.

After separation of the cells from an acidic (HCl) hemolytic medium containing 0.5 mM DTNA, the content of Ellman's reagent in this medium proved to be about 30% smaller than its initial content. Apparently, a considerable part of the reagent was bound to the cells. The separated cells were washed and studied in physiological salt solution (pH 7.4). Hereafter, these cells are referred to as the cells fixed by DTNA in an acidic medium.

It is known that intact erythrocytes are highly permeable to water and very poorly permeable to inorganic cations, because of which they act as linear osmometers in NaCl solutions in the salt concentration range 100–500 mM [21]. Figure 6 demonstrates that, under such conditions, the cells fixed by Ellman's reagent in an acidic medium also act as linear osmometers. This suggests the conservation of the semipermeability of their membranes and, in particular, the conservation of their barrier properties to

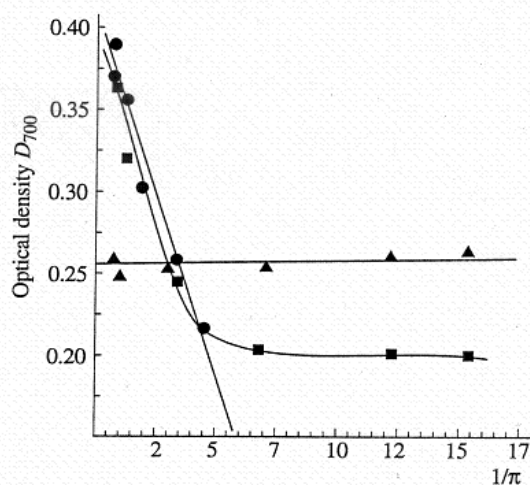
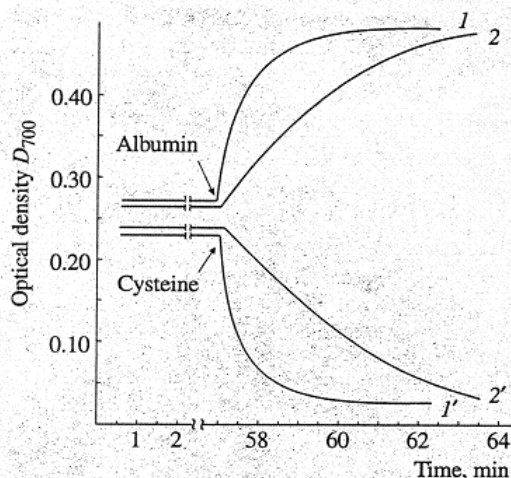


Fig. 6. The effect of the osmotic pressure  $\pi$  of a NaCl-containing medium on the volume of (circles) intact erythrocytes, and erythrocytes fixed by (triangles) glutaraldehyde and (boxes) DTNA in an acidic medium. The fixation of the cells by DTNA was performed in acidified physiologic salt solution (pH 3.2). The fixation of the cells by glutaraldehyde (1 vol %) was carried out at pH 7.4. In 5 min, the cells were separated, washed with physiological salt solution, and added in equal portions to a NaCl solution of a given tonicity. The changes in the volume were determined from the optical density  $D_{700}$  according to a published procedure [13].

inorganic cations. A similar change in the volume did not take place in the cells treated by 1% glutaraldehyde (Fig. 6). This membrane-penetrating bifunctional amino reagent is known to crosslink both cytosolic hemoglobin and membrane proteins, thus preventing the cell volume from changing. In this context, the data of Fig. 6 are also indicative of the fact that, in the cells fixed by Ellman's reagent in an acidic medium, hemoglobin remained mainly in the free state, which allows these cells to change their volume within the same limits as those for the intact cells.

In hypotonic media containing NaCl at concentrations from 100 down to 20 mM, the intact cells swelled to a volume above the critical volume and were lysed, whereas the cells fixed by DTNA in an acidic medium attained the same maximum volume, which remained invariable for several hours (Figs. 6, 7). This result corroborated that the membranes of the fixed cells retained their barrier properties to inorganic cations. This result also suggested that there were changes in the mechanical properties of the membranes of the fixed cells, in particular, in their



**Fig. 7.** The increase in the permeability of the membranes of the cells fixed by DTNA in an acidic medium in the presence of penetrating and nonpenetrating SH reagents. The cells were suspended (*1, 1'*) in 1.8 ml of a hypotonic medium containing 20 mM NaCl or (*2, 2'*) in the same volume of an isotonic medium containing 20 mM NaCl and sucrose. During continuous agitation, at the moment indicated by the arrowhead, the cell suspension was supplemented with 20  $\mu$ l of a solution of a nonpenetrating SH reagent (albumin to a final concentration of 30  $\mu$ g/ml) or a penetrating SH reagent (cysteine, dithiothreitol, or  $\beta$ -mercaptoethanol to a final concentration of 1%). The subsequent changes in the cell volume were determined from the optical density  $D_{700}$  of the suspension. The initial cell concentration was different in different samples.

modulus of isotropic expansion [22]. During hyper-tonic shrinkage, the membranes of these cells were deformed similarly to the membranes of the intact cells; but in hypotonic media, they were incapable of undergoing supercritical isotropic stretching, which in the intact membranes gives rise to pores. Such a change in the mechanical properties of the cell membranes can be due to the selective crosslinking of membrane proteins in the presence of Ellman's reagent at the prelysis step of acid hemolysis.

The effect of SH reagents on the membranes of the cells fixed by DTNA in an acidic medium was investigated under considerable ion concentration gradients across the cell membranes (Fig. 7, curves *1, 1'*). In reference experiments, the isotonicity of the extracellular environment with respect to the cell cytosol was restored by adding sucrose (Fig. 7, curves *2, 2'*). Addition of membrane-penetrating SH reagents (cysteine, dithiothreitol, or  $\beta$ -mercaptoethanol) to a suspension of these cells led to rapid hemolysis (Fig. 7,

curves *1', 2'*). Apparently, the reduction of SH groups of surface and intramembrane proteins of the fixed cells by these membrane-penetrating SH reagents completely lifted the fixation of the membranes and disturbed the barrier properties. Unlike this, introduction of the membrane-nonpenetrating SH reagent albumin [23] into the extracellular environment caused considerable shrinkage of the cells to a new equilibrium volume (by about 20% as follows from the change in the optical density) (Fig. 7, curves *1, 2*). This considerable change in the volume of the fixed cells could not be brought about by the osmotic pressure produced by albumin, since albumin at the final concentration (30  $\mu$ g/ml) induces insignificant osmotic pressure. This was corroborated in experiments with addition of the same albumin amount to a suspension of intact erythrocytes under isotonic conditions, which caused no change in the cell volume (data are not presented). Moreover, the equilibrium volume of the fixed cells was the larger, the lower was the NaCl concentration in the suspension medium (data are not presented); however, this volume was independent on the sucrose content until the isotonicity was attained (Fig. 7, curves *1, 2*). These results suggested that the albumin interaction with the outer surface of the cell membranes initiated release of cytosolic ions and their osmotically bound water according to the ion concentration gradient. This indicated the increase in the membrane permeability to ions with the simultaneous conservation of their fixation and barrier properties to sucrose. Such a specific rise in the ion permeability of the fixed cell membranes in the presence of albumin can be a consequence of the selective reduction of only SH groups exposed from the membrane into the outside.

Virtually complete and rapid hemolysis of the cells fixed by DTNA in an acidic medium also took place in a suspension alkalized to pH 12. The same result can be obtained under the joint action of two detergents, specifically, 4% pentanol and 0.4% Triton X-100, in a neutral medium (data are not presented). Bearing in mind that these detergents cannot destroy the covalent binding in proteins, one can assume that hemoglobin in the DTNA-fixed cells was in the free state and thus could leave the cells in the case of impairment of the barrier functions of the membranes.

The above methods of hemolyzing the cells fixed by DTNA in an acidic medium enabled one to study the DTNA distribution between cytosolic proteins and the cell membranes. The table shows that,

after hemolysis caused by the detergents, Ellman's reagent captured by the cells was located primarily in the membrane fraction. Prior to hemolysis, the DTNA content in the cell membranes seems to be five times as high as that in the cytosol, which is consistent with the inference that the crosslinking of membrane proteins occurs much more frequently than the crosslinking of hemoglobin. The data of the table also suggest that dithiothreitol, a reducer of SH groups, transferred much of membrane-captured DTNA into the hemolyzate. These results corroborated that, in the cells fixed by DTNA in an acidic medium, the reagent bound mainly to the membranes and primarily to membrane proteins containing SH groups. This conclusion is consistent with the result of the fractionation of the membranes with crosslinked membrane proteins according to a published procedure [16]. In the lower and upper layers containing lipids and glycophorin devoid of SH groups, respectively, there was almost no DTNA. At the same time, the middle layer, which contained integral proteins and spectrin having many SH groups, was intensely yellow.

It is known that Ellman's reagent in neutral and alkaline media performs the disulfide exchange with SH groups of proteins and remains in the free state after completion of the reaction [12]. This exchange probably sensitizes the cell membranes to the following hemolytic action of acid. However, the simultaneous treatment of the cells by DTNA and acid produced an additional inhibitory effect; and under certain conditions, hemolysis of all the cells did not occur (Figs. 4, 5). The inhibition of acid hemolysis under these conditions was related to the formation of covalent crosslinks between membrane proteins, in which the reagent itself was likely to participate (table). In the isolated cells, the crosslinking could partially be obviated by using a membrane-nonpenetrating SH reagent (albumin), or completely membrane-penetrating SH reagents, or alkalization of the medium. This crosslinking of membrane proteins can be explained with regard for the fact that DTNA is a dicarboxylic aromatic acid. Because of its very high hydrophobicity, DTNA was accumulated mainly in the cell membranes. In an acidic medium, DTNA was likely to acylate primarily SH groups of membrane proteins under acid catalysis [24], thereby causing the protein crosslinking. The transfer of the fixed membranes into an alkaline medium lifted the crosslinking because of the hydrolysis of the formed thioethers [24].

DTNA binding to cytosolic proteins and membranes of cells fixed by DTNA in an acidic medium

Hemolytic agent	Optical density $D_{700}$	
	Hemolyzate	Cell membranes
Dithiothreitol	0.707	0.327
4 vol % Pentanol and 0.4% Triton X-100	0.200	1.07

Note: The cells were isolated, washed with physiological salt solution, and then hemolyzed in a neutral medium by the said agents. The cell membranes were separated from the hemolyzate and resuspended in physiological salt solution whose volume was equal to the hemolyzate volume. The DTNA concentrations in the hemolyzate and the membrane suspension were determined from the optical density  $D_{400}$  at a wavelength of 400 nm after alkalization to pH 12 and tenfold dilution of the obtained solutions with distilled water (pH 12). The average data of three experiments are presented.

To explain the sigmoidal shape of the curves that characterize the inhibitory effect of DTNA (Fig. 4), let us proceed from the concept that hemolysis of an individual cell can be prevented when all the surface of its membrane is coated with a network of crosslinked proteins. If the concentration of DTNA was below 100  $\mu\text{M}$ , and its distribution among the membranes of suspended cells could be assumed to be approximately uniform, then the DTNA amount was apparently insufficient for complete fixation of the membranes of a noticeable amount of cells. At such concentrations, the reagent exhibited only its sensitizing properties. At DTNA concentrations above 100  $\mu\text{M}$ , a considerable part of the cells and, next, all the cells underwent complete crosslinking of proteins in their membranes and were not lysed. Probably, the pretreatment of the cells with DIDS decreased the efficiency of this fixation and diminished the maximum degree of inhibition of hemolysis (Fig. 4). The DIDS-inhibited anion channel was very likely to undergo changes that prevented it from involving in the network of proteins crosslinked by Ellman's reagent.

In the literature, there is ample evidence that a damage to the native structure of membrane proteins causes their lateral aggregation [25]. It is, however, unknown when the barrier functions of the membranes are disturbed: whether it takes place during denaturation of proteins, or it occurs during their following aggregation. During acid hemolysis, the crosslinking of membrane proteins by DTNA can prevent their aggregation but cannot eliminate their damage

caused by acidification of the suspension medium. The results of this work have shown that, taken alone, the disturbance of the structure of membrane proteins does not produce such barrier defects as are usually associated with hemolysis, if the subsequent aggregation of these proteins is impossible. Thus, one has to assume that the barrier defects responsible for acid- and, probably, alkali-induced hemolysis are largely the consequence of the aggregation proper. Under this assumption, the inhibitory effect of glycophorin on acid hemolysis [5] can be explained by the fact that glycophorin, being a highly stable membrane glycoprotein with a high surface charge, probably hinders the aggregation of the altered membrane proteins. The size of barrier defects in alkali hemolysis is small, and sucrose molecules (0.45 nm in size) cannot pass through them [4], whereas the size of barrier defects in acid hemolysis is about 15 nm [5]. One of the factors affecting the size of defects can be the peripheral spectrin network, which restricts the lateral diffusion of membrane proteins and impedes their aggregation. Since the isoelectric pH of spectrin is in the acidic pH range, the ability of this network to restrict the aggregation of altered proteins and the formation of barrier defects is likely to manifest itself mainly during alkali hemolysis. In addition, note that hemoglobin oxidation in an acidic medium is more intense than that in an alkaline medium, which probably leads to much more severe oxidative stress and more profound changes in membrane proteins during acid hemolysis.

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