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Biochimica et Biophysica Acta 1415 (1999) 349–360

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Low pH-induced hemolysis of erythrocytes is related to the entry of the acid into cytosole and oxidative stress on cellular membranes

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Received 2 September 1998; received in revised form 20 October 1998; accepted 22 October 1998

Abstract

HCl-induced lysis of mammalian erythrocytes, pretreated with DIDS, which is a specific inhibitor of the anion transport in their membranes, was markedly delayed. After acidification of a suspension of DIDS-inhibited cells, hemolysis was initiated by addition of a protonophore (Na-salicylate) at any moment chosen by will. These findings revealed that low-pH hemolysis depended on the rate of the transfer of acid equivalents into cytosole. Erythrocyte acid resistance was studied in a group of mammals and found to be inversely related to the rate of monovalent anion exchange in membranes which supported the above observations. In human erythrocytes, the critical level of cytosole acidification was found to be about pH 5.7 by measuring the acid equivalent absorbed by cells prior to hemolysis. HCl-induced hemolysis was also studied in human erythrocyte ghosts resealed with one-sixth of the initial hemoglobin content of cells. During the prelytic interval the ghosts suspended in isotonic NaCl/sucrose media shrunk, indicating an increase in ion permeability. The increase in prelytic permeability and hemolysis were strongly delayed in ghosts prepared from DIDS-treated cells, suggesting a uniform mechanism of lysing in cells and their ghosts. The prelytic increase in ion permeability was measured by the corresponding rate of ghost shrinkage and was found to be pH-dependent, with a high value below pH 3.4 and a very low one above pH 4.0. Compared to cells, the prelytic barrier impairment in ghosts had more mild character although it required greater concentration of cytosolic H^+. While finally complete, hemolysis of cells was strongly delayed in the presence of catalase (500–1500 U/ml) and superoxide dismutase (200–600 U/ml) in hemolytic media. In conclusion, the acid-induced hemolysis could be associated with an oxidative injury of membranes, mainly triggered by the entry of acid equivalents into the cytosole.

Keywords: Low pH-induced hemolysis; Mammalian erythrocyte; Anion channel inhibition; Oxidative injury; Barrier damage

1. Introduction

The main functions of erythrocytes are the transport of oxygen and carbon dioxide and the disposal of protons formed during tissue metabolism. Relatedly, the anion channels transfer acid and base equivalents across their membranes as an equimolar exchange diffusion of anions, mainly Cl^-, HCO_3^- and OH^- [1]. In acidic media, external Cl^- is exchanged with internal HCO_3^- or OH^- and the erythrocytes swell osmotically. The latter is due to the release of hemoglobin-bound alkali ions in exchange to protons.

The reasons for erythrocyte instability in low pH hemolytic media are still poorly understood [2]. The cell membrane is generally mooted as a critical target in the mechanism of pH-induced hemolysis. It is accepted that the outside aspect of cellular membranes alters in acidic media. These prelytic changes accumulate during the lag period and result in barrier

0005-2736/99/$ – see front matter
PII: S0005-2736(98)00202-8

defects and hemolysis. Ponder [3] has reported the base-induced hemolysis is of colloid-osmotic type. The barrier defects are of small size and, consequently, the lysis can be prevented by osmotic protectant with a proper molecular size. However, the acid-induced hemolysis cannot be inhibited in the same way. According to Arvinte et al. [4], acid media have exercised a biphasic effect on erythrocyte hemolysis, with a fast hemolysis between pH 3.6 and pH 4.7 and a slower one between pH 5.0 and pH 6.0. The cytoskeletal spectrin network precipitates at its isoelectrical pH 4.5, forming membrane particles [5] which are responsible for the shape changes in acidic media [6].

The deoxygenation of hemoglobin and its oxidation to methemoglobin can generate oxygen radicals which are normally eliminated by the intrinsic antioxidant system of erythrocytes. However, this system cannot encounter the increased rate of radical formation in cases such as reduced activity of an important enzyme, poisoning with pro-oxidative drugs, heavy metals, chemicals, etc. The oxyradicals, in turn, could precipitate an oxidative attack on membranes and cause hemolysis [7].

In acidic media, hemoglobin is readily oxidized thus causing oxyradical formation. Such acid-induced changes of hemoglobin can contribute to the mechanism of acid hemolysis, but this problem is as yet unstudied. The present work was aimed at studying the acid-induced hemolysis with respect to the low pH modification of cytosolic proteins, mainly hemoglobin, and related oxidative stress on membranes. The intracellular pH-stat of hemoglobin could be effectively isolated from the acidified extracellular media by slowing the movements of acid/base equivalents across the membranes [8]. Relatedly, the resistance against low pH-induced hemolysis has been determined in erythrocytes pretreated with DIDS as well as in various mammalian erythrocytes strongly differing in their rate of anion exchange [9]. DIDS is known to bind to the anion channel of erythrocyte membranes in a highly specific and irreversible manner and thus, to produce its inhibition [10]. On the other hand, catalase and superoxide dismutase, which dispose H_2O_2 and superoxide radicals, respectively, have been employed in hemolytic media to reduce possible oxidative injury to membranes. In addition, the hemolytic barrier damage in cells has been compared to that in their ghosts resealed with reduced content of hemoglobin.

2. Materials and methods

DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid), catalase (EC 1.11.1.6 from bovine liver), superoxide dismutase (EC 1.15.1.1 from bovine erythrocytes) and horseradish peroxidase were purchased from Sigma Chemical Co., St. Louis, MO, USA.

The erythrocytes were separated from fresh citrated blood of indicated species and washed three times in excess NaCl-saline before usage. Human cells were employed, when not otherwise indicated. One-step resealed ghosts were obtained from human erythrocytes according to Bodemann and Passow [11]. Briefly, packed cells were injected in hypotonic media containing 0.2 mM EGTA, 5 mM Tris–HCl buffer (pH 7.4) and 1 mM $MgCl_2$. The ratio of the volume of packed cells to the volume of hypotonic media was varied in order to obtain ghosts with different retention of hemoglobin. After 5 min incubation at 3°C, the isotonicity was restored to the final concentration of (a) 150 mM NaCl or (b) 75 mM NaCl and 5.5% (w/v) sucrose. The membranes were resealed at 37°C for 20 min and isolated by centrifugation. Prior to usage, the resealing was verified by two independent methods sensitive to the permeability barrier of membranes. The first one consisted of obtaining the Boyle van't Hof linear relation of ghost volume with inverse of osmotic pressure [12]. The other one represented recording of the derivative conductivity thermogram of a ghost suspension. With resealed ghosts, two isothermal peaks are recorded at 53°C and 66°C which are related to the heat denaturation of spectrin and to the heat-induced poration of membranes, respectively [13]. The addition of saponin (0.1 mg/ml final concentration) to a suspension of cells or ghosts was used to evoke hemolysis, detected photometrically at 700 nm.

The low pH-induced hemolysis of cells and ghosts was induced by adding 20 μl HCl-load to a continuously stirred 1.8-ml suspension. The final pH was determined by a pH-meter at similar conditions. The hemolysis was followed by recording the changes in optical density at 700 nm (OD_{700}) on a chart [2]. At

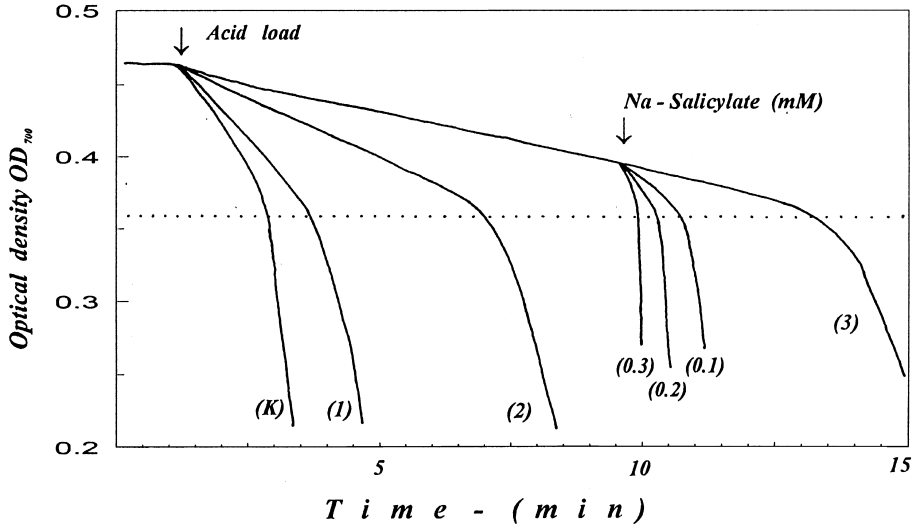


Fig. 1. Time course of HCl-induced hemolysis of intact (K) and DIDS-pretreated (1, 2 and 3) erythrocytes of calf. The pretreatment of cells with 10 μM DIDS was carried out in a media buffered at pH 7.0 (1), pH 7.6 (2) and pH 8.3 (3). Prior to usage, cells were washed in NaCl-saline and suspended in 1.8 ml NaCl-saline to obtain concentration as indicated by optical density (OD). Arrows indicate the addition of 20 μl acid (final pH 3.2) and Na–salicylate (final concentration as indicated in mM) to suspension. The number of cells remaining intact was followed by recording of optical density (OD_{700}) of continuously stirred suspension at 700 nm. Hemolysis commenced when optical density of suspensions fell beyond the dotted line.

this wavelength, the light absorption of hemoglobin is nil and the measured OD can be attributed mainly to the light scattering of cells still remaining intact. However, the swelling of cells decreases and shrinkage of cells increases the light scattering and, consequently, optical density of suspension [14]. Acid resistance of cells is defined as the time needed to lyse 50% of cells by the corresponding change in pH of the medium.

The inhibition of the anion transport in erythrocyte membranes was produced by the application of DIDS as a highly specific, covalent inhibitor of the anion channel [10]. The cells were incubated at 23°C in NaCl-saline, containing 10 mM buffer (phosphate or borate) and 10 μM DIDS, hematocrit 0.03, in the dark, for 15 min. In order to obtain different degrees of inhibition, the pH of the incubation media was changed from pH 7.0 to pH 8.3. Prior to usage, the treated cells were washed three times in excess NaCl-saline and the inhibition verified. It is known that the addition of erythrocytes to an isotonic 20 mM NaCl/sucrose medium results in rapid acidification of this medium due to the exchange of inner Cl^- with outer OH^- through the anion channel of erythrocyte membranes [15]. The inhibition of the anion channel was assessed by suspending DIDS-treated cells in this medium and recording the resulting acidification on chart. The acidification was slower and reduced in amplitude as compared to that produced with intact cells.

3. Results

The onset of acid-induced hemolysis was markedly detained in calf erythrocytes following the covalent modification of their anion channel by DIDS (Fig. 1). In addition, the inhibition of anion channel activity, that was produced during the preincubation of cells with DIDS, provoked an extension of the lag period of hemolysis. Both the extension of lag period and inhibition of anion transport depended on pH of the media, fixed during the pretreatment of cells with DIDS. In cells pretreated with DIDS at pH 7.0, the inhibition of the anion channel was low and the lag period was close to that in intact cells. When the pH of preincubation media increased, the inhibition of anion transport also increased (not shown) as well as the duration of the lag period (Fig. 1, curves 1–3). A similar effect of DIDS pretreatment on the lag period was also found in other mammalian erythrocytes. With respect to the lag period in intact cells, the

maximal retention of hemolysis by DIDS pretreatment was about 3.5-times longer in human and 4.5-times longer in rat and calf erythrocytes.

Another possibility to facilitate the transfer of acid loads through membranes of DIDS-treated cells was employed. Na-salicylate, dissolved in the hemolytic media, could permeate the lipid matrix of membranes in non-dissociated form thus introducing H^+ into the cytosole [16]. A rapid concentration-dependent hemolysis was caused by adding Na-salicylate to the acidified suspension at any moment during the prelytic period (Fig. 1). In contrast, the addition of Na-salicylate did not produce any hemolysis in neutral media (not shown). These results indicated that the access of cytosole to the introduced acid was important for the acid sensitivity of cells.

In intact erythrocytes of various mammals, the rate of anion exchange through the anion channel of their membranes has been shown to display strong species differences [9]. Considering the buffering capacity of hemoglobin differs to a smaller extent than

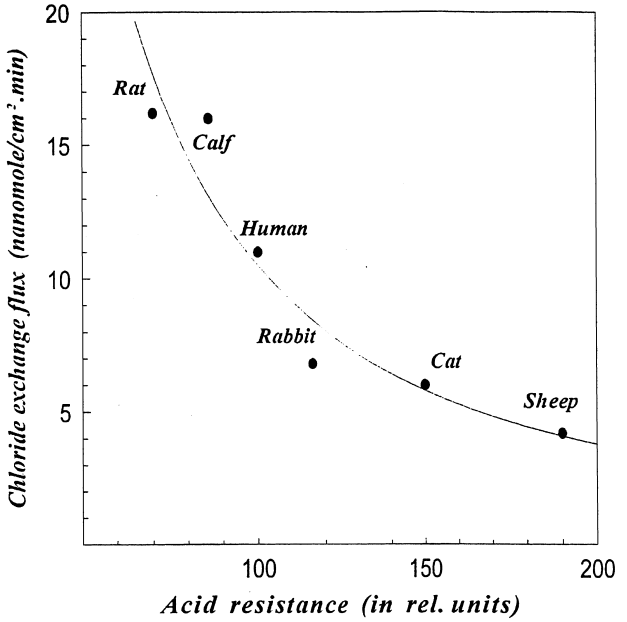


Fig. 2. Correlation between the acid resistance and exchange rate of monovalent anions in erythrocytes of different mammals. The acid resistance was measured as the time necessary to lyse 50% of cells at conditions described for Fig. 1 and was presented in relation to that of human erythrocytes. Data for the exchange rate of anions in erythrocytes of different species were adopted from Deuticke [9]. Mean values are shown for different number of measurements in sheep (4), cat (2), rabbit (2), human (5), calf (2) and rat (2).

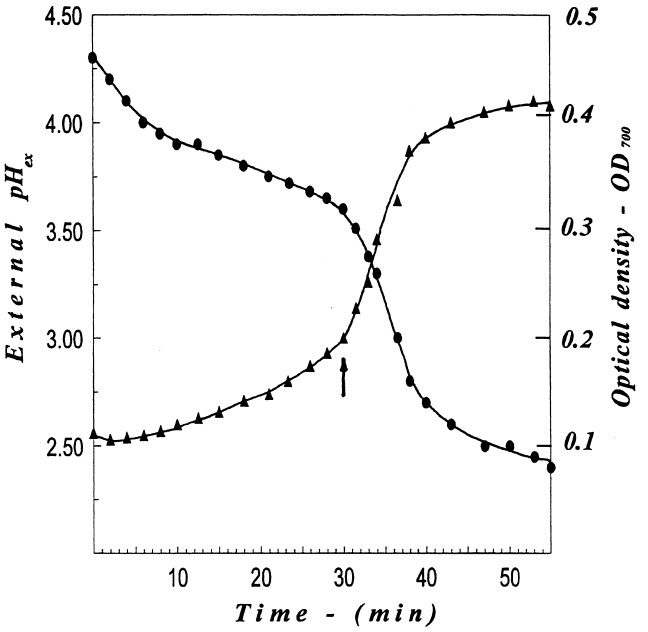
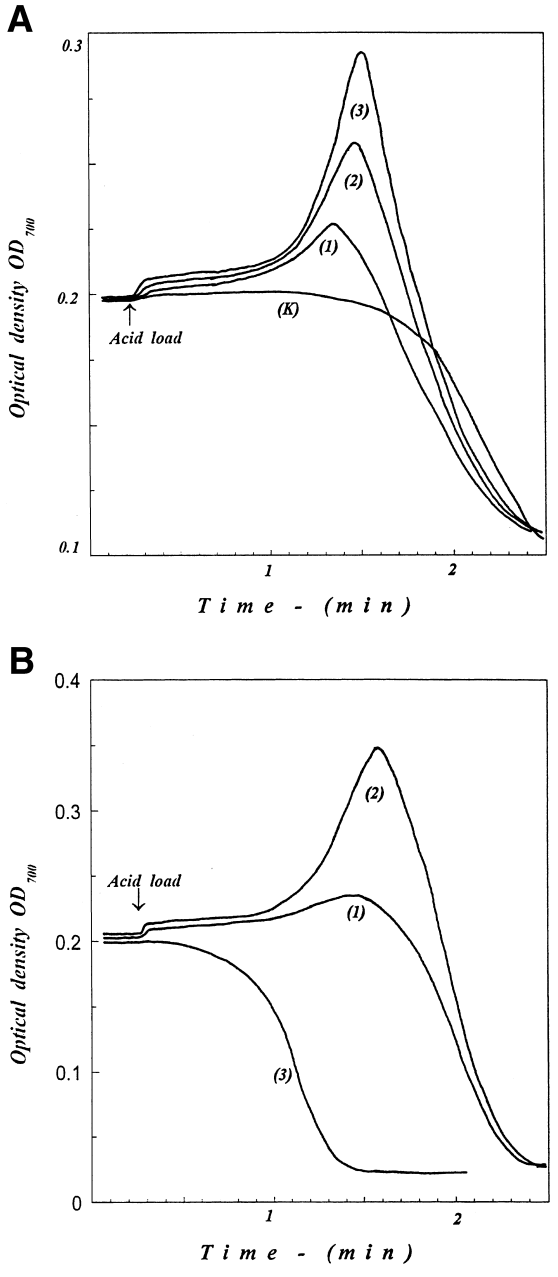


Fig. 3. Parallel data for hemolysis of DIDS-treated human erythrocytes (●) and changes in pH of hemolytic media (▲). The cells, pretreated with 10 μM DIDS at pH 8.3, were suspended in NaCl-saline at 3% (v/v) hematocrit. At time zero, a HCl load was added. The media pH (pH_{ex}) was measured and recorded on chart by a Radelkis pH-meter (Hungary), and hemolysis was determined photometrically at 700 nm after taking aliquots and diluting in NaCl-saline. The arrow indicates the start of hemolysis.

the anion exchange rate in different mammalian erythrocytes, one should expect a slow hemolysis in the case of a slow exchange rate. Indeed, Fig. 2 shows a marked correlation between the acid resistance and the exchange rate of monovalent anions in the erythrocytes from different mammals. Data for exchange rates were adopted from Deuticke [9]. In cells with greater exchange rates (rat, calf) the acid resistance was low, while cells with slow exchange of anions (cat, sheep) demonstrated strong resistance to acid impairment. This correlation strongly supported the assumption that acidification of cytosole was the main factor affecting hemolysis in human as well as in other mammalian red blood cells.

Increased structural stability of proteins is often accompanied by a parallel increase in their resistance to various denaturating agents. Relatedly, the covalent binding of DIDS to the anion channel has been shown to produce a strong structural stabilization of this protein [17]. The latter outcome could, in turn,

contribute to the structural stability of membranes and cells in acidic media when the affected protein constitutes a significant portion of membrane protein content. However, the weight ratio of anion channel/membrane proteins has been reported to be about 0.296 in human, 0.253 in cattle and only about 0.039 in rat erythrocytes [18]. Thus, the effect of DIDS on hemolysis did not correlate with the weight portion of the anion channel in mammalian erythrocytes, especially in rat erythrocytes, where this protein was a minor one. This indicated that the increased duration of lag period and increased acid resistance in DIDS-treated cells were possibly related to the reduced rate of anion exchange and reduced access of cytosole to the outside acid, respectively. The following experiments were carried out with human erythrocytes only.



Our results indicated that a substantial part of the lag period was occupied by a transfer of acid equivalents through cell membranes. The level of cytosolic pH (pH_{in}) associated with the onset of hemolysis in DIDS-treated cells can be determined by parallel recording of courses of both the hemolysis and pH of suspension media (pH_{ex}) after acidification (Fig. 3). The pH_{ex} was monitored by a submerged glass electrode, and the hemolysis was measured by changes in optical density of aliquots that were periodically taken from the suspension and properly diluted. During the prelytic interval, pH_{ex} linearly increased which indicated a constant uptake of H^+ by cells. The flow of H^+ across cell membranes could be calculated as 170 mM H^+/(hour per litter cells) at a transmembrane gradient of about 4.0 pH units. During the same time, optical density declined with no hemolysis under microscope. This indicated a prelytic swelling of cells that was apparently related to hemoglobin, whereas the latter buffered the acid equivalents entering into the cytosole as well as released osmotically active alkali cations [19,20].

As shown in Figs. 1 and 3, a similar time course in the decrease of optical density was commonly observed in different conditions of the acid-induced hemolysis. The respective curves of optical density

←

Fig. 4. HCl-induced hemolysis of ghosts at different transmembrane gradient of the concentration of ion. The ghosts were prepared from intact human erythrocytes and contained one-sixth of the initial hemoglobin content. (A) Ghosts resealed with 150 mM NaCl were suspended in isotonic media containing: (K) 150 mM NaCl; (1) 130 mM NaCl and 40 mM sucrose; (2) 110 mM NaCl and 80 mM sucrose; (3) 90 mM NaCl and 120 mM sucrose. (B) Ghosts resealed with 90 mM NaCl and 120 mM sucrose were suspended in isotonic media containing: (1) 90 mM NaCl and 120 mM sucrose (control); (2) 50 mM NaCl and 200 mM sucrose; (3) 130 mM NaCl and 40 mM sucrose. The arrow indicates the moment of acid load injection. The ascendant and consequent descendent portions of curves represent shrinkage and hemolysis of ghosts, respectively. Other details are the same as for Fig. 1.

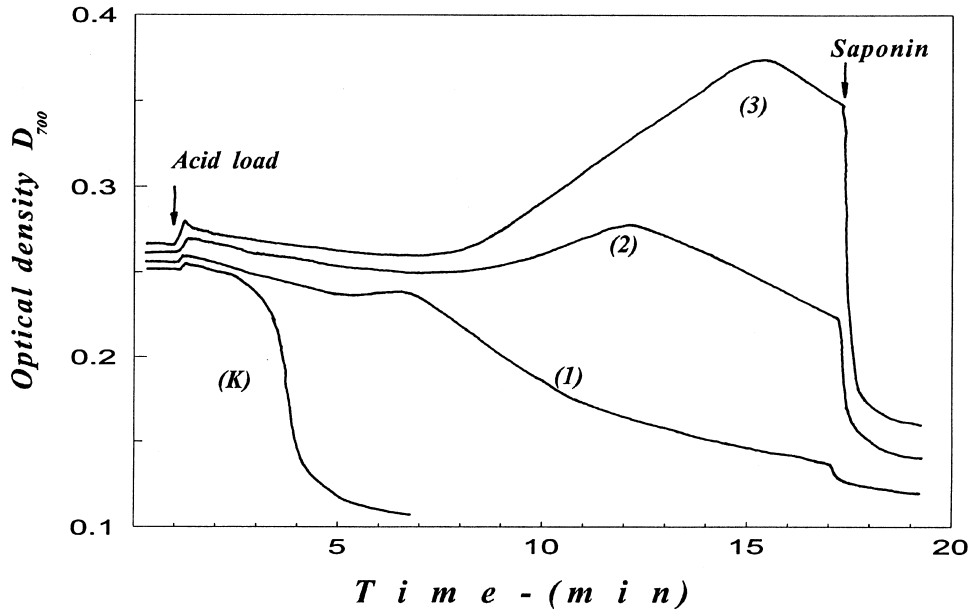


Fig. 5. HCl-induced hemolysis of DIDS-inhibited ghosts at different transmembrane gradients of ions. The ghosts contained NaCl-saline and one-sixth of the initial hemoglobin of cells, and were prepared from intact human erythrocytes (K) or erythrocytes pretreated with 10 μM DIDS at pH 8.3 (1, 2 and 3). The ghosts were suspended in NaCl-saline (K), and (1) NaCl-containing isotonic media with 60 (2) and 120 (3) mM sucrose. Each curve consists of an initial lag portion and a consequent descendent portion whereas the latter corresponds to hemolysis. In ghosts with outward gradient of the concentration of ion (curves 2 and 3), a prelytic increase in optical density, which indicates the osmotic shrinkage and the leakage of ions, was apparent. The arrow at the right part of the panel indicates the addition of saponin (final concentration 100 μg/ml) which induced instant and complete hemolysis of ghosts that remained intact till this moment. Other details are the same as for Fig. 1.

changes consisted of two portions: the initial one corresponding to the prelytic swelling of cells and the second one associated with the egress of hemoglobin (the steeper one). Thus, the beginning of the second portion indicated the start of hemolysis. According to the data, the hemolysis commenced after optical density changed to a same degree (Fig. 1, dotted line). This possibly indicated the same prelytic swelling and the same cytosolic acidification in both the intact and DIDS-inhibited cells. The result supported the conclusion that a certain amount of acid was necessary to be introduced into cytosole in order to initiate the hemolysis of cells.

According to data in Fig. 3, about 61% of the acid load was taken up and buffered by cells prior to the onset of hemolysis. This value was obtained determining the molar hydrogen ion concentrations in hemolytic media, i.e., 10^{-pHex}, at the start (C_{start}) and end (C_{end}) of the prelytic time interval (Fig. 3). The prelytic amount of acid buffered by cells was the product of suspension media volume and the concentration difference ($C_{start}-C_{end}$). On the bases of the buffer capacity of hemoglobin (62 mM H^+/(litter cells·ΔpH) [15], volume of the suspended cells, and the prelytic amount of acid buffered by cells, the cytosolic pH_{in} at the end of the prelytic interval was estimated to be pH 5.7. As evidenced by Figs. 1 and 3, a rapid egress of hemoglobin commenced upon reaching such a level of cytosolic pH_{in} (about pH 5.7) which indicated an instant collapse of permeability barrier. Hemoglobin coming out of cells continued to buffer the outer medium and when the remaining 39% of the acid was buffered, the final pH leveled off at pH 4.15. This observation confirmed the finding that at the onset of hemolysis, the inner pH_{in} was well above the final value of pH 4.15.

The acid-induced hemolysis was further studied in reconstructed erythrocytes (resealed ghosts) obtained from intact (Fig. 4) and DIDS-treated cells (Fig. 5). In general, the hemolysis of ghosts was found to be dependent on the preliminary imposed transmembrane gradients of NaCl. The hemolysis of ghosts resealed with NaCl-saline and suspended in the

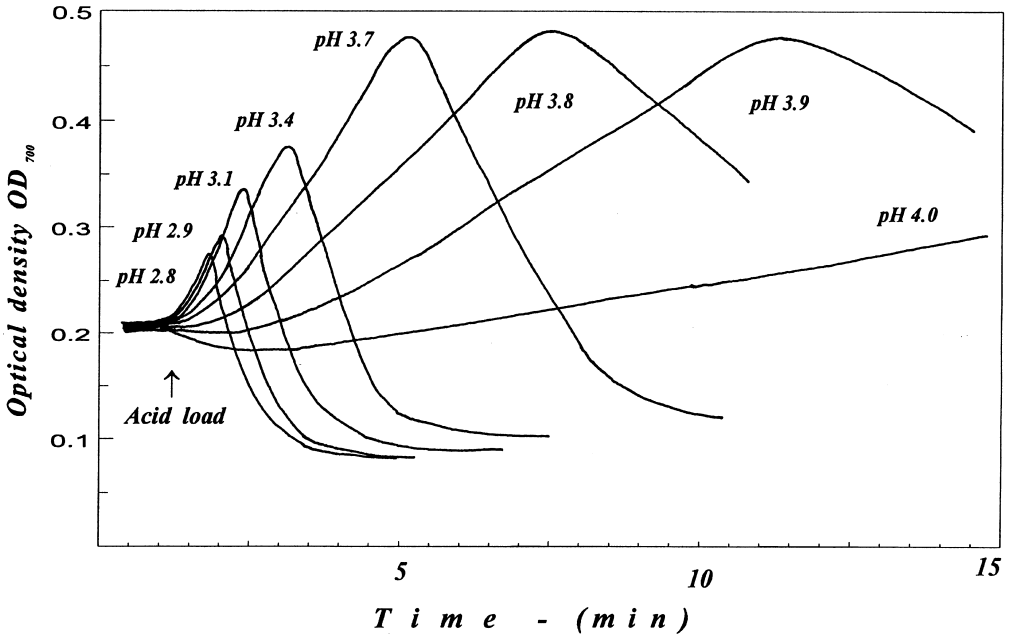


Fig. 6. Prelytic shrinkage and hemolysis of ghosts: dependence on pH of hemolytic media. Ghosts prepared from intact human erythrocytes were resealed with 150 mM NaCl and one-sixth of the initial hemoglobin content. To impose an outward gradient of the concentration of ion, they were suspended in isotonic media containing 90 mM NaCl and 120 mM sucrose. Under continuous stirring, various HCl loads were injected into the ghost suspension and the consequent change in optical density was recorded on the chart. The initial pH of suspension, as shown near the corresponding curve, was measured by a Radelkis pH-meter. Each curve consists of an initial lag portion and a consequent positive peak. The left and right part of the peak represent the shrinkage and hemolysis of ghosts, respectively. The rate of shrinkage and related ion permeability could be described by the slope of the ascendant linear portion of the curves.

same medium commenced after a lag period which was about twice as short in comparison to that in intact cells (Fig. 4A). During the lag period, the changes in optical density were smaller in ghost suspension as compared to that in cell suspension, which possibly was due to the reduced osmotic swelling related to hemoglobin buffering. Optical density remaining after the hemolysis of ghosts was greater since the released membranes were in greater concentration.

When sucrose was included in hemolytic media, an increase in optical density, proportional to sucrose concentration, was recorded during the prelytic time interval (Fig. 4A). According to Yang and Kamino [19] such a change in optical density might indicate a cell shrinkage related to a prelytic leakage of ions. This possibility was confirmed using ghosts resealed with isotonic NaCl/sucrose solution (Fig. 4B). The direction of transmembrane NaCl gradient in these ghosts could be changed depending on the outside NaCl concentration. Thus, when imposing an outward ion gradient, the ghosts shrank during the prelytic time interval (Fig. 4B, curve 2), while when imposing an inward ion gradient, the ghosts lysed, apparently swelling during the same time interval (Fig. 4B, curve 3).

Similar prelytic barrier disturbance was also detected in ghosts prepared from DIDS-pretreated cells (Fig. 5, curves 2 and 3). As shown in Fig. 5 (curves K and 1), in comparison to the intact cell ghosts, the DIDS-treated ghosts exhibited about 3-times slower time courses both for prelytic leakage of ions and for the hemoglobin egress. The retention of hemolysis of these ghosts was verified by a saponin hemolytic test (Fig. 5). These outcomes may be due to inhibition of anion movements through ghost membranes which slowed the equilibration of acid. These results confirmed also that the mechanism of acid-induced hemolysis, in both cells and their ghosts, contained a common stage. During this stage, the acid entered the cytosole and stipulated barrier impairment and hemolysis. The presence of sucrose in outside media

additionally retained the prelytic barrier impairment and did not allow the hemolysis to be completed (Fig. 5).

These results strongly suggest that the acid-induced hemolysis of ghosts was preceded by a formation of small barrier defects. The latter were initially permeable only for small ions inasmuch as the volume changes were found related to NaCl gradient and independent on the opposite sucrose gradient. Later on, larger barrier defects apparently appeared, allowing the equilibration of the sucrose gradient as well. Such a prelytic increase in the permeability for ions was not registered in intact cells (not shown). This indicated that the structural disturbance of membranes was more mild and slower developing in ghosts than that in intact cells.

The prelytic barrier impairment in ghosts was further studied employing different loads of HCl to induce hemolysis (Fig. 6). The ghosts were suspended with a fixed outward transmembrane gradient of the concentration of ion. The pH values of hemolytic media were measured by a glass electrode and did not differ substantially before and after hemolysis, probably due to the low hemoglobin content of ghosts. The injection of different acid loads resulted in different prelytic patterns of shrinkage. Surprisingly, as the acid load decreased, the prelytic shrinkage of ghosts increased to a saturation level. The magnitude of this maximal level of shrinkage depended linearly on the initial amplitude of the transmembrane gradient of the concentration of ion (not shown). These results possibly indicated that as acidity of hemolytic media decreased, the induced prelytic barrier defects became more selectively permeable for ions. This allowed a full equilibration of ions prior to hemolysis. Apparently, in such conditions (less acidic media and reduced content of hemoglobin) the enlargement of the barrier defects occurred at a lower rate.

The ascendant portion of curves in Fig. 6 presented the shrinkage of ghosts to a full equilibration of ions while the descendent portion of curves corresponded to hemolysis. The slope of the linear ascendant portion of these curves was assumed to be an indicator of the shrinkage rate and prelytic ion permeability of ghosts [21]. It may be concluded from this that prelytic ion permeability strongly depended on the initial pH of suspension media (Fig. 7). The time course of inner pH_{in} of ghosts was not measured after acidification and the conclusions were drawn only on the steady-state data of external pH_{ex}. According to Fig. 6, the prelytic increase in ghost permeability appeared to be strongly dependent on pH_{ex} with a high value below pH_{ex} 3.4 and a low one above pH_{ex} 4.0. Since ion permeability changed about 30-fold in a narrow range of pH (around pH_{ex} 3.7), permeabilization appeared as an all or nothing process taking place at around this pH.

Apparently, very different values of the cytosolic pH_{in} were needed to induce a lytic membrane alteration in intact cells and their ghosts. The hemolysis of cells commenced at pH_{in} 5.7 while the ghosts hemolysed at pH 3.7. and did not lysed at pH above 4.5. This indicated that membrane alteration was not a pH-induced structural transition at a certain level of pH_{in}. In addition, ghosts containing a portion of hemoglobin, comprising between 1/6 and 1/15 of the initial hemoglobin content of cells, were exposed to the same hemolytic medium. The latter experiments

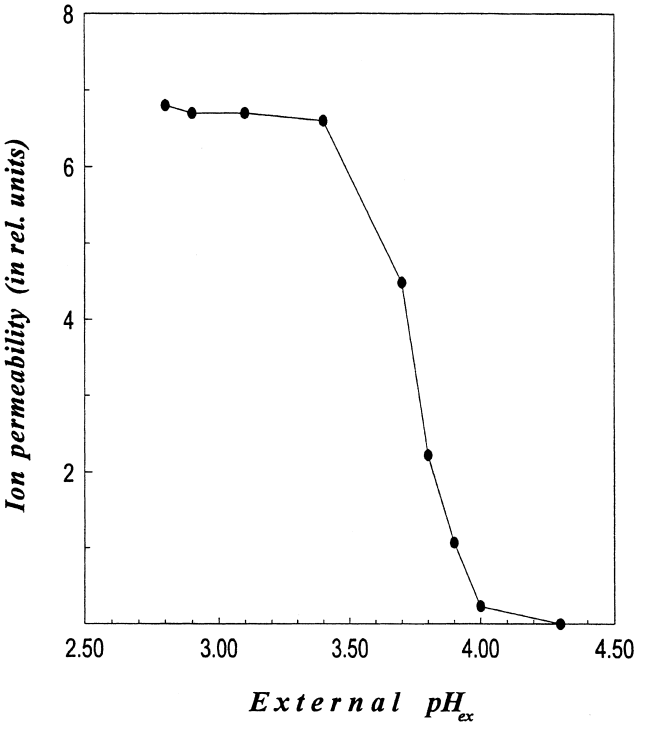


Fig. 7. Prelytic ion permeability of ghosts: dependence on pH of hemolytic media. Ion permeability was determined by the slope of the linear ascendant portion of curves as shown in Fig. 6.

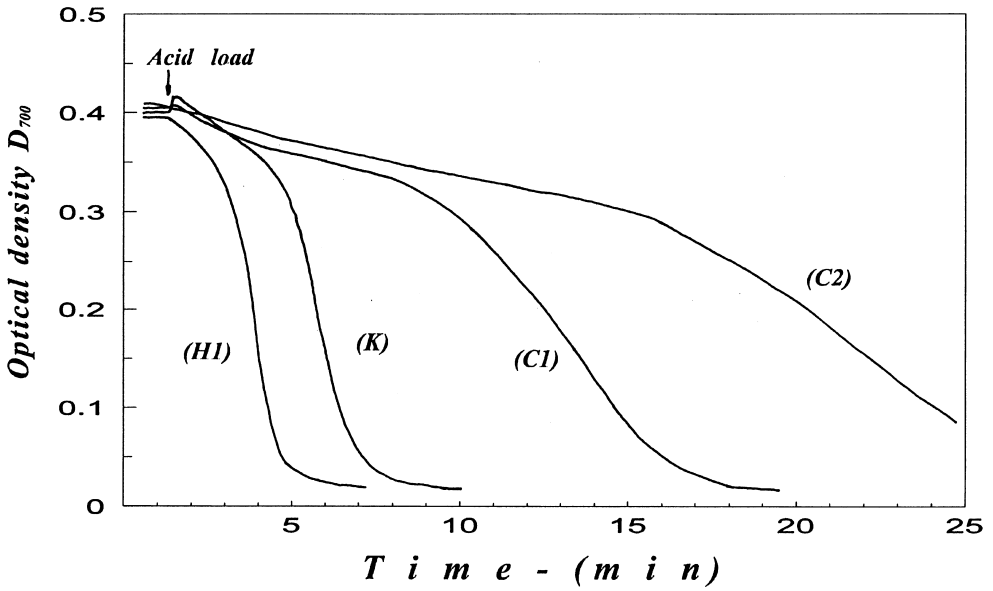


Fig. 8. Impact of pro-oxidative conditions on HCl-induced hemolysis of intact human erythrocytes. Cells were suspended in NaCl-saline that contained 2 mM H_2O_2 (H1) or catalase at different activity, 1000 U/ml (C1) and 1500 U/ml (C2). Each curve consists of two parts with different slopes, and the inflection point between them indicates the start of hemolysis. Other details are the same as for Fig. 1.

demonstrated that as the hemoglobin content of ghosts was diminished, the barrier impairment and hemolysis were progressively delayed (not shown). It should be emphasized that the different content of hemoglobin can be the reason for the different pattern of barrier defect formation, as well as the difference in the values of pH inducing hemolysis in cells and their ghosts.

The contact of cytosolic hemoglobin with acids has a potential to generate oxidative products capable of inflicting oxidative damage on membranes. Possible involvement of this process in acid-induced hemolysis was studied employing specific antioxidant enzymes known to prevent the oxidative stress in biological systems (Fig. 8). Catalase and superoxide dismutase, which reduce the concentration of hydrogen peroxide and superoxide, respectively, were present in the suspension media during the hemolysis of intact cells. Up to a concentration of 500 U/ml, catalase did not impact on hemolysis. Even though the final hemolysis was complete, at greater concentrations this enzyme strongly delayed hemolysis in a dose-dependent manner. Similar effects were obtained using superoxide dismutase which, at concentrations between 100 and 600 U/ml, markedly postponed hemolysis. About 400% increase in the acid resistance of cells was obtained in presence of 1500 U/ml catalase or 600 U/ml superoxide dismutase (Fig. 8). When hydrogen peroxide was added to the external medium, the result was an opposite one: the hemolysis was strongly accelerated at a concentration greater than about 2 mM (Fig. 8). At a concentration of 500 U/ml and more, peroxidase that oxidizes an organic substrate using H_2O_2 increased the acid resistance of cells only with 10% (not shown). Thus, even though peroxidase also reduced the concentration of H_2O_2, it was less successful in preventing hemolysis, probably because the outer aspect of membranes was additionally oxidized. These results demonstrated that the cellular injury at low pH was accompanied by an oxidative stress, the prevention of which inhibited hemolysis.

4. Discussion

According to above results, the acid-induced hemolysis involved a membrane alteration that was apparently related to an oxidative stress generated after the acidification of cytosole. This conclusion was based on the postponement of onset of hemolysis as obtained with the following conditions: (i) the

presence of antioxidant enzymes in outside media; (ii) reduced access of the outside acid to the cytosole of cells and ghosts; and (iii) reduced hemoglobin content of ghosts. This concept was in line with the fact that many other cells (white blood cells, gastrointestinal pathogens, *Helicobacter* species, etc.) and cellular organelles (mitochondria) which have low permeability for inorganic acids and no hemoproteid in such a vast amount, displayed a high resistance to acid-induced lysis in contrast to erythrocytes.

In general, the contact of intact and DIDS-treated erythrocytes to an acidified media could have induced an alteration in the physicochemical properties (surface charge, glycocalix structure, alignment of lipids and proteins) of the outer membrane aspect. The DIDS pretreatment, however, could hardly be able to prevent these alterations, especially in rat erythrocytes where the anion channel was a minor protein. Apparently, the hemolysis in rat erythrocytes was not elicited by such an alteration since the onset of hemolysis was significantly postponed following DIDS pretreatment. In addition, the hemolysis of DIDS-treated cells could be evoked by providing a protonophore at any moment chosen at will. These outcomes substantiated that such an alteration of outer interface region was either too small or insignificant to induce hemolysis during an interval that was 3- to 4-times larger than the lag period of hemolysis of intact cells.

Apparently, the uptake of acid via the anion channel of membranes preceded hemolysis and played the role of a rate-limiting process. This was clearly demonstrated by retention of hemolysis in cells with various extents of anion channel inhibition and in ghosts with inhibited anion transport. This was also revealed in erythrocytes of different mammals where acid resistance strongly correlated with the rate of anion transfer. On the other hand, kinetic studies had shown that the acid hemolysis was limited by a process having activation energy of 69.5 kJ/mol [22]. This value precisely coincided with the activation energy of Cl^-/bicarbonate diffusion through the anion channel (70 kJ/mol [23]) which agreed with the assumption that the transfer of acid equivalents could have been required for hemolysis.

The second stage of acid-induced hemolysis appeared to be a release of activated oxygen species in the cytosole and related oxidative modification of membranes. This was indirectly shown by the strong inhibition of hemolysis at the presence of free radical scavengers with highly specific action. Although applied in outside media, catalase and superoxide dismutase at overthreshold concentration markedly postponed the onset of hemolysis and reduced its rate. This could be due to antioxidant enzymes protecting cellular membranes against oxidative injury by eliminating a part of the oxidative radicals diffusing from cytosole to extracellular media. Apart from the H_2O_2 which readily diffused through the lipid bilayer, the superoxide radical was known to have had enough lifetime (several ms in aqueous media) and could have penetrated across the lipid bilayer or the anion channel of erythrocyte membranes [24]. Also, superoxide anions and H_2O_2, produced within a dialysis bag, were shown to diffuse rapidly through the membrane and could have been detected in outside media [25]. These reports were consistent with the assumption that oxidative species released within cytosole could diffuse to the outside media and be decomposed by the enzymes present outside. In DIDS-inhibited ghosts (Fig. 5) an additional postponement of the prelytic barrier damage and an inhibition of hemolysis were observed when sucrose, a radical scavenger of $OH^{\cdot}$, was present outside.

The oxidative injury of membranes at acidic pH was mainly related to cytosolic hemoglobin. This was demonstrated by comparing the hemolysis of intact with reconstructed erythrocytes. At pH below 4.0, ghosts with a reduced hemoglobin content displayed prelytic barrier damage of mild character inasmuch as the size of the respondent barrier defects was apparently less than the viscometric radius of sucrose (0.45 nm). Although the barrier disturbance in intact cells took place at much lower concentrations of cytosolic H^+ (pH_{in} 5.7) it had a severe character, because the cells lysed instantly and without prelytic volume changes. Arvinte et al. [4] reported 15 nm pores seen by electron microscopy in erythrocyte membranes after the hemolysis of intact erythrocytes at similar conditions. These data indicated that the barrier defects developed more rapidly and reached a larger size in the presence of greater amounts of hemoglobin.

Apart from low pH-induced hemolysis, there are many other factors known to evoke hemolysis by

inducing oxidative stress and alteration of membranes. Addition of the water soluble radical initiator, 2,2″-azobis(2-amidinopropane)dihydrochloride (AAPH) to erythrocyte suspension has induced a direct oxidative alteration of membranes and hemolysis that could be used to quantify the erythrocyte sensitivity to oxidative stress [26]. The tumor promoting agent, t-butylhydroperoxide, interacts with hemoglobin and methemoglobin within cells causing production of oxyradicals and hemolysis [27]. The anti-malaria drug, primaquine, causes an oxidation of hemoglobin within erythrocytes and hemolysis due to related oxidative stress [28]. Some bacterial hemolysins have shown to be capable of rapid hemoxidation (hemoglobin to methemoglobin) of human erythrocytes coupled to erythrocyte hemolysis [29]. Malaria parasites digest hemoglobin within host cells producing β-hematin (malaria pigment) which is a potent initiator of oxidative damage [30]. Killing of engulfed bacteria within phagolysosomes has appeared to depend on the combined action of acidic pH, superoxide ion, or further oxygen derivatives. Clearly, the mechanism of low-pH hemolysis has common features with many other cytolytic events of biological relevance, and might be used in further studies.

Acknowledgements

The author is grateful to the referees for their critical help in the presentation of experimental results obtained with DIDS and the demonstration of the involvement of oxidative stress.

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