Preferential Hemolysis of Postnatal Calf Red Cells Induced by Internal Alkalinization

ROBERT ZEIDLER and HYUN DJU KIM
From the Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona 85724

ABSTRACT Red blood cells from neonatal calves, but not from adult cows, rapidly hemolyze in buffered 300 mM solutions of a variety of nonelectrolytes and amino acids. Of these compounds, sucrose is chosen to elucidate the mechanism by which this preferential hemolysis takes place. As in other mammalian red cells, both calf and cow cells are found to be impermeable to sucrose and, in an isosmolar sucrose solution, to undergo volume shrinkage caused by the net loss of chloride ions with concomitant increase in intracellular pH. To test the potential role of intracellular pH change associated with chloride loss in promoting hemolysis, intracellular pH was altered by: (a) a direct addition of fixed acid or base to sucrose solution; (b) the removal of dissolved CO₂ from sucrose solution; and (c) the addition of cells to isotonic NaHCO₃ solution in the absence of sucrose. In all cases, only calf and not cow cells underwent hemolysis. Moreover, 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulfonic acid, a potent anion transport inhibitor, completely protected calf cells from hemolysis and caused a nearly total inhibition of both chloride loss and intracellular alkalinization. Furthermore, the hemolytic process is closely related to the integrity of a membrane protein, the band 3 protein, which can be cleaved to varying degrees by the combined treatment of pronase and lipase. Hemolysis is progressively inhibited as the band 3 protein undergoes proteolysis, until a total inhibition of hemolysis takes place when almost all of the band 3 protein is digested into smaller protein components with a mol wt of 65,000 and 35,000 daltons. These results suggest that the intracellular alkalinization process leading to a structural instability of the membrane band 3 protein is responsible for this calf cell hemolysis.

INTRODUCTION

The hemolytic process in which the cell membrane becomes permeable to hemoglobin has been extensively investigated during the last few decades. Various agents capable of inducing hemolysis in red cells include hypotonic solutions (Coldman et al., 1970), snake venom (Condrea, 1974), immunological complements (Li, 1975; Lauf, 1976), temperature extremes (Woolgar, 1974), and surface active agents (Gaetgens and Benner, 1975). The common process underlying these types of lysis is the ultimate osmotic swelling of the cell and disruption of the cell membrane. The hemolytic response to some of these agents varies among the different populations of red cells represented by vari-
ious species. This concept extends to newborn animal red cells which are physiologically quite different from red blood cells found in the adult animal. The red cells of newborn cattle (Frei et al., 1963), human infants (Danon et al., 1970; Luzzatto et al., 1970; Schettini and Mautone, 1973), and sheep (Widdas, 1951) are less susceptible to hypotonic osmotic hemolysis than are the cells from adult animals. However, red cells from the newborn animal are not always more resistant to hemolysis. For example, Holstein calf red cells are susceptible to hemolysis in isotonic Tris-HCl or histidine, whereas adult cow red cells are resistant to the lysis under these conditions (Luthra et al., 1975). The hemolysis of calf cells by Tris-HCl is thought to be due to osmotic lysis. Recently, Kim (1976) has examined in detail the mechanism by which this preferential hemolysis of calf cells takes place in solutions of amino acids. In contrast to Tris-HCl-induced hemolysis, the hemolytic mechanism by isosmolar histidine does not appear to be mediated by colloid osmotic swelling. In growing calf, the magnitude of histidine-induced hemolysis is found to be closely related to the fetal cell population, so that when all the fetal cells disappear at 2–2.5 mo after birth hemolysis ceases to occur.

In this communication, we report our further attempts to elucidate the mechanism of hemolysis by isotonic sucrose in calf red cells. Some of the results presented in this study parallel those found for the hemolysis produced by histidine. As in other mammalian red cells, both calf and cow red cells are impermeable to sucrose and undergo volume shrinkage in iso-osmolar sucrose solution due to the net loss of chloride with concomitant alkalinization of the intracellular compartment. Evidence is presented to show that the common mechanism underlying hemolysis of calf red cells exposed to nonelectrolytes or amino acids is brought about by intracellular alkalinization, which ultimately causes a disruption of the cell membrane.

MATERIALS AND METHODS

Preparation of Red Blood Cells
Blood was drawn from either the tail vein or the jugular vein of newborn Holstein calf or adult Holstein cow. The blood was collected into heparinized tubes in ice buckets. The plasma and the buffy white coat were removed usually within 1 h and the red cells were washed several times by alternate centrifugation and resuspension in ice-cold 165 mM NaCl or 110 mM MgCl₂.

Measurement of Hemolysis
Unless otherwise stated, hemolysis of red blood cells was measured at 37°C by suspending 0.3 ml packed cells into 10.0 ml of 300 mM disaccharides or amino acids. The sucrose solution was buffered with 10.0 mM Tris-HEPES or sodium phosphate at pH 7.4. At frequent intervals, a sample of cell suspension was taken, from which the hemoglobin appearing in the supernate as well as in the whole suspension was determined on a Zeiss spectrophotometer (model PMQ2) according to the method of Drabkin (1944).

Ion Measurements
Red cells washed in isotonic MgCl₂ were suspended at a hematocrit of approximately 10% in 300 mM sucrose buffered with Tris-HEPES, pH 7.4, at 37°C. In the case of calf cells, a
rapid succession of samples were taken within 10-15 min of incubation before the
initiation of hemolysis. These were used to measure the rate of appearance of K, Na, and
Cl in the supernatant medium in relation to the total cell content of these ions.

**Measurement of pH**

Immediately after the washed red cells had been suspended in the sucrose medium, the pH
of the whole suspension was measured with an acid-base analyzer (Radiometer Copen-
hagen, Type E 5021a) by use of a capillary glass electrode, type G297/G2. Intracellular
and extracellular pH measurements were accomplished by suspending the packed red
cells in a medium at a 10% hematocrit and centrifuging 0.4 ml of this suspension in a
microcentrifuge (Beckman 3200, Beckman Instruments, Inc., Palo Alto, Calif.) for 30 s.
The extracellular pH was determined from this supernatant medium. Immediately after
measurement of the extracellular pH, the remaining separated medium and packed red
cells were quickly frozen in a bath consisting of dry ice and ethanol. After repeated
freezing and thawing, the frozen packed red cells were cleaved from the frozen medium
by cutting the plastic tube just below the top of the packed red cells. These hemolyzed
cells were thawed and the pH of this hemolysate was quickly determined at a temperature
of 37°C.

**Pronase Treatment**

Washed calf and cow red cells were incubated at 37°C in a medium containing 150 mM
NaCl, 5 mM KCl, 0.1 mM MgCl₂, 0.1 mM CaCl₂, 5 mM sodium phosphate buffer, pH 7.4,
and varying amounts of pronase (grade B, Calbiochem, San Diego, Calif.) for 45 min. At
the end of this incubation, the cells were spun down and washed four times. These cells
were reincubated for an additional 10 min in the above medium containing 0.75 mg/ml
lipase (Steapsin, ICN Pharmaceuticals, Inc., Cleveland, Ohio) without pronase. The
exact concentration of pronase and lipase in the medium was determined by measuring
protein concentrations by means of the method of Lowry et al. (1951). This protein
determination is necessary because pronase does not dissolve completely at high concen-
trations. Cells were exhaustively washed no fewer than five times in isotonic NaCl before
use.

**Polyacrylamide Gel Electrophoresis**

The red cell membranes were prepared according to the procedure of Dodge et al. (1963)
as modified by Burger et al. (1968). The membrane protein patterns were determined by
the Fairbanks method (Fairbanks et al., 1971). The membrane proteins (20-180 µg per
gel) were overlaid on 1% SDS-5.6% acrylamide gels and electrophoresis lasting ~4.5 h
was performed with 3.3 mA per 9.0-cm tube. The protein bands were stained with
Coomassie blue. The molecular weights of these protein bands were determined with the
following standards: β-galactosidase, mol wt 130,000; phosphorylase A, mol wt 94,000;
bovine serum albumin, mol wt 68,000; glyceraldehyde-3-phosphate dehydrogenase, mol
wt 36,000; carbonic anhydrase, mol wt 29,000; cytochrome c, mol wt 11,000. Densitometry
of the gels was accomplished at a wavelength of 500 nm by the use of a spectrophotometer
(model 2000, Gilford Instrument Laboratories, Oberlin, Ohio) which was equipped with a
linear transport accessory.

**Preparation of Bicarbonate-Free Sucrose Solution**

Dissolved CO₂ was removed from the buffered 300 mM sucrose solution and from the red
cells by bubbling for 30 min a gas mixture of 90% N₂ and 10% O₂ through the sucrose
solution and through a 10% hematocrit suspension of red cells in a balanced salt solution.
Control cells and medium were similarly bubbled with compressed air. After bubbling, the cells were collected and centrifuged in an air-tight centrifuge tube for 15 min at 8,000 rpm at 4.0°C. In all subsequent manipulations, exposure of the cells to air was kept to a minimum. The packed red cells were suspended at a 10% hematocrit in a flask which was continually being gassed with 90% N₂ and 10% O₂ through outlets in the rubber stopper. Samples were obtained for pH and hemolysis measurements by withdrawing a portion from the flask with a syringe.

RESULTS

The osmotic fragility response and the critical hemolytic volume of calf and cow red cells are shown in Fig. 1. As found by Frei et al. (1963) calf red cells are more resistant to hypotonic hemolysis than are cow cells. However, both calf and cow cells have the same critical hemolytic volume of 1.25 which is considerably smaller than the critical hemolytic volume of 1.6 seen in human red cells. The results reported elsewhere (Kim, 1976; Luthra et al., 1975) have shown that newborn calf red cells but not adult cow red cells undergo hemolysis in a variety of iso-osmolar solutions including Tris-HCl, amino acid, and a nonelectrolyte. In contrast to Tris-HCl solution in which cell swelling always preceded the onset of hemolysis, cell shrinkage rather than swelling was observed in iso-osmolar amino acid before hemolysis.

In Fig. 2, the hemolysis of calf red cells but not cow red cells occurring in a variety of disaccharides and a trisaccharide is shown. Although it is difficult to assess the true hemolytic effect of each sugar, maltose produced the most rapid...
rate and greatest extent of hemolysis, and lactose caused hemolysis to a much lesser extent. In view of the common use of sucrose, it was chosen as a representative probe to investigate the mechanism of this preferential hemolysis of calf red cells. The result of measuring the calf and cow red cell volume upon exposure to isotonic sucrose is depicted in Fig. 3. The calf cells suspended in sucrose respond similarly to histidine exposure (Kim, 1976) in that these cells appear to shrink slightly before hemolysis. Cow red cells exhibit an initial volume decrease followed by a slow return to their original cell volume. These

![Graph showing hemolysis of calf and cow red cells in various saccharide solutions.](image)

Figure 2. Calf and cow red blood cell hemolysis in 300 mM saccharide solutions. Red cells were incubated at 37°C in 300 mM of the following saccharide solutions buffered with 10 mM sodium phosphate buffer: (○) maltose, pH 7.4; (□) trehalose, pH 7.3; (▲) sucrose, pH 7.4; (△) lactose, pH 7.4; (■) melezitose, pH 7.3; (×) melibiose, pH 7.2; (□) cellobiose, pH 7.2. The pH was measured immediately after the cells had been placed in the saccharide solution.

results thus indicate that the respective cell volumes do not approach the critical hemolytic volume at the time of initiation of the hemolytic process. These results are consistent with [14C]sucrose uptake measurements which indicate that both calf and cow red cells, like human red cells, are impermeable to sucrose. Hence, a mechanism other than osmotic lysis is suggested to account for the hemolysis observed in calf cells.

The hemolytic effect of sucrose is accentuated by an increase in temperature. The results on temperature dependency depicted in Fig. 4 show that a maximum hemolysis of 96% in 0.5 h occurs at a temperature of 49°C, whereas at room temperature, little hemolysis takes place in the first 0.5 h followed by a slow but significant rate of hemolysis. Increasing the temperature augments the rate of hemolysis with Q10 of 3.9 between the temperatures of 45.0°C and 29.5°C. The apparent energy of activation (Ea) determined from the Arrhenius plot is 22,000 cal/mol, as compared to 19,000 cal/mol in histidine hemolysis (Kim, 1976).
Another critical parameter governing the hemolytic process is the variation of pH. First, the pH of sucrose medium can be altered by adding fixed acid or base to a desired value. As shown in Fig. 5, the maximum rate of hemolysis takes place at a pH value above 7.5, while a pH of 6.7 produces the low rate of hemolysis of 18% in 1 h at 38°C. Data in Fig. 5 show that the membrane component responsible for hemolysis can be titrated with an apparent pK of 7.1. Since in red cells intracellular pH closely approximates pH of medium, an increase in medium pH is expected to raise intracellular pH. Second, the change in pH was examined by removing dissolved CO₂ from medium and cells. To obtain CO₂-free medium and cells, both the cell suspension and iso-osmolar

**Figure 3.** Calf and cow cell volume in buffered isosmolar sucrose (V) relative to the original cell volume (V₀) in whole blood. The cell volume in sucrose was determined by hematocrit measurement.

**Figure 4.** A, Temperature dependence of calf red cell hemolysis. Calf cell hemolysis was monitored in 300 mM sucrose buffered with 10 mM sodium phosphate (pH 7.4) maintained at various temperatures. B, Arrhenius plot of the log of the rate of hemolysis determined from the linear portion of Fig. 4 a, plotted against the reciprocal of the temperature (°K).
sucrose medium were gassed with a 90% N₂ and 10% O₂ mixture for 30 min or more at room temperature. The CO₂-free cells were recovered by centrifugation in a capped centrifuge tube to minimize exposure to air and suspended in CO₂-free sucrose solution at 10% hct. The result of measuring intracellular pH and the rate of hemolysis in CO₂-free conditions are given in Fig. 6. In the absence of dissolved CO₂ and external HCO₃⁻, the intracellular alkalization process was distinctly slower than in sucrose medium gassed with air, resulting in a corre-

![Figure 5](image_url)

**Figure 5.** Dependence of calf red cell hemolysis upon the pH of the sucrose medium. Red cells were incubated in 300 mM sucrose with 10 mM Na-phosphate buffer from pH 6.70 to pH 9.60 and hemolysis subsequently followed.

spondingly slower rate of hemolysis. This finding is in line with the observation of Cousin et al. (1975) who found that the Cl⁻ and HCO₃⁻ exchange flux is greater than the Cl⁻ and OH⁻ exchange in cow red cells. These findings suggested that the increase in internal pH is an important factor in promoting hemolysis. Third, to investigate further the role of the intracellular alkalization process in promoting hemolysis, cells were suspended in isotonic NaHCO₃ solution in the absence of sucrose. In Fig. 7 the measurement of intracellular pH and the rate of hemolysis are given. Clearly, the intracellular alkalization occurs rapidly in both calf and cow red cells suspended in isotonic NaHCO₃. Again, only calf cells and not cow cells were hemolyzed in isotonic NaHCO₃ solution. The results shown in Figs. 6 and 7 indicate that the critical intracellular pH in producing hemolysis is pH 7.5, the value of which is near the normal intracellular pH of 7.15 of calf cells in their own plasma. Moreover, the common underlying mechanism by which the calf cells undergo hemolysis irrespective of whether the hemolytic agent is a nonelectrolyte or an amino acid appears to be the intracellular alkalization brought about by Cl⁻ and HCO₃⁻ and/or OH⁻ exchange across the cell membrane.

If the intracellular alkalization process is the mechanism responsible for the induction of hemolysis, it should be possible to abolish hemolysis by interfering
with the Cl− and HCO3− and/or OH− exchange mechanism with an anion transport inhibitor. The inhibitory effect of several well-known transport inhibitors on calf cell hemolysis is depicted in Fig. 8. 4-Acetamido-4′-isothiocyanato-2,2′-stilbene disulfonic acid (SITS), which has been shown to be effective in inhibiting

Figure 6. The effect of dissolved CO2 and HCO3− on intracellular alkalinization and on the rate of hemolysis of cells suspended in buffered isosmolar sucrose. To obtain CO2-free medium and cells, both the cell suspension and isosmolar sucrose medium were gassed with a 90% N2-10% O2 mixture for 30 min at room temperature. The nearly CO2-free cells were recovered by centrifugation and suspended in CO2-free sucrose solution at a hematocrit of 10%.

Figure 7. Intracellular pH measurements and hemolysis of calf and cow cells suspended in a variety of hemolytic media. Hemolysis of calf and cow cells was determined in red cells suspended in buffered 300 mM sucrose, 300 mM histidine, and 150 mM NaHCO3.

anion transport in cow red cells (Cousin and Motais, 1976) as well as human red cells (Knauf and Rothstein, 1971; Rothstein et al., 1976), completely protected the calf cells from hemolysis. The results shown in Figs. 11 and 12 indicate that the inhibitory effect of SITS on hemolysis is attributable to total inhibition of the chloride efflux, with resultant blockage of the intracellular alkalinization proc-
Unlike the SITS effect on human red cells, the inhibitory action of SITS on Cl\(^{-}\) efflux from calf and cow red cells is a reversible reaction, since the inhibitory effect can be abolished by washing the cells with isotonic salt solutions. Phloretin was shown to have no effect on chloride efflux in human cells suspended in sucrose (Gerhardt and Schnell, 1975). Because we found a similar effect of phloretin in calf cells, hemolysis is not expected to be present. This expectation is well founded in results depicted in Fig. 8. On the other hand, para-chloromercuribenzenesulfonate (PCMBs) to a large extent inhibited hemolysis, although not as effectively as SITS. In the presence of PCMBs, calf cells become leaky with respect to cations (Zeidler and Kim, unpublished results). The partial inhibition of hemolysis by PCMBs may be accounted for by the loss of cations which, in turn, is expected to affect the intracellular alkalinization process.

Another way in which the anion transport of red cells can be altered is by utilizing the proteolytic enzyme pronase. It has been well established that treatment of human red cells with pronase results in the cleavage of the membrane protein called band 3 into two smaller components having 35,000 and 65,000 mol wt, and that pronase at high concentration inhibits the anion transport process (Passow, 1971; Cabantchik and Rothstein, 1974). It was therefore of interest to examine the effect of pronase on the calf cell anion transport and hemolysis. To ascertain the effect of pronase, gel electrophoresis was carried out on SDS-solubilized membrane proteins from calf and cow red cells. The result is
summarized in Table 1. The SDS gel electrophoresis profile revealed a remarkable similarity not only between the calf and cow red cells but also between these cells and human red cells. As in human red cells, pronase treatment of calf cells produces hydrolysis of the band 3 proteins into two smaller components with the mol wt of 39,000 and 65,000 daltons. However, unlike SITS-treated cells, the chloride net efflux continues to occur, albeit to a lesser extent, in pronase alone or in pronase- and lipase-treated cells (see Fig. 12). Thus, the pronase treatment is not expected completely to protect cells from hemolysis. As shown in Fig. 9, pronase treatment results in a partial inhibition of hemolysis. Also shown in Fig.

TABLE 1
Calf and Cow Erythrocyte Membrane Protein Patterns

| Band | Calf Mean molecular weight ± SE | Coomassie blue-stained membrane proteins % | Cow Mean molecular weight ± SE | Coomassie blue-stained membrane proteins % |
|------|---------------------------------|------------------------------------------|---------------------------------|------------------------------------------|
| I    | 210,000 ± 16,000               | >20                                      | I                               | 215,000 ± 58,000                        | >18                                      |
| II   | 188,000 ± 15,000               | >20                                      | II                              | 193,000 ± 20,000                        | >18                                      |
| III  | 103,000 ± 6,000                | 18                                       | III                             | 103,000 ± 8,000                         | 18                                       |
| IV   | 79,000 ± 7,000                 | 8                                        | IV                              | 81,000 ± 7,000                          | 8                                        |
| V    | 48,000 ± 3,000                 | >22                                      | V                               | 49,000 ± 3,000                          | >19                                      |
| VI   | 36,000 ± 6,000                 | 7                                        | VI                              | 43,000 ± 6,000                          | 8                                        |
| VII  | 23,000 ± 2,000                 | 7                                        | VII                             | 27,000 ± 1,000                          | 8                                        |

* n = 4.
‡ n = 3.

9 is the effect of lipase which is included in an effort to gain more insight into the role of the membrane lipids in relation to this hemolytic phenomenon. It was found that a crude lipase prepared from the pancreas at concentrations above 1.0 mg/ml in itself causes hemolysis of calf cells in isotonic NaCl solution. However, at subhemolytic concentration the crude lipase delays the intracellular alkalinization process associated with chloride efflux, resulting in a partial inhibition of hemolysis of calf cells (Figs. 9 and 12). It was also found that treating cells first with pronase followed by lipase protected the cells to a greater extent than when cells were pretreated with only one enzyme (Fig. 9). In the presence of a constant lipase concentration and varying concentrations of pronase, it is possible progressively to inhibit hemolysis. The inhibitory effect of these enzymes on hemolysis is found to be linearly related to the extent to which the band 3 membrane proteins undergo hydrolysis. The result is shown in Fig. 10. When more than 90% of the band 3 proteins are cleaved by the treatment of pronase and lipase, hemolysis no longer occurs. These results suggest that the band 3 membrane protein is intimately involved with the hemolytic process. It seems that the intracellular alkalinization somehow causes certain conformational changes of this protein in the intact cells. At the same time, the findings do not allow us to rule out a lipid involvement since the pronase digestion could expose more membrane sites which, in turn, could be attacked by the residual
Figure 9. Determination of intracellular pH and rate of hemolysis upon pre-treatment of calf cells with pronase (3.0 mg/ml), lipase (0.75 mg/ml), or with pronase (3.0 mg/ml) followed by lipase (0.75 mg/ml). Cells were incubated for 30 min with pronase or 10 min with lipase and then washed extensively in ice-cold isotonic NaCl containing 0.1% bovine serum albumin. These cells were then used immediately for determination of intracellular pH and hemolysis. For combined treatment of both enzymes, the cells were first treated with pronase which was removed by exhaustive washing. This was followed by treatment with lipase and the cells were again washed thoroughly before use. Control cells were subjected to identical procedures.

Figure 10. A, Effect of pronase and lipase digestion upon hemolysis. B, Relationship between the band 3 membrane protein content of calf red cells as revealed by gel electrophoresis and the rate of hemolysis. Red cells were incubated at 37°C for 45 min in various concentrations of pronase shown in the left panel, followed by incubation in 0.75 mg/ml lipase for 10 min. Cells were subjected to a procedure identical to that described in Figure 9.
lipase that was not able to be removed from the membranes by washing. The inhibitory effect by the combined action of pronase and lipase cannot be attributed to the inhibition of Cl⁻ efflux with the resultant blockage of intracellular pH change (Figs. 11 and 12). The results shown in Fig. 11 indicate that the critical intracellular pH of 7.5 can be reached by the addition of 6 mM HCO₃⁻ without causing hemolysis. Apparently, once the membranes of calf cells are digested by pronase-lipase, the alkalinization process alone can no longer cause membrane instability culminating in hemolysis.

![Figure 11. Effect of pronase-lipase pretreatment and of SITS upon the intracellular pH of calf red cells. Arrow on control cell curve indicates beginning of hemolysis. Arrow on pronase-lipase curve indicates addition of KHCO₃ to sucrose solution to a final concentration of 6.0 mM. Under these conditions, neither SITS nor pronase-lipase-treated cells were hemolyzed.]

In Fig. 12, chloride efflux in a variety of inhibitory conditions is compared. In cells suspended in buffered sucrose, chloride efflux exceeds what can be accounted for by the exchange of OH⁻ and HCO₃⁻ which is present at approximately 0.1 mM (Jennings, 1976). As in calf and cow cells suspended in amino acid, few cations follow the chloride efflux in sucrose solution, suggesting that a cation other than Na⁺ or K⁺ must be accompanying the chloride ion, in this case presumably a proton.

**DISCUSSION**

The hemolysis of red cells by a variety of hemolytic agents is generally thought to occur as a result of a loss of membrane selectivity to cations, resulting in Donnan-mediated osmotic swelling. Another type of cell lysis is brought about by hemolytic agents which directly inflict injury to the cell membrane architecture. Several of these lytic substances have been shown to combine with or to absorb onto the cell membrane surface. Among these are vitamin A and lysolecithin (Dingle and Lucy, 1962), the basic protein component of snake venom (Condrea et al., 1965) and polyoxyethylene glycol monododecyl ethers (Kondo and Tomizawa, 1968). Other lytic substances appear to combine with more specific moieties in the cell membrane. For example, protamine combines with a lipoprotein
fraction of the cell stroma (Becker, 1960), phosphorylase A2 from snake venom reacts with phospholipids (Martin et al., 1975), and some aromatic molecules react with the lipid portion of the cell membranes (Rogers, 1969). In a similar manner, preferential hemolysis of calf cells in the presence of isotonic amino acid or a nonelectrolyte does not appear to be mediated by the classical colloid osmotic swelling of cells leading to hemolysis. On the other hand, even though cell swelling is never seen in the prehemolytic phase, colloid osmotic swelling as

![Graph showing effects of pronase and lipase and of SITS upon chloride efflux from calf red cells suspended in sucrose. Control (○); pronase 4.5 mg/ml (●); 0.75 mg/ml lipase (▲); pronase (4.5 mg/ml) plus lipase (0.75 μg/ml) (△); and 75 μg/ml SITS (○). Percent equilibration refers to the ratio of chloride content in the supernatant fluid to the chloride content of the whole suspension.](image)

the underlying mode of hemolysis cannot be entirely ruled out, since the volume determination by hematocrit measurement does not permit us to assess the events immediately preceding hemolysis. It is possible that at the very onset of hemolysis, cell swelling may, in fact, occur in much the same manner as in immune hemolysis (Lauf, 1976). Currently, more sophisticated measurements of the cell volume upon exposure to sucrose are underway, utilizing a specially designed optical cell equipped with a visual monitoring system for determination of cell size.

Before discussing the underlying hemolytic mechanism, it is instructive to consider the following events that occur consequentially to sudden exposure of red cells to a nonelectrolyte medium: (a) red cells suspended in isotonic impermeable nonelectrolytes have outward directed ion gradients. Since chloride is more permeable than alkali cations, the membrane potential becomes highly positive inside. Independent of potential, the electrically silent exchange of chloride for external HCO₃⁻ or OH⁻ occurs, driving the inside alkaline at the expense of external acidification. Cotransport of Cl⁻ and H⁺ out of the cell on the titratable
carrier (Gunn, 1972) may contribute to these pH shifts (Jennings, 1976). Indeed, the exchange of intracellular Cl\(^{-}\) with extracellular HCO\(_3\)\(^{-}\) and/or OH\(^{-}\) ions is manifested by an increase in intracellular pH from 6.9 to 7.5. As the intracellular pH rises, the pH of the extracellular buffered sucrose medium concomitantly decreases from pH 7.4 to 6.5. As shown in Fig. 6, Cl\(^{-}\) exchanges much faster with HCO\(_3\)\(^{-}\) than with OH\(^{-}\) ions and, hence, the rate of intracellular alkalinization is more rapid when HCO\(_3\)\(^{-}\) is present in the medium. These relative rates of HCO\(_3\)\(^{-}\) and OH\(^{-}\) ion permeability are similar to results found in cow red cells by Cousin et al. (1975); (b) this intracellular alkalinization process would favor a proton dissociation of hemoglobin which, in turn, would assume a greater role in the conformation of the electrical neutrality of the intracellular compartment. The consequence of a polyvalent anion replacing monovalent anions is to decrease the total number of ions in the cell, thereby causing a reduced internal osmotic pressure and a shrinkage of cell volume; (c) the membrane potential, which is governed by the diffusion gradient of chloride, is now expected to be reversed so that the cell inside becomes positive with respect to the outside. The potential would undergo a considerable change, the magnitude of which would be governed by the transient change in the chloride ratio. As a result, both Na and K ions would diffuse down their electrochemical potential gradients. This is indeed what happens when human red cells are suddenly suspended in a nonelectrolyte medium (Davson, 1939; Donlan and Rothstein, 1969). Unexpectedly, however, both calf and cow red cells lost few cations in either a nonelectrolyte medium or in an amino acid solution (Kim, 1976). A detailed investigation on the permeability of cations and anions will be published elsewhere.

In contrast, cells suspended in isotonic NaHCO\(_3\) are expected to maintain nearly normal membrane potential, the inside negative with respect to outside after Cl\(^{-}\) and HCO\(_3\)\(^{-}\) exchange have both come to steady-state distribution. Yet, the calf cells in isotonic NaHCO\(_3\) rapidly hemolyzed, suggesting that the reverse of the membrane potential per se is not closely related to the hemolytic process. Moreover, under the hemolytic conditions employed in this study, the intracellular alkalinization process is always accompanied by chloride efflux. To determine whether chloride efflux per se is an obligatory step in inducing hemolysis, calf cells were suspended in isotonic NaBr and Na acetate. No evidence of hemolysis was noted, which suggests that the drastic loss of chloride ion in itself is not what causes the calf cell hemolysis.

Evidence presented herein suggests that the intracellular alkalinization process appears to be the common mechanism underlying the preferential hemolysis of calf cells, regardless of the chemical composition of the hemolytic agents, including nonelectrolytes, amino acid, or NaHCO\(_3\). At the moment, exactly how this intracellular alkalinization causes hemolysis of calf by not cow cells is a matter of conjecture. The inhibitory effect of SITS and pronase-lipase digestion points to a possible involvement of the band 3 membrane proteins. The stilbene analogs have been shown to bind mostly the 65,000-mol wt component of the band 3 proteins (Cabantchik and Rothstein, 1974). It is not clear whether the inhibitory effect of SITS is attributable directly to the chemical modification of the band 3 proteins or secondarily to the inhibition of chloride efflux, resulting in the blockage of the intracellular alkalinization process as shown in Figs. 11 and
12. The interpretation of pronase-lipase digestion data is even more complicated. Lipase or pronase treatment alone protects calf cells from hemolysis to a much lesser extent than treatment of cells with pronase followed by lipase. Since the protective effect is closely related to the extent to which the band 3 proteins undergo hydrolysis, as shown in Fig. 10, the band 3 proteins are implicated in the hemolysis mechanism. It is of interest to note that the band 3 membrane proteins are also implicated for transport of anion (Cabantchik and Rothstein, 1974), glucose (Lin and Spudich, 1974), and water (Brown et al., 1975) across human and other mammalian red cells. However, the above findings do not offer unequivocal support for the notion that the band 3 proteins in calf cells are involved in hemolysis. Another interpretation is also possible. For example, involvement of lipid in hemolysis cannot be ruled out on the basis of the available data. The augmented protection in higher pronase concentrations could simply be the result of the exposure of more lipid sites on which the lipase might then act. More work is needed to identify the membrane site involved in hemolysis.

The hemolytic technique has frequently been used in studies on the permeability of rapidly permeating molecules. Furthermore, analyses of osmotic fragility curves have provided useful data about the homogeneity, shape, surface-to-volume ratio (Canham, 1969), and pathology of red cells (Prankerd, 1961). This preferential hemolysis of fetal red cells by sucrose may be utilized to isolate newly formed postnatal red cells in growing newborn calves. The common technique of separating red cells according to their density does not allow the separation of fetal cells from postnatal red cells, since both cells share a similar density profile (Kim et al., unpublished results). With this new preferential hemolysis technique now available, it may be possible to determine more accurately the developmental events occurring in the postnatal calf red cell population with respect to changes in activities of enzymes such as acetylcholinesterase and Ca^{2+}-activated ATPase (Hanahan, 1973) and to the transition of red cells high in potassium to cells low in potassium content (Israel et al., 1972).

The authors are indebted to the competent technical assistance of Mr. Paul Cook and Mr. Bak Young Si. Special thanks are due Mr. Robert Schoenberger of the University of Arizona Research Farm who supplied the blood samples used in this study.

A preliminary report of these data has been presented at the 60th Annual Meeting of the Federation of American Societies of Experimental Biology, 1976, at Anaheim, Calif.

The work is supported by National Institutes of Health grant AM 17723. R. B. Zeidler is supported by a National Institutes of Health grant HL 05884.

Received for publication 7 June 1976.

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