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Transitory Postnatal Hemolysis of Calf Red Cells by Amino Acids*

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Summary. Among the amino acids which can be solubilized to give a concentration of 300 mM at near physiological pH, histidine and proline caused a complete hemolysis of newborn calf but not of adult cow red cells within 20 to 30 minutes at 38 °C. While hydroxyproline, valine, and serine resulted in a partial lysis of calf cells, threonine, glutamine, and glycine were ineffective. In this communication, emphasis has been focused on the mode of the lytic process by histidine, which was found to be affected by several governing parameters including the pH, temperature and the extracellular salts in the solution. Unlike human red cells suspended in isotonic histidine, both calf and cow cells lost little Na and K ions. In the presence of 300 mM histidine, both calf and cow cells displayed an instantaneous uptake of histidine amounting to 20 to 45 μmoles/ml RBC followed by a slow influx rate of 0.25 to 0.5 μmoles/ml RBC × min. The extent to which histidine entry was allowed by the cell was counterbalanced by Cl^- efflux, resulting in little change in cell volume prior to hemolysis. Moreover, histidine-induced hemolysis can be prevented by 1 mM or lower PCMBS without a discernible effect on histidine influx suggesting a possible membrane lesion or damage at the outer surface of the cell.

Hemolysis induced by histidine decreased substantially when a calf reached two months of age at which time the red cells containing the fetal hemoglobin are virtually depleted. The results of hemoglobin electrophoresis obtained during this postnatal period revealed that those cells resistant to histidine hemolysis almost invariably contain the adult type hemoglobin suggesting a selective, specific action of the amino acids on the fetal cells.

The red cells of newborn animals have distinct physiological characteristics which are often different from those of adult animals. In the newborn Holstein calf, red cells contain a high concentration of K relative to Na ions, whereas a reverse trend occurs in adult cow cells (Christinaz & Schatzmann, 1972; Israel, Macdonald, Bernstein & Rosenmann, 1972). The transition from high to low potassium type cells which takes place gradually during the first two months after birth is a reflection of removal of the fetal cells by newly fabricated cells which have fundamentally

* A preliminary report of these data has been presented at the 19th Annual Meeting (1975) of the Biophysical Society, Philadelphia, Pennsylvania.

different membrane transport characteristics (Israel *et al.*, 1972). The activity of certain membrane-bound enzymes, such as acetylcholine esterase and Ca^{++}-activated ATPase, also undergoes a considerable reduction during this neonatal period (Hanahan, 1973).

In addition, these two cell types display characteristically different osmotic fragility responses to hypotonic media; the newborn calf cells are more resistant to hypotonic hemolysis than those of adults (Frei, Perk & Danon, 1963). Recently, Luthra, Ekholm, Kim and Hanahan (1975) reported a finding in which isotonic solutions of either Tris-HCl buffer, pH 7.6, or histidine were found to cause hemolysis of newborn Holstein calf red cells but not of adult cow cells. In the present communication, experiments were designed to gain more insight into the mechanism of the amino acid-induced hemolysis. It was found that while isotonic Tris resulted in cell swelling prior to hemolysis, histidine-induced hemolysis occurred unaccompanied by cell swelling. That calf cell hemolysis by histidine can be entirely prevented in the presence of PCMBS without a discernible effect on histidine uptake suggests that histidine selectively damages the external or outer surface of the red cell membrane. Moreover, in contrast to the adult cow cells, the newborn calf cells became unstable in the absence of the external salt solution so that exposure to a variety of nonelectrolytes also resulted in a rapid hemolysis.

Materials and Methods

Red Cell Preparation

Blood was drawn into a heparinized Vacutainer (Becton-Dickinson Co.) from either the neck or the tail vein of restrained Holstein calves and cows housed at the University of Arizona Research Farm. The blood sample was brought back to the laboratory in an ice bucket usually within 15 min. Red cells were separated from the buffy coats and washed several times with cold 0.165 M NaCl. Since the extracellular salt solution was found to have an inhibitory effect on the histidine-induced hemolysis, cells were finally collected at 7,000 rpm in a Beckman centrifuge (Model L3-50) for 15 min to facilitate the removal of the washing solution. The resultant hematocrit was 96–98%.

Measurement of Hemolysis

A 300 mM histidine solution is relatively difficult to prepare at room temperature with the amino acid not going into the solution completely. However, a brief heating to 60–80 °C usually results in complete solubilization. The isotonic histidine solution was always freshly prepared, since storage at room temperature causes a turbid solution within

two or three days. Red cells were suspended at 3% hematocrit in a prewarmed histidine medium at 38 °C. The suspension pH was determined at 38 °C on a Radiometer pH meter (model PHM71) utilizing a capillary electrode (type G297/G2). To estimate hemolysis, frequent samples were taken from which supernatant solution together with the whole suspension were used for the hemoglobin determination according to the procedure of Drabkin (1944).

Hemoglobin Electrophoresis

To an aliquot of red cells or hemolysate was added an equal amount of water. The resultant hemolysate was frozen and thawed once. To remove membranous particulate matter, an aliquot of the hemolysate was vigorously mixed with toluene (20% v/v) and subsequently centrifuged in a Brinkman centrifuge (Model 3200) to recover the hemoglobin fraction. The purified hemoglobin solution was applied to a cellulose acetate membrane and subjected to electrophoresis in a Beckman Microzone electrophoretic cell under a voltage gradient of 140–180 V utilizing (I=0.075) diethyl barbituric acid-sodium diethyl barbiturate buffer, pH 8.6.

Measurement of Histidine Uptake

In order to remove endogenous histidine from red cells, cells were preincubated in a balanced salt medium consisting of 5 mM KCl, 10 mM Na-phosphate buffer, pH 7.4, and 150 mM NaCl at 38 °C for one hour. Red cells were recovered by centrifugation and washed once with 0.165 M NaCl. Cells were then suspended at 10% hematocrit in 300 mM histidine concentration supplemented with ^{14}C-histidine (0.1 μC/ml). Then, 0.4 ml of the whole suspension was taken at frequent intervals and quickly layered on 0.8 ml of dibutylphthalate. The mixture was centrifuged for 1 to $1^1/_2$ min in a Brinkman centrifuge (Model 3200) which effectively separated the cells from the incubation medium. An aliquot of the clear supernatant solution was saved for radioactivity determination and the remainder was aspirated. The side of the centrifuge tube was repeatedly washed by means of a continually applied water stream coupled with simultaneous evacuation of the water by suction. During this phase of washing the bulk of the phthalate oil is usually removed. The side of the tube and the remainder of the phthalate was wiped dry by a cotton tip. The red cell pellet was extracted as follows: 0.35 ml water was added to the pellet to effect a complete hemolysis and then 0.8 ml of a chloroform-methanol mixture (2:1) plus a drop of concentrated HCl was added and the tube vigorously vortexed. The mixture was centrifuged for 1 min and the upper phase was used for the radioactivity determination. The radioactivity counts in the cell were corrected for a trapped extracellular volume of 3.5% which was determined separately by using an impermeable marker, H^3-methoxy-inulin. The radioactivity determination was made on a Nuclear Chicago liquid scintillation counter, utilizing a counting cocktail composed of PPO (2 g), POPOP (100 mg), toluene (800 ml), ethanol (200 ml) and Triton X-100 (500 ml). Histidine uptake was estimated from the specific activity of the medium and the radioactivity counts in the cell.

Ion Measurement

Chloride was measured on a Buchler-Cotlove chloridometer and Na and K were determined on a Beckman atomic absorption spectrophotometer.

Sources of Materials

All of the amino acids used in this study and the following chemicals were obtained from Sigma: 55′-Dithiobis-(2-nitrobenzoic acid) (DTNB), *ρ*-chloromercuribenzoic acid (PCMB), *ρ*-chloromercuriphenylsulfonic acid (PCMBS), and N-ethyl maleimide (NEM). Phlorizin and ouabain were obtained from ICN K & K Laboratories, Inc. 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS) was obtained from Nutritional Biochemical Corporation. The uniformly labeled ^{14}C-histidine was obtained from New England Nuclear.

Results

The data gathered on the hemolysis of calf and cow red cells in those amino acids which can be solubilized to give concentrations of 300 mM at near physiological pH's are shown in Fig. 1. The calf cells refer to cells derived from newborn Holstein calves no more than 4 to 5 days old and in most cases within 48 hr after birth. Histidine and proline caused a rapid hemolysis of calf cells reaching a maximum in 20 to 30 min. Hydroxyproline, valine, and serine, on the other hand, induced a partial lysis, whereas the other three amino acids caused an insignificant amount of hemolysis (Fig. 1*a*). It is evident that none of these amino acids caused hemolysis of adult cow red cells during this 60-min incubation (Fig. 1*b*). However, if the incubation period is prolonged, a small amount of hemolysis of cow cell does take place in histidine medium.

Of these amino acids, histidine was chosen for further studies on red cell hemolysis because of its pronounced effect and the ease with which the cell suspension pH of 7.4 can be maintained at 38 °C without the aid of other added buffers. It was found that the absolute rate of hemolysis in histidine depends greatly upon a variety of experimental conditions including the change in pH (Fig. 2), temperature (Fig. 3) and the external salt concentration (Fig. 4).

As shown in Fig. 2*a*, no hemolysis occurs at pH values below 6.7 and as the pH is raised, the lytic process is accentuated reaching a maximum at pH 8.0. For the purpose of comparison, the percent hemolysis of calf and cow cells at 30 min are replotted as a function of pH (Fig. 2*b*). In sharp contrast to the calf cells, cow cells exhibited no sign of lysis within a pH range of 6.7 to 8.0.

Again for the comparative purpose, the arbitrary hemolytic values at 30 min in the two cell types are plotted as a function of temperature in Fig. 3. At room temperature, a small amount of hemolysis of the calf cells occurred but not of the cow cells (Fig. 3*a*). In fact, cow cells

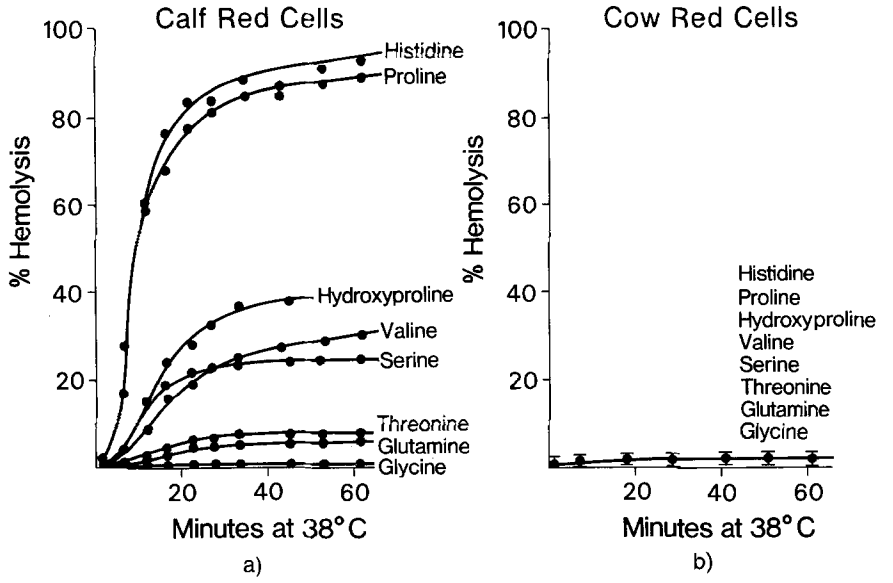

Fig. 1. Hemolysis of newborn calf (*a*) and adult cow (*b*) red cells in 300 mM amino acids. A 3% cell suspension was incubated at 38 °C and frequent samples were taken for the determination of % hemolysis. The cell suspension pH values varied from 6.78 in glutamine to 7.49 in histidine medium

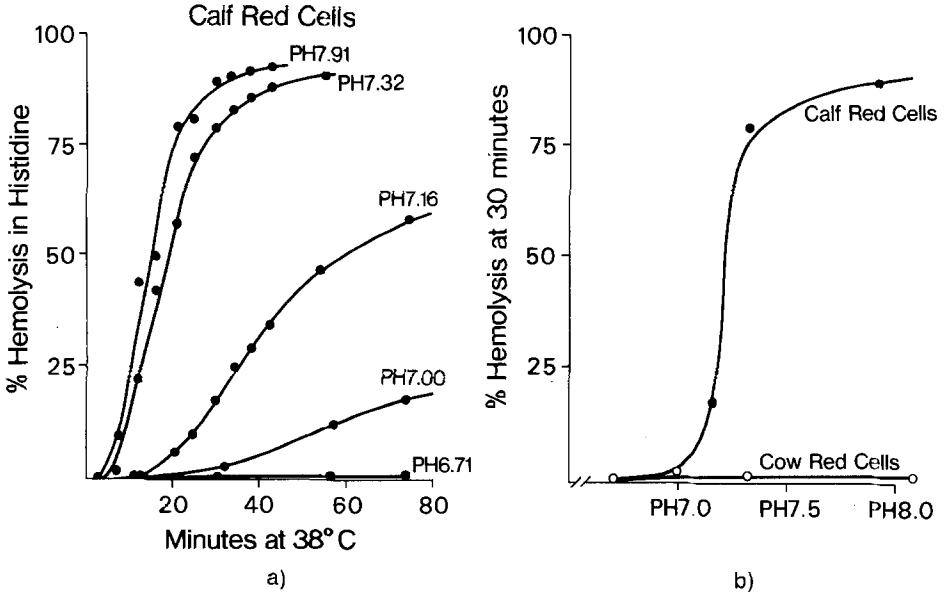

Fig. 2. Newborn calf cell hemolysis in isotonic histidine (*a*) and a comparison between the hemolysis of calf and cow cells (*b*) as a function of pH. For the purpose of comparison, the arbitrary hemolytic values at 30-min incubation in isotonic histidine at 38 °C are used. The hemolytic conditions are the same as in Fig. 1

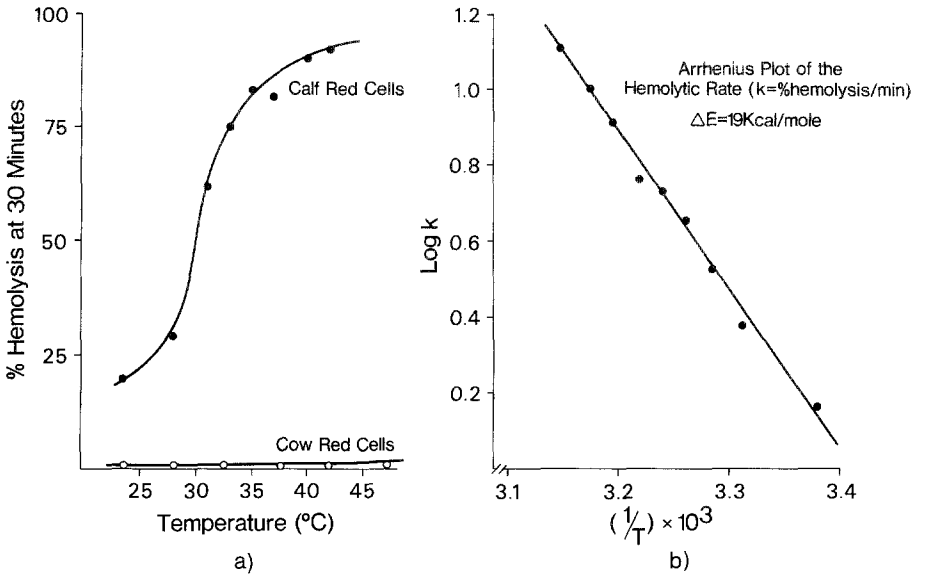

Fig. 3. Hemolysis of calf and cow red cells in isotonic histidine as a function of temperature (*a*) and an Arrhenius plot of calf cell hemolysis (*b*). The hemolytic rate (*k*) in the unit of % hemolysis/minute was used to construct the Arrhenius curve. The calculated apparent activation energy is 19 kcal/mol. The hemolytic conditions are the same as in Fig. 1

exhibited a total resistance toward lysis by histidine in a wide temperature range from 23° to 48 °C, whereas the lytic process of the calf cell was temperature dependent and increased markedly within this range as the temperature was raised. This is most clearly shown when the hemolytic rates in the unit of % hemolysis is used to construct an Arrhenius plot (Fig. 3*b*). The calculated apparent activation energy was 19 kcal/mol.

The extracellular salt concentration is another critical parameter determining histidine-induced hemolysis. This effect is depicted in Fig. 4 in which different aliquots of isotonic histidine are replaced by isotonic $CaCl_2$, $MgCl_2$ and NaCl. Divalent cations, particularly Ca^{++}, are more effective in preventing hemolysis than monovalent cations.

When the extracellular salts are entirely replaced by nonelectrolytes, such as sucrose, calf cells, but not cow cells, became unstable culminating in hemolysis as shown in Fig. 5. Again, inclusion of a small amount of salt stabilized the cells and with 30 mM NaCl, hemolysis in sucrose can be totally prevented. However, if histidine is added instead of salt, the cells become even more hemolytic than seen in isotonic sucrose alone.

In view of the distinct difference between the two cell types, obvious questions arise as to the postnatal transition from a histidine-sensitive

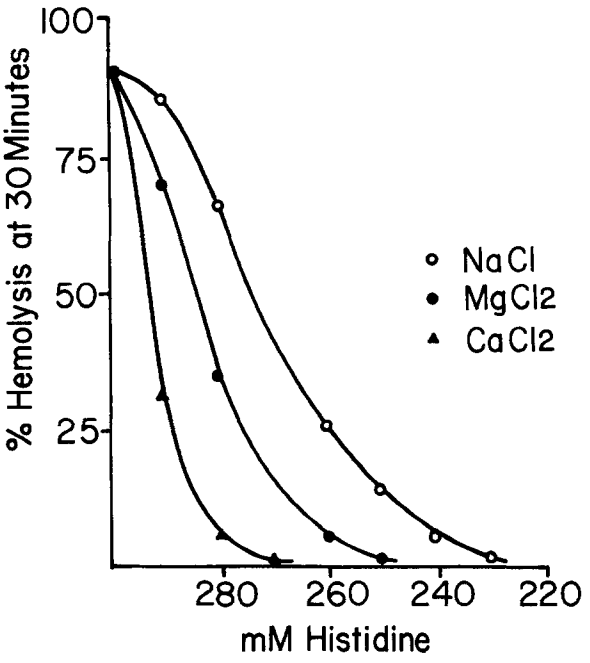

Fig. 4. Histidine-induced hemolysis of calf red cells as a function of the extracellular salt concentrations. Three aliquots of calf cells were washed several times in each of the following isotonic salt solutions: 0.165 M NaCl (○), 0.11 M $MgCl_2$ (●) and 0.11 M $CaCl_2$ (▲). Cells were resuspended at 38 °C in various histidine concentrations plus salt which were prepared by replacing different aliquots of isotonic histidine by isotonic salts. Thus, the resultant incubation medium was always maintained at isotonic concentration, while the histidine content was varied from 300 to 230 mM

cell type to an insensitive cell type and to the mechanism of hemolysis. In an attempt to answer the question of how soon do the cells fabricated after birth assume the adult cell characteristics, the rate of fetal cell disappearance was compared with the extent of histidine hemolysis in growing animals. The rate of fetal cell depletion was estimated by the cellulose acetate membrane electrophoresis of hemoglobin. The result is shown in Fig. 6. At birth, there was predominantly one type of electrophoretic band in the first animal (# 1041), presumed to be the fetal type, whose intensity decreased as the animal grew old. Soon after birth, an additional band, presumed to be the adult type, appeared whose intensity increased. No trace of fetal type is seen about two months after birth. In the second animal (# 1042), a small but significant amount of adult type hemoglobin is already seen even at the time of birth. Here the removal of fetal type is virtually completed in about $1^1/_2$ months. These results on the depletion of fetal cell type after birth in calves

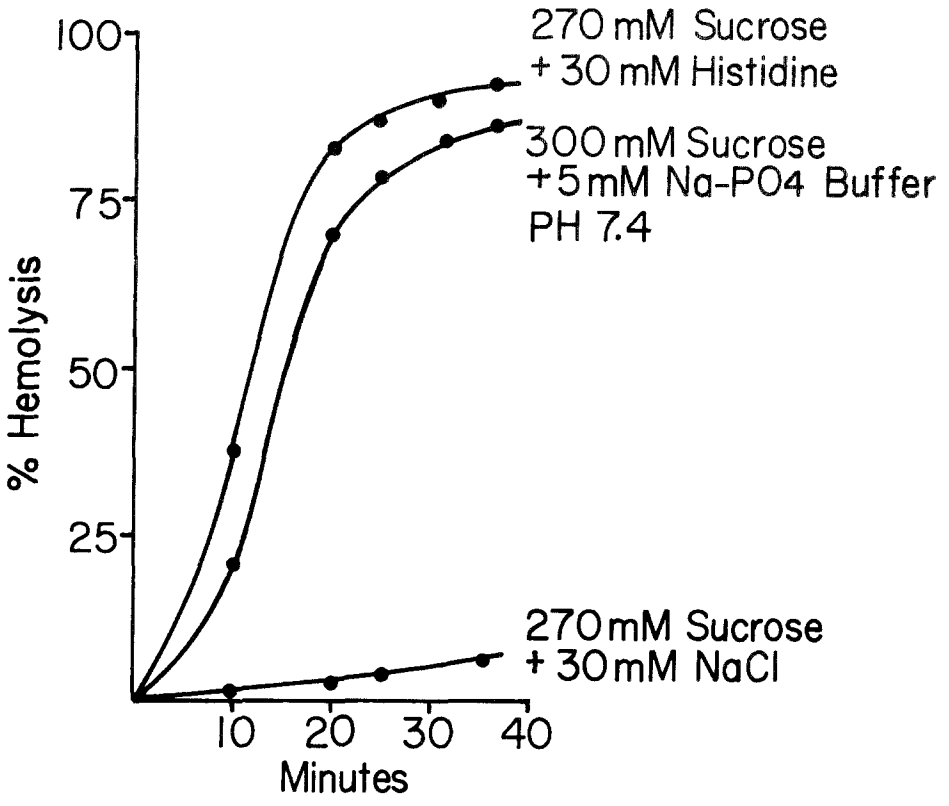

Fig. 5. Calf cell hemolysis in isotonic sucrose solution. The different aliquots of isotonic sucrose were replaced by either isotonic NaCl or histidine as in Fig. 4

are in reasonably good agreement with the observation reported by Israel *et al.* (1972). Frequent blood samples were taken from the growing second animal and were exposed to histidine as shown in Fig. 7. The unhemolyzed cell fractions were recovered and subjected to electrophoresis. At the sixth day, the whole blood clearly exhibited two bands. After histidine exposure, only the adult type survived. At the tenth day a fetal band still constitutes a major part of the red cell populations. After exposure to histidine, again only the adult type survived. Clearly, this observation holds true for all cases, suggesting a selective, specific hemolysis of fetal cells by histidine. However, the result is not a rigorous proof of selectivity since the extent of hemolysis of cells containing adult type hemoglobin would have been undetected. To circumvent this difficulty, the percent hemolysis in histidine was plotted as a function of animal age. The result is shown in Fig. 8. The choice of 20 min stems from the observation that the maximum hemolysis occurred in 20 min at the time of birth

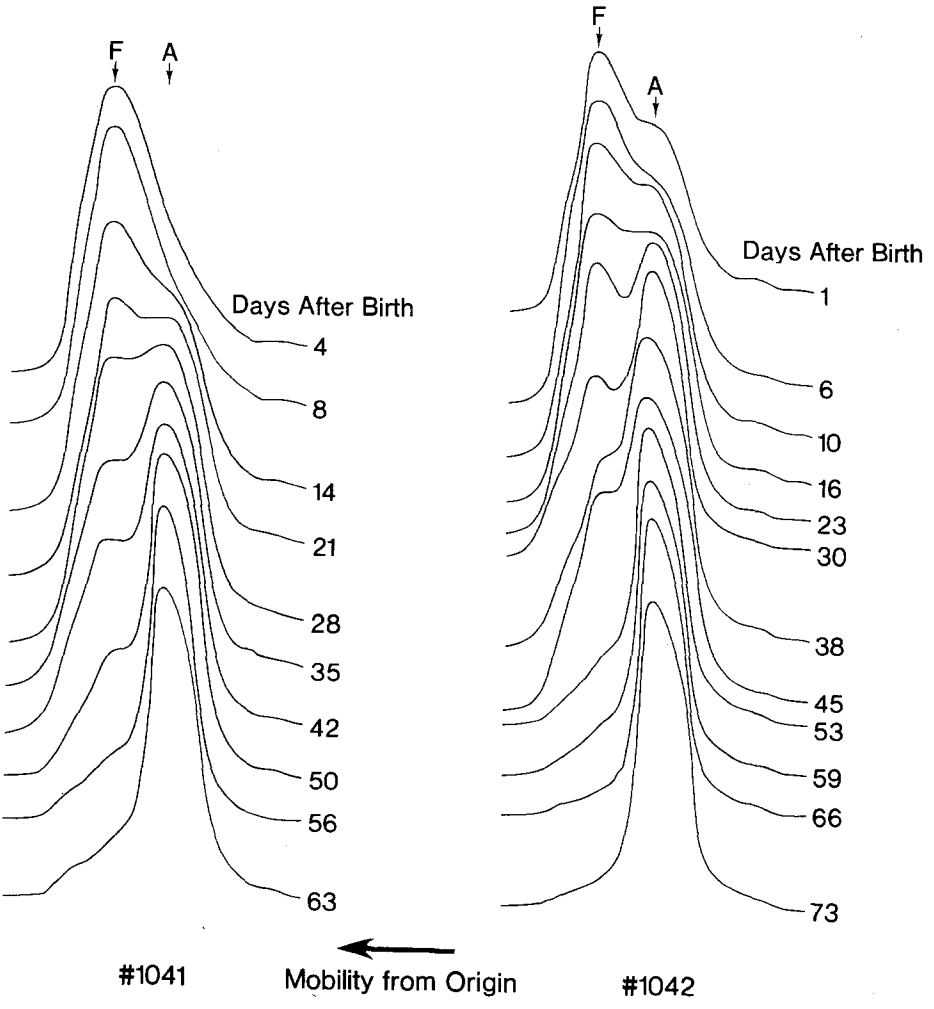

Fig. 6. Electrophoretic patterns of hemoglobin in growing calves. Hemoglobin electrophoresis was carried out on a cellulose acetate membrane utilizing (I=0.075) diethyl barbituric acid-sodium diethylbarbiturate buffer, pH 8.6. The density of the electrophoretic bands were determined on a Beckman Microzone Digital Integrator (Model R-111). While the first peak designated by (F) is presumed to be fetal hemoglobin, the second peak denoted as (A) is presumed to be the adult type hemoglobin. The University of Arizona calf numbers: the first animal (#1041), and the second animal (#1042)

in both animals. The second animal (# 1042) is the one that contained a small amount of adult type hemoglobin at the time of birth.

Two points need to be stressed:

1. Extension of the slope of the lines suggest that it would be some $2^1/_2$ to 3 months after birth until the cells would display a total immunity toward histidine.

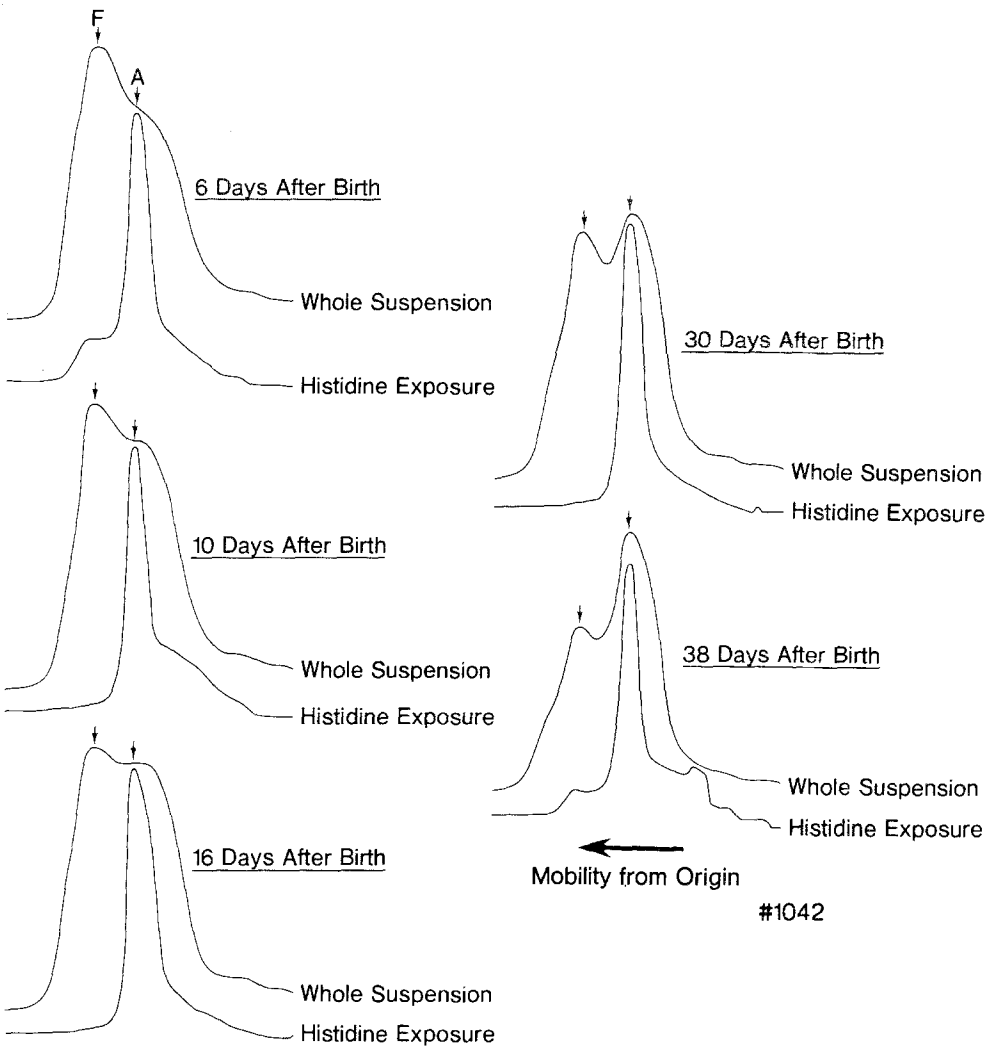

Fig. 7. The hemoglobin electrophoretic patterns of a growing calf (#1042) before and after exposure to histidine. Calf cells were suspended in 300 mM histidine at 38 °C for 1 hr and the unhemolyzed cells were recovered by centrifugation. The hemoglobin electrophoresis was carried out on both the whole suspension and the unhemolyzed cells denoted by "Histidine exposure" in the Figure

2. Since the fetal cells are virtually depleted by $1^1/_2$ to 2 months, the 20 to 25% hemolysis noted at 2 months must represent the hemolysis of newly made postnatal red cells. In this regard, early postnatal cells are again different from adult cells, suggesting the animal's age as an important determinant for the red cells' function (Kim & Duhm, 1974).

In regard to the mechanism of hemolysis, it would seem conceivable that the two cell types could have different membrane permeability to

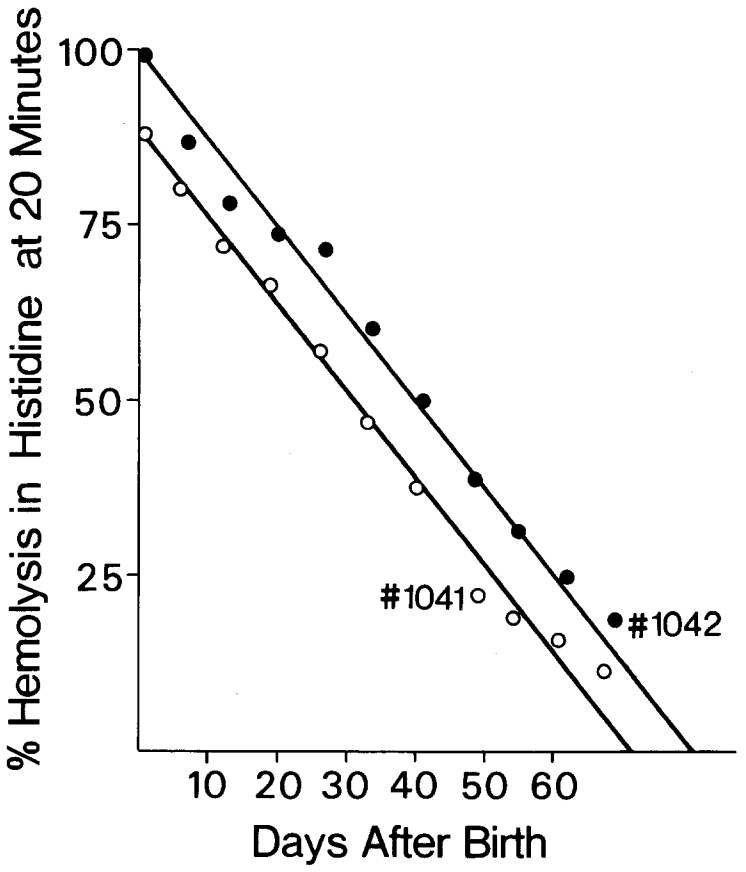

Fig. 8. Postnatal calf cell hemolysis in histidine with advancing age of the animals. A complete hemolytic event in histidine was first followed on each occasion of blood sampling in two growing calves from which the hemolytic values at 20 min of incubation were obtained. The choice of 20 min stems from the observation that the maximum hemolysis occurred in histidine at the time of birth in both animals

histidine. If the calf red cells are more permeable to histidine than the cow red cells, the calf cell hemolysis by histidine could simply be mediated by cell swelling. To determine the membrane permeability of histidine, both calf and cow cells were suspended in 300 mM histidine augmented with ^{14}C-histidine. The result of histidine uptake, shown in Fig. 9, revealed that there was an instantaneous uptake and/or binding of histidine in both calf and cow cells, the magnitude of which varied considerably from 20 to 45 μmoles/ml RBC among different animals. Following the extraordinarily rapid initial uptake, the histidine influx slowed down markedly to an average rate of 0.25 to 0.5 μmoles/ml RBC × min. Thus, the variations in membrane permeability to histidine could not be invoked to account for the transitory postnatal hemolysis.

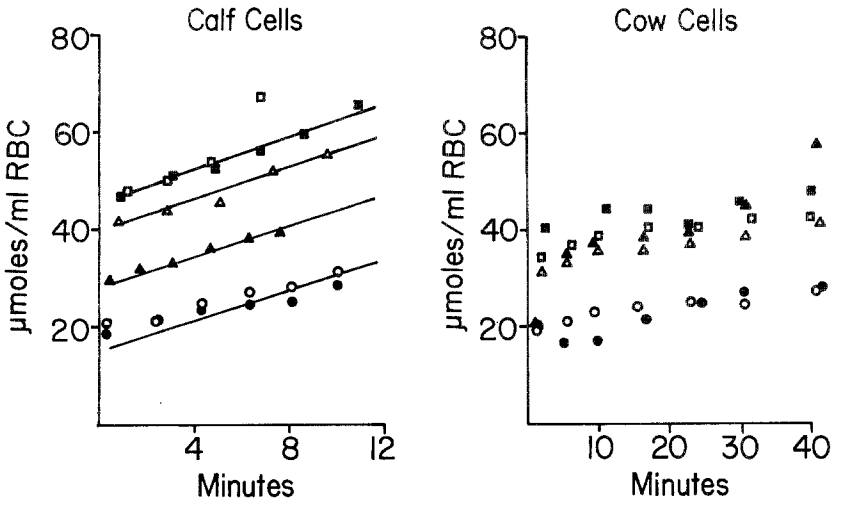

Fig. 9. Histidine uptake in calf and cow red cells. Cells were preincubated for 1 hr at 38 °C in balanced salt medium to allow the removal of endogenous amino acids. Cells were then resuspended at 10% Hct in prewarmed 300 mM histidine supplemented with 0.1 μC/ml ^{14}C-substrate at 38 °C. The amount of histidine taken up by the cell was computed by taking into account the trapped extracellular volume of 3.5%

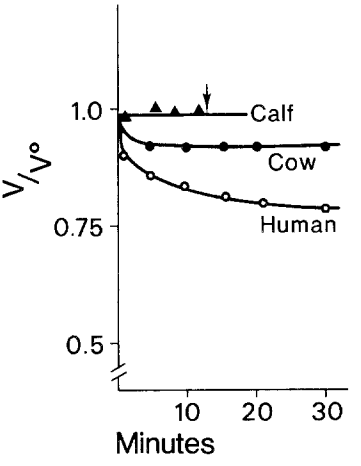

Fig. 10. The cell volume in isotonic histidine (V) relative to the original cell volume (V^0). The cell volume was determined by hematocrit measurement and related to the original cell volume computed from hemoglobin determination of the suspension and mean corpuscular hemoglobin concentration (MCHC) value obtained on whole blood. The first sign of hemolysis noted is indicated by the arrow

Despite the relatively large uptake of histidine in the presence of isotonic histidine, the cell volume as determined by hematocrit measurement did not increase; if anything it decreased as shown in Fig. 10. Therefore, it seems unlikely that the histidine-induced hemolysis is medi-

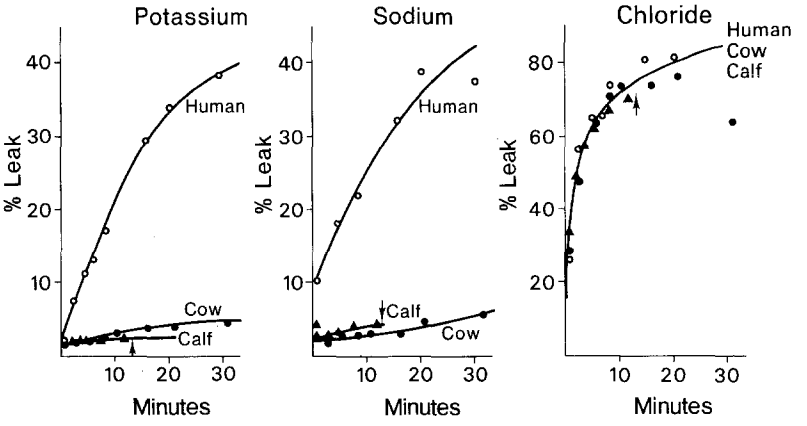

Fig. 11. K, Na and Cl^- leakage in isotonic histidine medium. Calf (▲), cow (●) and human (○) cells were exhaustively washed in 0.11 M $MgCl_2$ at 4 °C and resuspended in 300 mM histidine at a Hct of 15% at 38 °C. % leak refers to a ratio of ion content in the supernatant fluid over the whole suspension. The cation and chloride contents in the cell were as follows:

	Na	K	Cl
		(μM/ml RBC)	
Calf	21.2	93.6	101
Cow	120	17.5	91
Human	19.7	102	92

ated by a colloid osmotic swelling. To account for the observed cell volume change, K, Na and Cl levels were measured when 0.11 M $MgCl_2$-washed red cells were suspended in isotonic histidine medium. The result is shown in Fig. 11. Human cells, included for the purpose of comparison, lose a substantial number of cations in much the same manner as that well-established by other investigators either in nonpenetrating nonelectrolyte or nonpenetrating anion medium (Donlon & Rothstein, 1969; Cotterrell & Whittam, 1971). In nonpermeable nonelectrolyte, human cells initially became leaky to Cl ions. The resultant membrane potential governed by the new Cl^- ratio would impose a large electrical driving force for the cation leakage. Indeed, human cells as well as calf and cow cells lost Cl^- rapidly in histidine medium. Unexpectedly, however, both calf and cow cells lost few cations in histidine. Apparently, the extent to which Cl^- left the cell was more or less balanced by histidine uptake resulting in a maintenance of calf and cow cell volume not grossly different from the original cell.

In contrast, human cells exhibited a striking amount of shrinkage as a result of a net exodus of salts in histidine medium (Fig. 10). However,

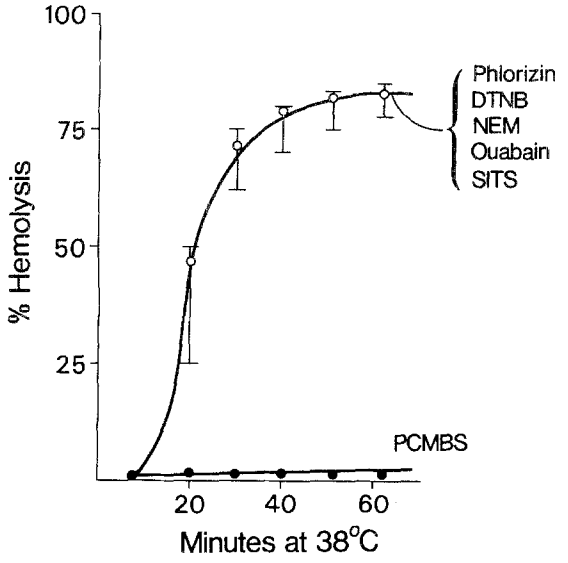

Fig. 12. Effect of inhibitors on histidine-induced hemolysis. The type and concentration of inhibitors used were as follows: 1 mM phlorizin, 3 mM DTNB, 1 mM NEM, 1 mM ouabain, 75 μg/ml SITS and 1 mM PCMBS

in a prolonged incubation, cell volume would eventually be returned to the original level due to a continual influx of histidine (Luthra *et al.*, 1975).

Although histidine-induced calf cell hemolysis does not appear to be mediated by colloid osmotic swelling, flooding the cell interior with a large amount of histidine could have a deleterious effect. If an agent which either inhibits histidine influx or prevents hemolysis could be found, it could prove to be a useful probe toward elucidation of the mechanism of hemolysis. To this end, several transport inhibitors have been tested for their effectiveness in preventing hemolysis. As shown in Fig. 12 it was found that 1 mM or less PCMBS can completely protect cells from hemolysis, provided that the cells were preincubated in the presence of the inhibitor for 30 min or longer at 38 °C.

Furthermore, PCMBS protection is a reversible reaction as shown in Fig. 13, in which cells are preincubated in 1 mM PCMBS at various times as indicated by the arrows, and subsequently resuspended in isotonic histidine alone. Clearly, the longer the exposure, the better the protection of cells from hemolysis. When the preincubated cells were washed several times in 0.165 M NaCl, and resuspended in isotonic histidine alone, hemolysis could no longer be prevented.

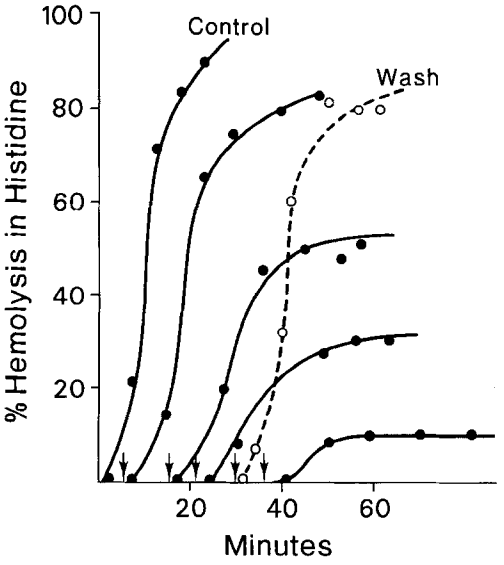

Fig. 13. The reversibility of PCMBS protection. Cells were preincubated as indicated by the arrows in 1 mM PCMBS, 5 mM K, 150 mM NaCl, and 10 mM Na-phosphate buffer, pH 7.4 at 38 °C. Cells were recovered and resuspended in isotonic histidine for the subsequent measurement of hemolysis. An aliquot, preincubated for 30 min in 1 mM PCMBS (○), was washed three times in 0.165 M NaCl and resuspended in isotonic histidine

To determine whether PCMBS protection was brought about by an inhibition of histidine entry to the cell, histidine uptake was measured in PCMBS-preincubated cells. The results shown in Fig. 14 indicate that histidine influx was indistinguishable from the control value. That the histidine-induced hemolysis can be prevented without affecting the amount of histidine taken up by the cell suggests that histidine somehow damages the external or outer surface of the cell membrane.

Discussion

In response to mechanical and osmotic stress, red blood cells become permeable to hemoglobin and culminate in hemolysis. Hemolysis of red cells is brought about basically in two ways, depending on whether the mechanism is one of simple osmotic swelling or a direct injury to the membrane architecture by the hemolytic agents (Ponder, 1948). The osmotic lysis technique has frequently been used in the comparative studies on the permeability of rapidly permeating molecules and more recently has been extended to the measurement of organic anion permeation in cow red cells (Aubert & Motais, 1975). Moreover, analysis of

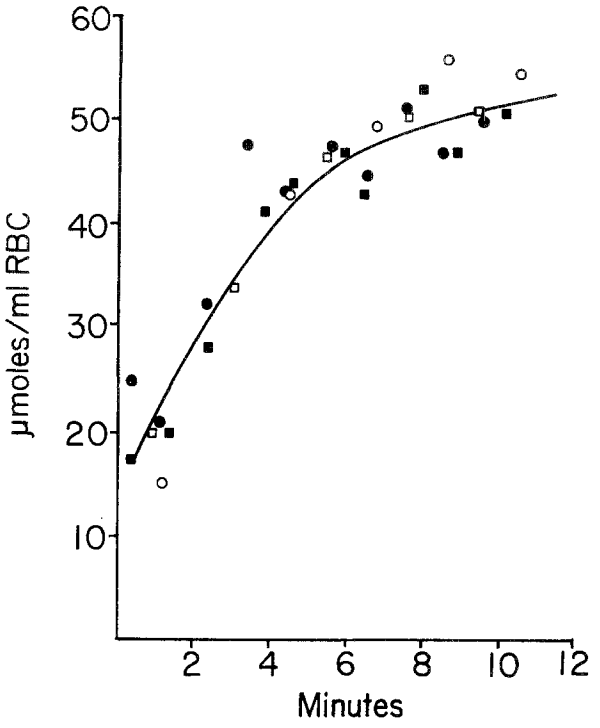

Fig. 14. Effect of PCMBS on histidine uptake by calf red cells. The control cells (●, ■) were suspended at 11% hematocrit in 300 mM histidine and frequent samples were taken for the measurement of histidine uptake. Another aliquot was preincubated in a medium consisting of 1 mM PCMBS, 5 mM KCl, 10 mM Na phosphate buffer, pH 7.4, and 150 mM NaCl for 30 min at 38 °C. PCMBS-treated cells (○, □) were then recovered by centrifugation and resuspended in 300 mM histidine for the subsequent influx measurement

osmotic fragility in which the degree of hemolysis is plotted against decreasing NaCl concentration has provided immeasurably valuable information about the homogeneity, the shape, the surface to volume ratio (Canham, 1969) and the pathophysiology of red cells (Prankerd, 1961).

In recent years much evidence has been accumulating in support of the concept that the colloid osmotic swelling mediated by the alteration of the membrane permeability of cations is the common feature of all kinds of hemolysis (Davson & Ponder, 1940; Wilbrandt, 1941; Green, 1956; Miller & Buhler, 1974). Indeed, detailed electron-microscopic studies on a variety of hemolytic situations revealed that functional holes are produced in the membrane, resulting in the cell swelling due to the Donnan influence of hemoglobin (Seeman, 1967; Iles, Seeman, Naylor & Cinader, 1973). As expected, hemolysis could be delayed if a nonpenetrating substance such as sucrose was added to the medium to counteract the osmotic effect of hemoglobin (Wilbrandt, 1941).

In this regard, histidine-induced hemolysis must be fundamentally different, since the calf cell membrane maintained an effective barrier with respect to Na and K prior to hemolysis. Even if the normally sluggish cation permeability in the calf cell were to be altered by histidine, it would not have contributed to the cell swelling by virtue of the fact that the external cations were entirely replaced by the amino acid. Thus, the cell swelling could take place only if the cell membrane were readily permeable to histidine. The result obtained on histidine influx showed that there was an extraordinarily rapid uptake of histidine amounting to 20–45 μmoles/ml RBC followed by a slow influx rate. However, the extent to which histidine entered the cell was counterbalanced by a concomitant efflux of Cl ions. As a result, cell volume did not increase in isotonic histidine prior to hemolysis, even though the calf cell can reach the critical hemolytic volume of 1.25 during a hypotonic hemolysis. Ordinarily, the red blood cell membrane potential is governed by the chloride ratio. However, in view of the rapid Cl^- and histidine exchange, the estimation of the resultant membrane potential is difficult to make without the benefit of precise knowledge of the internal pH, permeability coefficient of histidine, etc. In view of the close proximity of the isoelectric point of histidine (7.58) to the normal cell pH, it would seem likely that histidine entering the cell bears a net negative charge, the extent of which depends upon the internal alkalinization brought about by Cl and OH exchange. Thus, the electrical driving force for cation diffusion may be appreciably small as opposed to the situations in which cells were suspended in nonpenetrating nonelectrolytes. In any case, the calf cells lost little Na and K prior to hemolysis irrespective of whether the suspending medium was histidine or sucrose (Cook and Kim, *unpublished results*). These results suggest that histidine might block or interfere with the cation diffusion which is said to be occurring across the aqueous channels limited by positive charges. Alternatively, histidine entry is independent of the cation diffusion path, and that the small amount of cation leak in histidine medium actually represents the magnitude of normal cation diffusion since the Na+K stimulated membrane pump activity must have been either drastically reduced or abolished under this zero-trans condition. Further work is needed to resolve this unexpected finding.

As emphasized by McManus (1967), the red blood cell of each mammalian species must be regarded as an altogether distinct biological entity. Indeed, the human red cell is a striking contrast to that of other species in its response toward histidine. In confirmation of a previous observation

(Luthra *et al.*, 1975), the cell membrane became exceptionally leaky with respect to Na and K ions, resulting in a transitory cell shrinkage.

The exact mechanism by means of which histidine causes the hemolysis of calf but not of cow red cells remains obscure. However, it may be worthy to recall certain pertinent observations made on histidine. First of all, histidine is a well-known chelating agent (Sundberg & Martin, 1974). As such, it could bind or interact with divalent cations, particularly Ca^{++}, which might be an integral part of the membrane matrix. Among the numerous mono- and divalent cations and anions tested, Ca^{++} is the most effective agent in preventing the histidine-induced hemolysis. Secondly, histidine is the substrate for the so-called "histidine binding protein J" which is a component of the high affinity histidine transport system in *Salmonella typhimurium* (Kustu & Ames, 1974). To the best of the author's knowledge, the presence of such proteins in the red blood cell membrane has not been reported. It would seem reasonable that the calf cell might serve as an ideal model to examine the binding proteins which might be involved in the puzzling histidine uptake kinetics and hemolysis.

In an effort to delineate the structural requirement for the protective effect of PCMBS, various phenyl analogs including *p*-toluene sulfonic acid, sulfosalicylic acid and PCMB were tested. Of these, only PCMB was found to be as effective as PCMBS in protecting against histidine-induced lysis, suggesting a critical role for membrane sulfhydryl group. Yet, simple washing alone completely abolished the PCMB or PCMBS protection (Fig. 13). This is in sharp contrast to the well known effects of the specific sulfhydryl reactive-reagent PCMBS on red cells including the inhibition of H_2O transport (Naccache & Sha'afi, 1974), Na exchange diffusion (Motais & Sola, 1975) and the increase in passive cation permeability (Sutherland, Rothstein & Weed, 1967), all of which required the dissociation of a mercaptide bond with PCMBS by cysteine or dithiothreitol.

The spectacular difference seen in the response to histidine between the calf and cow red cell are reminiscent of the well-documented postnatal metabolic adaptation of red blood cells occurring in many newborn animals. An outstanding example is found in the newborn pig in which the glycolytic capacity is lost as the fetal cell population is replaced by the adult red cells which are impermeable to glucose (Kim, McManus & Bartlett, 1972). Similarly, the newborn calf red cell is endowed with ample metabolic machinery, much of which is lost in the adult stage. For example, glyceraldehyde, dihydroxyacetone, ribose and ribulose are

all metabolized to lactate by the calf but not the adult cow cells (Labda and Kim, *unpublished results*). The assessment of the extent to which cells made after birth assume the adult characteristics would require the physical separation of postnatal cells from the fetal cell population. To this end, the studies reported herein on histidine hemolysis might be efficaciously employed for the isolation of those red cells released into the circulation after birth in growing animals. This technique has advantages over the conventional technique based on the red cell density profile which cannot readily be applied for postnatal cell populations because of the appreciable density overlap between the fetal and postnatal cell populations. Moreover, the finding that the cation content is kept near normal physiological levels during histidine hemolysis seems to provide sufficiently adequate conditions for the study of membrane transport processes in postnatal red cells.

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