Calcium-promoted Changes of the Human Erythrocyte Membrane

IN VOLVEMENT OF SPECTRIN, TRANSGLUTAMINASE, AND A MEMBRANE-BOUND PROTEASE*

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DARRELL R. ANDERSON, J. LAWRENCE DAVIS, and KERMIT L. CARRAWAY
From the Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74074

Erythrocytes suffer a contraction and loss of deformability upon ATP depletion and accumulation of calcium, an effect which can be mimicked by hemolysis of the cells in the presence of calcium, followed by resealing or by introduction of calcium into the cells with the ionophore A23187 (Kirkpatrick, F. H., Hillman, D. G., and LaCelle, P. L. (1975) Experientia 31, 653-654). At high calcium concentrations several significant changes are noted in the polypeptides of membranes isolated subsequent to the calcium treatment, including the formation of a polypeptide aggregate, loss of a component of molecular weight 85,000 (IVa), and formation of a component of molecular weight 180,000 (II). Aggregate formation and loss of IVa are strongly correlated with the activity of the cytoplasmic enzyme transglutaminase, as indicated by experiments using activators or inhibitors of the enzyme. The mechanism apparently involves cross-linking of membrane spectrin (polypeptides Ia and Ib) into a supramolecular aggregate along with IVa and possibly some other components. The formation of II occurs as a result of activation or release of a membrane-bound protease and is correlated with the loss of component Ic, indicating a precursor-product relationship.

Extraction of membranes from calcium-treated cells shows two additional changes. A substantial fraction of spectrin is not extracted by EDTA from membranes of treated cells. In contrast the spectrin of membranes of untreated cells is almost completely extracted. Two smaller polypeptides (M, = 60,000 and 25,000) are found in association with membranes of treated cells and can be extracted from the membrane by EDTA. Examination of the calcium concentration dependence of these various changes indicates that the change in spectrin extractability and proteolytic cleavage of Ic occur at lower calcium concentrations than the aggregate formation.

To circulate and transport oxygen an erythrocyte must maintain membrane flexibility, a process which requires energy in the form of ATP. ATP-depleted erythrocytes become rigid and echinocytic (1), leak cations (2), accumulate calcium (3), and are eventually destroyed in the microcirculation. If cells are repleted with ATP, the loss of deformability is reversible (1) as long as calcium accumulation is minimal. The deformability loss and cell shape changes can be mimicked by hemolysis and resealing of erythrocytes or by treating them with an ionophore in the presence of calcium (4). Deformability is reversible at low calcium concentrations but becomes essentially irreversible at concentrations near 1 mM. The mechanism of deformability loss is of considerable interest, since similar changes in membrane flexibility occur in diseases such as sickle cell anemia (5) and hereditary spherocytosis (6), as well as the normal erythrocyte aging process (7).

We have previously shown two prominent changes in protein components that occur in erythrocyte membranes from cells treated with calcium during hemolysis (8, 9) with or without resealing. One of these involves an irreversible aggregation of proteins, while the second apparently results from proteolysis of membrane polypeptides in the presence of calcium (8). The present investigation sought to clarify the mechanisms of aggregation and proteolysis and to describe altered association patterns of polypeptides with the membrane. A combination of extraction, chemical modification, enzyme analysis, and electrophoresis techniques has been used to describe changes that occur under conditions which promote deformability loss. A model for irreversible deformability loss is suggested based on the stabilization of an altered membrane state by cross-linking catalyzed by the enzyme transglutaminase.

EXPERIMENTAL PROCEDURES

Materials—Human blood was obtained fresh from Stillwater Municipal Hospital or Dallas Community Blood Bank. Ionophore A23187 was a generous gift of Dr. Robert Hosley of Lilly Research Laboratories. [14C]Putrescine and [3H]acetic anhydride were from New England Nuclear. All other chemicals were reagent grade or highest purity available.
Calcium Treatments and Membrane Analyses—Calcium treatments were performed by a modification of the procedures of Triplett et al. (8). One volume of packed red cells was hemolyzed in 10 volumes of 10 mM Tris (pH 7.4) containing varying amounts of CaCl₂. The hemolysate was incubated at 37℃ for 30 min. When resuspending was necessary, the isotonicity was restored to 0.674 M with 3 M NaCl prior to the incubation step. Membranes were obtained by washing in 10 mM Tris (pH 7.4) with centrifugation at 17,000 × g for 20 min.

Calcium was also introduced into erythrocytes by use of the ionophore A23187, as described previously by Kirkpatrick et al. (4). After ionophore and calcium treatments membranes were prepared by hemolysis and washed with 10 mM Tris, pH 7.4.

Electrophoresis of membranes solubilized in dodecyl sulfate was performed on polyacrylamide gels in 0.1% detergent as previously described (10). Polyepitope bands were designated according to a system outlined previously (8, 11).

Membrane Extractions—Membranes obtained from erythrocytes hemolyzed in the presence of increasing concentrations of CaCl₂ were extracted in 5 volumes of buffer containing 5 mM glycine, 5 mM mercaptoethanol, and 1 mM EDTA (pH 9.5) at 4℃ for 24 h (11, 12). Aliquots of extracts were taken for electrophoresis in dodecyl sulfate along with aliquots of extracted residual membranes. Protein concentrations were determined by the procedure of Lowry et al. (13).

14CPutrescine Assay for Transglutaminase—The 14Cputrescine assay for guinea pig transglutaminase was modified for use in studying the red cell enzyme by observing incorporation of putrescine into endogenous erythrocyte protein. The assay mixture containing 0.6 μl of fresh erythrocyte hemolysate (1.6 mg of protein), CaCl₂ as specified, 4 μM 14Cputrescine (2.5 μCi/μl), and 10 mM Tris (pH 7.4) was incubated for 30 min at 37℃, and the reaction terminated by the addition of 2 ml of ice-cold 5% trichloroacetic acid. After washing six to seven times with cold trichloroacetic acid, the precipitates were solubilized in 0.5 ml of NCS and counted. Blank samples containing no Ca²⁺ or iodoacetamide were also included. The assay was linear for up to 30 min.

Inhibition of Aggregation Process by Iodoacetate Modification—Fresh human blood was washed three times in Krebs-Ringer buffer (pH 7.4) and reacted with 0 to 5 mM iodoacetic acid for 60 min at 37℃ and a hematocrit of 10. After washing three times in isotonic saline containing 10 mM Tris (pH 7.4), one-half of each cell suspension was hemolyzed in 10 mM Tris containing 5 mM CaCl₂. The remaining samples were hemolyzed in the same buffer without CaCl₂ for controls. The suspensions were incubated for 30 min at 37℃ and washed three additional times in 10 mM Tris to remove hemoglobin. The washed ghosts were immediately solubilized in 4% sodium dodecyl sulfate and placed on 5% polyacrylamide gels in the presence of dodecyl sulfate.

Localization of Aggregating Factor from Erythrocyte Hemolysates—One volume of packed cells was hemolyzed in 10 volumes of 10 mM Tris (pH 7.4) for 10 min at 4℃. The membranes were sedimented by centrifugation at 1,500 × g for 5 min and the supernatant was removed. The pellet membranes were resuspended in 10 mM Tris (pH 7.4) and then washed until free of hemoglobin. The washed, packed, hemoglobin-free ghosts were resuspended in the original hemolysate which had been centrifuged at 27,000 × g for 60 min and the suspension was made 5 mM in CaCl₂. The mixture was incubated at 37℃ as in previous calcium treatments. An identical sample of membranes was mixed with hemolysate in the absence of added Ca²⁺ and incubated as a control. The membranes were again washed free of hemoglobin and dissolved in 4% sodium dodecyl sulfate. The membrane patterns were analyzed by dodecyl sulfate gel electrophoresis for aggregation and other changes.

[3H]Acetic Anhydride Labeling (14) of Membrane Proteins for Quantitation of Aggregate and Component II Formation—To quantify aggregate formation, washed membranes from 1 ml of packed erythrocytes were solubilized in 2% sodium dodecyl sulfate (0.8 ml, 10 min, 100℃). Solubilized membranes (0.5 ml) were labeled by treating with 0.26 mCi of [3H]acetic anhydride (29 mCi/mg) in 5 μl of dimethylsulfoxide for 20 min at room temperature. The samples were dialyzed overnight against 0.1% dodecyl sulfate at room temperature, and 100 μg of protein (15) were applied to each electrophoresis gel.

Quantitation of component II formation was obtained by labeling intact ghosts under the same reaction conditions. Membrane suspensions were washed four times with 100 mM Tris (pH 7.4) and solubilized for electrophoresis.

Radioactivity associated with protein bands was measured after slicing the bands from Coomassie blue-stained gels and incubating in NCS.

RESULTS

Correlation of Calcium-promoted Membrane Polypeptide Aggregation with Hemolysate Transglutaminase Activity—The stability of the calcium-promoted aggregate and absence of calcium in purified aggregate (9) suggested that covalent bond formation might be occurring during calcium treatment. Two additional experiments were particularly informative regarding the nature of the reaction. (a) Addition of hemolysate and Ca²⁺ to washed erythrocyte membranes caused aggregate formation. No aggregate was formed in the absence of hemolysate. (b) Iodoacetate treatment of erythrocytes before hemolysis prevented aggregate formation. These facts suggested the involvement of a cytoplasmic enzyme. Since transglutaminase is known to require calcium (15), to cross-link membrane proteins (16), and to be present in erythrocyte hemolysates (17), we investigated the relationship between the activity of this enzyme and the aggregation that occurred.

Transglutaminase activity in hemolysates could be easily quantified by incorporation of 14Cputrescine into hemolysate protein. Fig. 1 shows the calcium dependence of the activation of the enzyme. Quantitation of the aggregate reaction was more difficult. Since the aggregate remained near the top of electrophoresis gels, scanning the gels was not practical. A radioactivity assay was devised which involved labeling membranes which had been solubilized in sodium dodecyl sulfate with the nonspecific reagent [3H]acetic anhydride (14). After dialysis, electrophoresis, and staining the aggregate bands were sliced from the top of the gels and counted. Radioactivity incorporated into the aggregate was proportional to the amount of membrane protein applied to the gel over a range of membrane concentrations. This radioactivity assay indicated an increase in aggregate formation with increased calcium incorporated into erythrocytes. Fig. 2 shows the strong correlation between hemolysate transglutaminase activity and aggregate formed as calcium concentration is increased. A similar correlation could be found using strontium, which also activates the enzyme, but is approximately 10-fold less effective as an activator.

Additional evidence for the involvement of transglutaminase in the cross-linking was obtained using specific inhibitors of the enzyme. Several amino compounds were used as competitive inhibitors. Fig. 3 shows the effects of unlabeled putres-
FIG. 2. Correlation of aggregate formation with transglutaminase activity of erythrocytes over a range of calcium concentrations. Transglutaminase activities and aggregate formation were measured as described under “Experimental Procedures” over a series of appropriate concentration of inhibitor.

![Graph showing correlation between aggregate formation and transglutaminase activity](image)

FIG. 3 (left). Effects of glycinamide (●) and histamine (■) on [14C]putrescine incorporation and unlabeled putrescine (△), glycinamide (○), and histamine (□) on aggregate formation. [14C]Putrescine incorporation and aggregate formation were measured after a 20 min incubation at 37° with 5 mM CaCl₂ in the presence of an appropriate concentration of inhibitor.

![Graph showing effects of glycinamide and histamine](image)

The second major change caused by calcium is the appearance of a polypeptide of molecular weight of 180,000. Although we had previously suggested that it arose via a proteolytic cleavage, there were still questions about its origin and the nature of the protease. To show that II did not arise by adsorption from the cytoplasm, membranes were labeled with [3H]acetic anhydride and incubated with unlabeled hemolysate in the presence or absence of calcium. Gels from this experiment show an increase in component II and loss of Ic in the presence of calcium (Fig. 5). Band II of these gels was excited and counted. The increased radioactivity in this band (Table II) with Ca²⁺ treatment indicates that it came from membrane polypeptides rather than the hemolysate. Further evidence that Band II was originally a membrane polypeptide was obtained by incubating washed erythrocyte ghosts in the presence of calcium. The loss of component Ic and formation of II are clearly demonstrated by this experiment (Fig. 6), which indicates that hemolysate is not required for this process. Thus, it appears that Ca²⁺ acts to activate or release a membrane factor responsible for the Ic → II conversion. These results do not necessarily indicate that this factor is not present in hemolysate, since it could have been partially adsorbed onto the membranes during the hemolysis procedure. There is also a question concerning the specificity of the calcium requirement for the proteolysis. Our previous results (8) showed that Mg²⁺ at 20 mM would not promote formation of II when introduced into membranes by hemolysis and resealing. Calcium was effective at concentrations as low as 0.5 mM. However, incubation of washed ghosts with isotonic KCl or 20 mM MgCl₂ will also produce some formation of II without Ca²⁺ addition (data not shown). Thus, calcium may not be directly involved in the protease activity, but could serve another function, such as release of the enzyme from the membrane, which is less strictly specific.

Two lines of evidence indicate that proteolysis is responsible for Band II formation. First, the formation of II is prevented by some protease inhibitors. Weise and Ingram (19) have shown that proteolytic degradation of membrane polypeptides during isolation of chick erythrocyte membranes in a calcium-containing buffer can be inhibited by bisulfite or tetrathionate. If human erythrocytes are preincubated with these agents before treatment with calcium, formation of II is inhibited (Fig. 5). The effect is not simply due to the pH decrease caused by the addition of the bisulfite, since addition of hydrochloric acid to give a similar pH change is ineffective. Our results correspond exactly to those of Weise and Ingram (19) in that bisulfite and tetrathionate inhibit the formation of II (P, by their nomenclature), but phenylmethylsulfonyl fluoride is ineffective. Second, the formation of II is directly correlated with the loss of component Ic. This can be shown over a range of calcium concentrations, times, temperatures, and with the protease inhibitors. Results of our previous studies (8, 9) also show this
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**Fig. 5.** Inhibition of formation of component II by incubation of \([^{3}H]\)acetic anhydride-labeled ghosts, 2 mM Ca\(^{2+}\), hemolysate, and inhibitors for 20 min at 37\(^\circ\)C. A, ghosts and hemolysate; B, ghosts, hemolysate, and Ca\(^{2+}\); C, ghosts, hemolysate, Ca\(^{2+}\), and 50 mM sodium bisulfite; and D, ghosts, hemolysate, Ca\(^{2+}\), and 5 mM sodium tetrathionate. Each 3.5% polyacrylamide gel contained 100 \(\mu\)g of protein. The nomenclature used for the polypeptide bands is that reported previously from our laboratory (8-11). Bands Ia, Ib, Ic, and VII correspond to Bands 1, 2, 2.1, and 5 in the system of Steck (18).

**Table II**

<table>
<thead>
<tr>
<th>Calcium added</th>
<th>(^{3}H) in II</th>
<th>dpm</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td></td>
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<tr>
<td>2 mM</td>
<td></td>
<td>340</td>
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**Fig. 6.** Formation of component II by incubation of washed ghosts with Ca\(^{2+}\) for 30 min at 37\(^\circ\)C in 10 mM Tris, pH 7.4, alone (A) or with 5 mM CaCl\(_2\) (B). Gel B contains twice as much protein as Gel A.

**Extraction of Membranes from Calcium-treated Erythrocytes**

Additional insights into the effects of calcium could be obtained by extracting membranes under conditions used for removing spectrin and actin from the membrane. Fig. 7 shows the results obtained when membranes from cells treated with high calcium concentrations are extracted. Gels A to E represent the material remaining in the membrane following extraction in pH 9.5 EDTA buffer. Gels F to J are the corresponding patterns of soluble material extracted from the membranes. Neither the protein aggregate nor component II is extracted from the membranes of the treated cells. Likewise, component Ic is not extracted from membranes of untreated cells, again emphasizing the relationship of Ic and II. The most noteworthy effect is the change in extractability of spectrin with increased calcium concentration. Essentially all of the spectrin is extracted from membranes of untreated cells, but a substantial fraction remains unextracted in membranes of the treated cells. This material has not undergone intermolecular covalent cross-linking, since it still shows the same polypeptide molecular weight. It simply shows an enhanced affinity for the membrane after the calcium treatment. The failure to extract some of the spectrin of treated membranes might
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FIG. 7. Effects of calcium addition during hemolysis on proteins of the human erythrocyte membrane. Patterns are shown for extracted residual membranes and EDTA extracts from cells hemolyzed in the presence of increasing concentrations of CaCl\textsubscript{2} in 10 mM Tris (pH 7.4). Residual membrane samples (Gels A to E) and extracts (Gels F to J) were subjected to electrophoresis on 5% acrylamide gels. Concentrations were 0, 2.0, 3.0, 4.0, and 5.0 mM Ca\textsuperscript{2+} for Gels A to E and Gels F to J, respectively.

thus be due to a failure of those samples to reach equilibrium during the extraction. It is interesting to note that there is no enhanced binding of the erythrocyte actin (VII) to the membrane.

The membrane extracts show increased quantities of two polypeptides with lower molecular weights (25,000 and 60,000). It seems likely that these are cytoplasmic proteins which adsorb to the membrane surface as a result of the calcium treatment, although it is possible that the smaller of these is the missing fragment from the Ic \rightarrow II conversion. The larger may be catalase, in view of its molecular weight and a previous report that membrane binding of catalase is enhanced by increased intracellular erythrocyte calcium (20).

Concentration Dependence of Calcium Effects—To understand the relationship of the various cellular changes to molecular events, it is necessary to separate the latter into individual processes. This can best be accomplished by studying the effects over a range of calcium concentrations. To obtain reproducible results at low calcium concentrations and to minimize the effects of extracellular agents, a different procedure for introducing calcium into the erythrocyte was investigated. As reported by Kirkpatrick et al. (4), deformability changes can be induced in erythrocytes using 5 \textmu M A23187 and calcium concentrations as low as 50 \textmu M. At high calcium concentrations the effects on membrane polypeptides are the same for the ionophore with calcium as for the hemolysis system. Although the ionophore itself causes perturbations of the erythrocyte membrane, these effects do not cause significant changes in the membrane polypeptide patterns or in extractability of spectrin. However, membrane spectrin extraction is altered if the cells are treated with ionophore and calcium, even at low calcium concentrations (Fig. 8). Higher calcium concentrations do not appear to increase the amount of unextracted spectrin. The conversion of Ic to II is also seen at low Ca\textsuperscript{2+} concentrations, but it is incomplete unless the calcium concentrations are raised to higher levels. Finally, the loss of IVa is not seen until higher concentrations are reached and appears to correlate more strongly with aggregate formation than with proteolysis, as previously suggested (8). Aggregate formation does not become important until the Ca\textsuperscript{2+} concentration approaches 0.5 mM.

DISCUSSION

The involvement of transglutaminase in the Ca\textsuperscript{2+}-promoted membrane polypeptide aggregate formation is clearly indi-
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cated by the studies reported here. Erythrocyte transglutaminase activity can be monitored by a standard assay involving putrescine incorporation (21). The responses of the erythrocyte enzyme toward a variety of divalent metal ions, substrate-type inhibitors, and sulfhydryl agents strongly resemble those of the well studied transglutaminase of guinea pig liver (15). Previous studies have indicated the ability of this latter enzyme to cross-link erythrocyte membrane proteins (16). There is a strong correlation between the activity of the erythrocyte enzyme in hemolysates and the formation of a stable protein aggregate in the membrane. This correlation extends over a variety of transglutaminase activators and inhibitors tested.

This cross-linked product is of interest in trying to understand the organization of proteins in erythrocyte membranes. Previous studies indicate that spectrin is a predominant component of the aggregate (9). The present studies indicate that IVa is also involved along with spectrin. This result suggests a closer association between IVa and spectrin than is present among the other membrane polypeptides. Whether this association is real or an artifact of the enzymic cross-linking is unknown. Likewise, it is not known whether this association is present in native erythrocytes or whether it results from Ca\(^{2+}\) perturbations.

At higher calcium concentrations or longer incubation times, a loss of Band III is also observed (8). It may also be cross-linked into the aggregate under these more strenuous conditions. It is interesting to note that no cross-linking of erythrocyte actin has been observed under any conditions tested.

The postulate of the formation of II from Ic by a proteolytic cleavage is also well supported by results using inhibitors or varied concentrations of calcium. The protease is associated with isolated erythrocyte membranes but it also may be present in hemolysates. A similar situation exists for the enzyme glyceraldehyde-3-phosphate dehydrogenase (22), which distributes between the membrane and hemolysate. This type of behavior might serve to explain the varied contents of component II observed with different membrane preparations. The specificity of this proteolytic cleavage is noteworthy, since it appears to be limited to Ic and cleaves at a specific point. Whether this is due to the specificity or localization of the protease or to the localization of Ic is unknown at present. Nothing is really known about the properties or functions of this protein. Fairbanks and Avruch (23) have shown that it (labeled 2.1 by their nomenclature) is phosphorylated by a cyclic AMP-dependent protein kinase. It may be the same polypeptide, the catecholamine-sensitive phosphorylation of which appears to regulate ion movements in avian erythrocytes (24). Further studies of the properties of this protein are clearly needed, and the changes that occur with Ca\(^{2+}\) treatment may provide an opportunity in this area. The effects of Ca\(^{2+}\) concentration on the individual processes indicate clearly that proteolysis and aggregation are not correlated. Studies with inhibitors of these processes agree with that conclusion. Proteolysis can be inhibited by bisulfite with little effect on aggregation. Aggregation can be inhibited by a number of agents with no effect on proteolysis.

The extraction studies show that introduction of calcium into erythrocytes also promotes a change in the spectrin association with the membrane and enhanced association of some smaller polypeptides. It is particularly interesting to try to correlate some of these effects with results on the decrease of cell volume and deformability (4). At low concentrations where only spectrin extractability is altered, the primary morphological effect is a volume decrease. The change in deformability encompasses the range of concentrations over which cleavage of Ic and association of the smaller polypeptides are observed. The onset of polypeptide aggregation occurs at calcium concentrations at which essentially all of the erythrocytes have lost deformability. The irreversible phase of deformability loss has a calcium concentration dependence similar to the aggregation reaction, since both require approximately 1 mM Ca\(^{2+}\). The accumulated results suggest a model to explain irreversible membrane deformability. At least two stages are necessary. The first stage occurs at low calcium concentrations, is reversible, and involves a reorganization of the membrane or a change in spectrin that alters the spectrin association with the membrane. This stage appears to correlate with loss of cell volume and may be a necessary prerequisite to deformability loss. The precise membrane changes that are involved in deformability loss are still obscure. Whether these require proteolysis or the altered spectrin association is not known. Both proteolysis and the binding of cytoplasmic proteins may be side reactions. The final stage of the irreversible deformability loss stabilizes this altered membrane organization by introducing covalent cross-links between the membrane polypeptides. In this way the membrane is essentially "fixed" into a rigid morphology.

The relationship of the protein changes described here to physiologically important events in abnormal red cells is still unclear. Riggs and Ingram (25) have shown the presence of polypeptides of $M_r = 25,000$ and $63,000$ in membranes of erythrocytes from sickle cell anemia homozygotes. A polypeptide of $M_r = 60,000$ was found in membranes from patients with hereditary spherocytosis and identified as catalase (20). It was also increased in membranes of normal cells loaded with calcium. There does not appear to be a formation of protein aggregate in the hereditary spherocytosis and sickle cells, but it might be present in irreversibly sickled cells, which are difficult to purify for analysis (26). However, it should be noted that Baker et al. (27) have shown that procaine hydrochloride can restore deformability of "irreversibly" sickled cells, so extensive cross-linking appears unlikely. The function of cytoplasmic transglutaminase, whether in erythrocytes or more complex cells (15), is still a mystery. The possibility that it might regulate cell or membrane organizational properties is intriguing (28) but in need of experimental justification.

After this work was essentially completed, a report appeared by Lorand et al. (29) indicating a correlation between protein aggregate formation in erythrocyte membranes and transglutaminase activity. The results are in complete accord with the transglutaminase studies reported here.

REFERENCES

2. LaCelle, P. L. (1969) Transfusion 9, 238-245
Calcium-promoted changes of the human erythrocyte membrane. Involvement of spectrin, transglutaminase, and a membrane-bound protease.
D R Anderson, J L Davis and K L Carraway


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