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# Hypertonic Cryohemolysis: Ionophore and pH Effects

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*Summary.* Human erythrocytes suspended at 37 °C in hypertonic solution of either electrolytes or nonelectrolytes undergo hemolysis when the temperature is lowered toward 0 °C (Green, F.A., Jung, C.Y. 1977 *J. Membrane Biol.* **33**:249). In the present studies this hypertonic cryohemolysis was profoundly affected by the pH of incubation, and was completely abolished at pH 5. In hypertonic NaCl, there was an apparent pH optimum at 6–6.5. In hypertonic sucrose, on the other hand, hemolysis increased progressively with increasing pH between 6 and 9. Amphotericin B inhibited hypertonic cryohemolysis in NaCl or KCl solution. No inhibiting effect of amphotericin B was observed when hypertonicity was due to sodium sulfate or sucrose. Valinomycin also inhibited hypertonic cryohemolysis in KCl, but did not affect the process in NaCl or sucrose solution. SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate) and phloretin interfered with this valinomycin effect, whereas phlorizin did not. These results indicate that dissipation of an osmotic gradient across membranes may be responsible for the inhibition of the hemolysis by these ionophores. Iso-osmotic cell shrinkage induced by valinomycin in 150 mM NaCl solution did not result in cryohemolysis.

In a previous communication (Green & Jung, 1977), we reported that human erythrocytes suspended in hypertonic solution at 37 °C were hemolyzed when the temperature was lowered to 0 °C. Erythrocytes were severely crenated during the incubation, and no obvious prelytic cell swelling was observed, indicating that a mechanism other than simple membrane stretching (LaCelle, 1970) may be operating in this form of hemolysis. Two distinct temperature-sensitive stages were involved, the first being incubation at some temperature above 20 °C with an increasing effect up to 45 °C, and a second stage consisting of lowering the temperature to below 15 °C with increasing hemolysis down to 0 °C. Both hypertonicity and the temperature shift were essential. Nonelectrolytes and electrolytes both induced this form of hemolysis, although the presence of ions reduced the extent of the lysis in hypertonic sucrose.

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The effect of the temperature shift in inducing this hemolysis may well be due to the phase transitions in membrane lipids (Fox & Tsukagoshi, 1972), but the precise effect of hypertonicity is not clear (Woolgar & Morris, 1973). The present study represents a continuing effort to elucidate the molecular events underlying this interesting phenomenon. The data indicate that under certain conditions ionophores such as amphotericin B and valinomycin protect the cells from this form of hemolysis. It is proposed that the ionophore-induced dissipation of the osmotic gradient across a portion of the membrane is responsible for this protective effect. The study also demonstrates that cold-induced hypertonic hemolysis is sensitive to the pH of the incubation and that the pH profile in the case of hypertonicity due to sucrose is quite different from the pH effect with sodium chloride.

## Materials and Methods

Freshly drawn human erythrocytes were washed three times in physiological saline, stored in a cold room and used within five days. Unless otherwise indicated, cells were suspended at the final concentration of 2 % v/v in the indicated hypertonic solution at pH 7.4. For the experiments where effects of pH were studied, pH of 4.9–8.6 in the incubation medium was buffered using Tris maleate and sodium hydroxide at a final concentration of 50 mM.

The standard production of hypertonic cryohemolysis used in the present study consisted of a 10 min incubation at 37 °C (stage *1*), followed by another 10-min incubation at 1 °C (stage *2*) of cell suspension, with indicated hypertonicities used throughout (Green & Jung, 1977). Hemolysis was measured by assaying hemoglobin release as detailed elsewhere (Green & Jung, 1977). Cell shrinkage was measured in terms of hematocrit of 20 % (v/v) cell suspensions, using Van-Lab capillary tubes and an Adams Microhematocrit Centrifuge.

Amphotericin B (Fungizone, Intravenous; Squibb), glutaraldehyde (Sigma), and SITS (K & K) were dissolved in the incubation medium from appropriate aqueous stock solutions. Valinomycin (Sigma), cytochalasins B and E (Aldrich), phloretin (K & K), and phlorizin (K & K) were maintained as ethanol stock solutions and added into the incubation media keeping the final ethanol content less than 1 % (v/v).

## Results

### *Effect of pH on Hypertonic Cryohemolysis*

Cryohemolysis induced by hypertonicity revealed a marked pH dependency. With hypertonic sodium chloride (Fig. 1), the extent of the hemolysis was minimal at pH 5. It increased 5- to 10-fold and gave an apparent maximal effect at pH 5.7–6.5 with an apparent half-maximum at approximately pH 5.5. The slope of this increase indicates that two

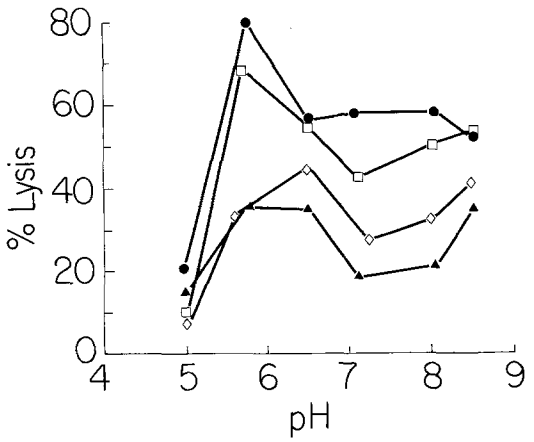


Fig. 1. Cryohemolysis of human erythrocytes in hypertonic NaCl solution, as a function of pH. Two percent (v/v) erythrocytes in the indicated hypertonic NaCl solution at varying pH buffered with 50 mM Tris maleate were incubated at 37 °C (stage *1* incubation) for 10 min then at 0 °C (stage *2* incubation) for another 10 min. Both 1.03 M (● and ◇) and 1.37 M (▲ and □) NaCl solutions were used, each symbol representing a single independent experiment

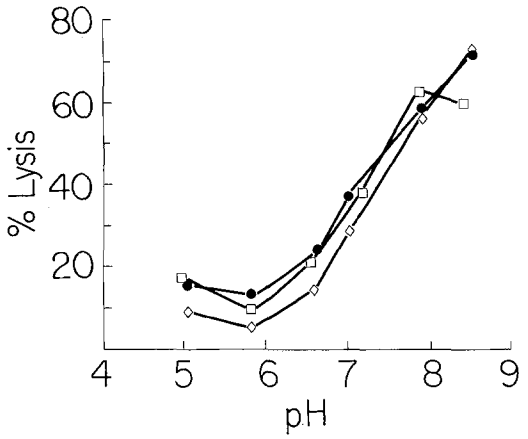


Fig. 2. Cryohemolysis in hypertonic sucrose solution, as a function of pH. Erythrocyte suspensions in Tris maleate-buffered 0.86 M sucrose solution at varying pH were incubated at 37 °C for 10 min, then at 0 °C for 10 min. Four independent experiments are shown with different symbols

dissociable protons were involved in this effect. A further increase in pH to 7.0 resulted in a significant reduction in hemolysis. There was a trend toward slightly increasing hemolysis with increasing pH between 7 and 8.5. With hypertonic sucrose (Fig. 2), the pH profile appeared to be much simpler. The hemolysis was minimal at pH between 5 and 6. It increased with increasing pH, and gave a maximal effect at pH 8–8.5 with an apparent

inflexion point at pH 7.2. The slope of the curve suggested that a single proton dissociation was involved in this effect. The cryohemolysis induced by sodium chloride thus differed from that induced by sucrose, both in its apparent pK and stoichiometry with respect to putative proton dissociation.

### *Iso-osmotic vs. Hyperosmotic Shrinkage*

Severe reduction in packed volume of erythrocyte suspension was evident during cold-induced hypertonic lysis (Table 1). Microscopic observations also revealed severe crenation and other forms of shrinkage of erythrocytes which appeared to persist up to lysis. For example, in hypertonic NaCl solutions, a significant degree of hemolysis was accompanied by 45–55% reduction in cell volume, which occurred at 1.03 M NaCl concentration. However, 0.68 M NaCl solution resulted in a 40–45% reduction in cell volume without causing any appreciable cold-induced hemolysis. Valinomycin induced iso-osmotic shrinkage of erythrocytes suspended in isotonic NaCl solution (Knauf *et al.*, 1977). This ionophore at the concentrations of $10^{-6}$ and $10^{-5}$ M, each in isotonic NaCl solutions, reduced the cell volume by 25 and 35 %, respectively. In neither case did iso-osmotic cell shrinkage result in cold-induced hemolysis (Table 1).

Table 1. Iso-osmotic and hyper-osmotic cell shrinkage and cryohemolysis

| Incubation media | First stage incubation (37°)[a] | | Second stage incubation (0°)[b] | |
|---|---|---|---|---|
| | Hematocrit (%) | Hemolysis (%) | Hematocrit (%) | Hemolysis (%) |
| 0.17 M NaCl | 20.0 | 0.3 | 20.0 | 0.8 |
| 0.68 M NaCl | 12.2 | 2.6 | 11.5 | 4.6 |
| 1.03 M NaCl | 11.2 | 3.1 | 9.3 | 22.1 |
| 1.37 M NaCl | 11.0 | 2.8 | 9.0 | 28.5 |
| 1.71 M NaCl | 11.0 | 2.6 | 10.5 | 18.4 |
| 0.17 M NaCl + $10^{-6}$ M valinomycin | 14.7 | 0.7 | 15.5 | 1.9 |
| 0.17 M NaCl + $10^{-5}$ M valinomycin | 13.3 | 1.8 | 13.0 | 3.2 |

Each value for both hematocrit and hemolysis represents the mean of duplicate determinations carried out at the same time. The variability was never greater than 10% of the average value shown in each set of determinations.

[a] At the end of first stage incubation alone.

[b] First followed by second stage incubation.

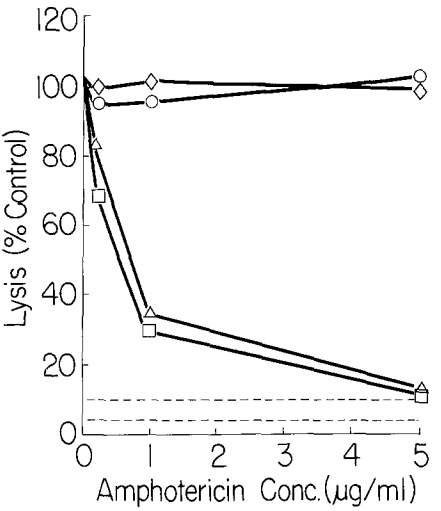


Fig. 3. Cryohemolysis in hypertonic solutions of different solutes as a function of amphotericin B concentration. Erythrocytes were suspended in phosphate buffer, pH 7.4, containing 0.86 M sucrose (○), 0.8 M $Na_2SO_4$ (◇), 1.37 M NaCl (△), and 1.03 M KCl (□), in the absence or in the presence of varying concentrations of amphotericin B. Each of these was subjected to a 10-min stage *1* incubation at 37 °C followed by a 10-min stage *2* incubation at 0 °C. Hemolysis was expressed as percent of control in which no amphotericin B was added. Dotted lines indicate overall range of hemolysis observed when the stage *2* incubation was omitted

### *Effect of Amphotericin B*

Amphotericin B at a concentration of $5 \times 10^{-6}$ M completely prevented the cryohemolysis when hypertonic sodium chloride was used (Fig. 3). This effect required the presence of the ionophore in Stage *1* incubation; amphotericin B added at the beginning of Stage *2* was much less (20 %) effective *(not shown)*. This effect was dose-dependent with a $K_i$ (an apparent concentration of amphotericin B giving half-maximal effect) of approximately 0.5 μg/ml. A similar effect was also observed with the hemolysis induced by hypertonic KCl, with a $K_i$ value slightly higher than that in hypertonic NaCl. No amphotericin B effect was observed, however, when the hemolysis was induced by hypertonic sucrose or sodium sulfate (Fig. 3). Hypertonic D-mannitol also induced cryohemolysis which could not be prevented by amphotericin B *(not shown)*.

### *Effect of Valinomycin*

Valinomycin, an ionophore specific to potassium and rubidium (Andreoli, Tieffenberg & Tosteson, 1967; Tosteson *et al.*, 1967) prevented

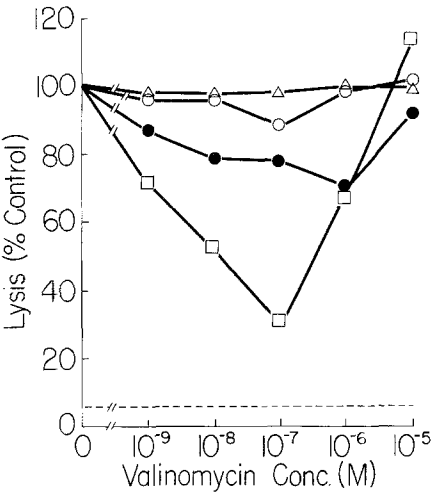


Fig. 4. Cryohemolysis in hypertonic solution of different solutes, as a function of valinomycin concentration. Erythrocytes were suspended in phosphate buffer, pH 7.4, containing 1.03 M KCl (□), 1.37 M KCl (●), 1.37 M NaCl (○), and 0.86 M sucrose (△), in the absence and in the presence of varying concentrations of valinomycin. Methodology and data presentation are otherwise identical to those in Fig. 3

hypertonic cryohemolysis when 1.03 M KCl was used (Fig. 4). This effect revealed a biphasic dose-response with respect to the ionophore. The effect required very low concentrations of ionophore, 30 % of the maximum effect being exhibited by $10^{-9}$ M valinomycin. The effect was increased with an increasing concentration of the ionophore, and reached a maximum of 70 % prevention at $10^{-7}$ M. With the valinomycin concentrations higher than $10^{-7}$ M, the effect was reversed, and the prevention of the hemolysis diminished progressively with increasing valinomycin concentration. No prevention, or occasional augmentation, of the hemolysis was observed at $10^{-5}$ M valinomycin. The extent of the protective effect of valinomcyin observed at lower concentrations is affected by changes in KCl concentration. Although a similar biphasic dose-response of the valinomycin effect was observed with the hemolysis in 1.37 M KCl, the effect was much reduced, a maximum of less than 30 % occurring at $10^{-6}$ M valinomycin. The exact nature of the valinomycin effect is not clear, and one can speculate that two independent processes may be involved, but of opposing directions and partly overlapping in the concentration range between $10^{-8}$ and $10^{-7}$ M valinomycin. Valinomycin at even higher concentrations did not produce hemolysis when the cold incubation was omitted *(not shown)*, indicating that this possible lytic effect of the ionophore at higher concentrations is

also specific for the temperature shift. A valinomycin effect was minimal, if any, when cryohemolysis was induced in 1.37 M sodium chloride (Fig. 4). With 1.03 M sodium chloride, the ionophore was also totally ineffective in preventing hemolysis *(not shown)*. Valinomycin was also without effect on the cryohemolysis in hypertonic sucrose solution (Fig. 4).

### *Effects of SITS, Phlorizin and Phloretin*

SITS, an inhibitor of anion flux in human erythrocytes (Knauf & Rothstein, 1971), exerted no effect at concentrations up to $10^{-5}$ M on the cryohemolysis induced by hypertonic KCl, NaCl, or sucrose (Table 2). Phlorizin and phloretin are also known to affect the anion movement in human erythrocytes (Lepke & Passow, 1973; Wieth *et al.*, 1973; Kaplan & Passow, 1974). Neither of these biphenolic compounds affected cryohemolysis in 1.03 M KCl (Table 2). Phloretin, but not phlorizin, however, reduced the protective effect of valinomycin on hypertonic cryohemolysis (Fig. 5).

Table 2. Effects of membrane-reacting reagents on cryohemolysis

| Reagents | Suspension media | Hemolysis (percent) | | | |
|---|---|---|---|---|---|
| | | After stage 1 | | After stage 2 | |
| | | Without reagent | With reagent | Without reagent | With reagent |
| Glutaraldehyde (0.1 %) | 1.37 M NaCl | 4.1 | 2.8 | 58.4 | 2.8 |
| | 0.98 M Sucrose | 4.7 | 3.1 | 60.4 | 0.7 |
| (1.0 %) | 1.37 M NaCl | 3.8 | 1.2 | 57.6 | 0.9 |
| | 0.98 M Sucrose | 4.6 | 0.6 | 61.3 | 0.3 |
| Cytochalasin B ($10^{-5}$ M) | 0.17 M NaCl | 0.7 | 0.8 | 1.5 | 1.3 |
| | 1.37 M NaCl | 2.2 | 2.1 | 30.5 | 26.8 |
| Cytochalasin E ($10^{-5}$ M) | 0.17 M NaCl | 0.6 | 1.2 | 2.2 | 1.8 |
| | 1.37 M NaCl | 1.8 | 1.6 | 33.5 | 29.8 |
| SITS ($10^{-5}$ M) | 1.37 M NaCl | 1.8 | 1.8 | 36.7 | 32.3 |
| | 1.03 M KCl | 1.9 | 2.2 | 36.5 | 37.1 |
| | 0.98 M Sucrose | 3.3 | 2.9 | 61.3 | 58.4 |
| Phloretin ($10^{-4}$ M) | 1.03 M KCl | – | – | 28.6 | 34.2 |
| ($10^{-3}$ M) | 1.03 M KCl | – | – | – | 35.5 |
| Phloridzin ($10^{-4}$ M) | 1.03 M KCl | – | – | 35.8 | 40.1 |
| ($10^{-3}$ M) | 1.03 M KCl | – | – | – | 33.8 |

Each reagent was added prior to stage *1* and allowed to remain through stages *1* and *2*. Each value represents an average of three to four determinations, which varied not more than ±12 % from average value. The same batch of erythrocytes was used for each reagent.

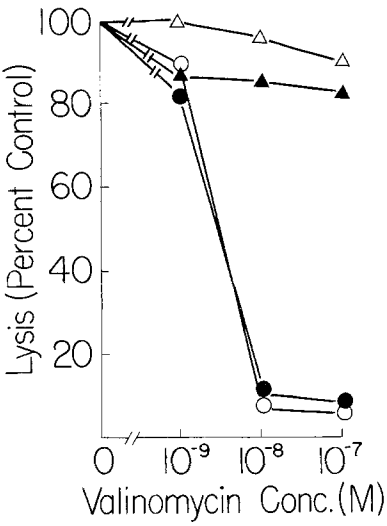


Fig. 5. Effect of SITS, phlorizin, and phloretin on cryohemolysis in hypertonic KCl solution in the presence of varying concentrations of valinomycin. Erythrocytes were suspended in 1.03 M KCl solution, pH 7.4, buffered with 50 mM phosphate containing specified concentrations of valinomycin, in the absence (●) or in the presence of $4 \times 10^{-4}$ M SITS (△), $10^{-3}$ M phlorizin (○), and $10^{-3}$ M phloretin (▲). Procedures and data presentation were otherwise as in Fig. 3

SITS showed a similar effect under the identical experimental conditions (Fig. 5), but these results were not entirely reproducible.

### *Effect of Other Membrane-Reacting Reagents*

Cytochalasin B, an inhibitor of cytokinetic functions (Wessells *et al.*, 1971), is also known to increase susceptibility to hypotonic lysis as well as to decrease erythrocyte deformability (Beck, Jay & Saari, 1972). Neither cytochalasin B nor E at concentrations of $10^{-5}$ M in isotonic NaCl solution induced cryohemolysis (Table 2). We have previously shown that colchicine ($10^{-6}$ M) was not effective (Green & Jung, 1977). Glutaraldehyde effectively prevents cold-induced hypertonic hemolysis (Table 2). This effect is most likely due to cross-linking of membrane lipids and proteins (Vassar *et al.*, 1972).

## Discussion

The magnitude of cold-induced hypertonic hemolysis is very pH-dependent (Figs. 1 and 2). The pH-profile of cryohemolysis in hypertonic

sucrose and that in hypertonic NaCl are significantly different, suggesting that molecular events underlying cryohemolysis in these two different solutes may be different at least in one stage in its mechanism. In the case of sucrose, the pH-profile is strikingly similar to the pH-profile reported for the release of hemoglobin (Mitchell *et al.*, 1965; Jung, 1971) from human erythrocyte membranes at low ionic strength or spectrins (Hoogeveen *et al.*, 1970; Bennet & Branton, 1977). The release of bulk membrane proteins, on the other hand, is known to be much less sensitive to pH (Mitchell, Mitchell & Hanahan, 1965). Membrane-bound hemoglobin as well as spectrins have been considered as key structural components in maintaining membrane integrity (Ponder, 1955; Weed, Reed & Berg, 1963).

In the case of hypertonic NaCl, the pH-profile of the cold-induced hemolysis showed a pH-maximum at approximately 6. The general pattern of this pH-profile is reminiscent of that for anion movement across human erythrocyte membranes (Dalmark, 1975). Both the sulfate and chloride exchange flux in erythrocytes are strongly pH-dependent and exhibit pH-maxima at pH 6.2 and 7.8, respectively, the exact position of these pH-maxima as well as the shape of the pH-profile being known to be affected by experimental conditions (Dalmark, 1975, 1976). It may be interesting to examine whether anion movement (net or exchange) across the erythrocyte membrane in hypertonic NaCl or sucrose exhibits pH-maxima similar to that for hemolysis.

Amphotericin B protects the cells from cold-induced lysis in hypertonic NaCl and KCl. It is, on the other hand, not effective in preventing the hemolysis in hypertonic sodium sulfate, mannitol, or sucrose (Fig. 3). Amphotericin B induces channels which are known to be permeable to $Na^+$, $K^+$, $Rb^+$, and $Cl^-$, but are virtually impermeable to sulfate, mannitol, or sucrose (Andreoli, Dennis & Weigl, 1969). It is thus apparent that the ionophore prevents the cold-induced hypertonic hemolysis only with those solutes to which the drug-induced channel is permeable and that the movement of both cations and anions is required.

The results of the valinomycin experiment also support this conclusion. Valinomycin inhibits cold-induced hemolysis in hypertonic KCl without affecting the hemolysis in NaCl or sucrose (Fig. 4). This reagent specifically increases $K^+$ permeability of erythrocytes, its effect on $Na^+$ permeability being negligible by comparison (Tosteson *et al.*, 1967; Andreoli *et al.*, 1967). Valinomycin does not increase anion permeability (Simon & Morf, 1973). With increasing concentrations of valinomycin up to $10^{-7}$ M the net KCl flux increases due to increasing $K^+$ permeability, but reaches a value which itself becomes limited by net $Cl^-$ movement, at approximately $10^{-6}$ M

valinomycin (Knauf *et al.*, 1977). The inhibition by valinomycin of the cold-induced hypertonic hemolysis in KCl solution (Fig. 4) correlates well with expected ionophore-induced KCl movement.

If the valinomycin effect is indeed secondary to the KCl movement, agents which affect net $Cl^-$ flux and thus the ionophore-induced KCl movement would be expected to alter the valinomycin effect on hemolysis. SITS is known to inhibit net $Cl^-$ movement (Knauf *et al.*, 1977). In the present study, SITS interfered with the valinomycin effect (Fig. 5), as might be predicted. The effect of phloretin in reducing the protective effect of valinomycin (Fig. 5), on the other hand, cannot be fully evaluated by this reasoning, since the effect of phloretin on net $Cl^-$ movement is not well documented (Wieth *et al.*, 1973). The lack of any significant effect of phlorizin except for possible slight enhancement on the valinomycin effect may be due to the fact that this compound enhances net $Cl^-$ movement (Kaplan & Passow, 1974). These results suggest that dissipation of the salt gradient across membranes may be responsible for the inhibition of cold-induced hemolysis by these ionophores.

The exact role of hypertonicity in cryohemolysis is not clear and one can only speculate at this moment. One obvious consequence of hypertonic incubation is osmotic cell shrinkage. Some 40–50 % volume reduction was observed under conditions leading to hypertonic cryohemolysis (Table 1). This shrinkage may produce abrupt folding of membranes with localized changes in surface force (Kregenow, 1971; Poznansky & Solomon, 1972). This shrinkage also implies approximately 70 % cell water loss. Neglecting some possible salt leak, this would cause a large (about threefold) increase in intracellular ionic strength at the initial stage of cell shrinkage. Amphotericin B and valinomycin would reduce cell shrinkage by dissipating the osmotic gradient across membranes in hypertonic solution of appropriate solutes. This could reduce the membrane folding or moderate the increase in intracellular ionic strength, thus preventing hypertonic cryohemolysis. It is tempting to suggest, as a working hypothesis, that this severe cell shrinkage may dislocate or disarrange certain membrane proteins such as the actin-spectrin system, which is thought to play a key role in maintaining material-like properties of cell membrane (Evans & Hochmuth, 1977; Bennett & Branton, 1977).

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