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Inhibition of Erythrocyte Membrane Shape Change by Band 3 Cytoplasmic Fragment

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The ATP-dependent transformation of crenated white human erythrocyte ghosts into smoothed disc and cup forms is inhibited by the soluble 40–45-kilodalton (kDa) cytoplasmic portion of the major transmembrane protein, band 3. The band 3 fragment was prepared by chymotryptic treatment of inverted vesicles stripped of peripheral proteins. When present at $\geqslant 0.2$ mg per mg membrane protein (ie, $\geqslant 2$ mol fragment per mol endogenous band 3), the fragment significantly reduced the rate of shape change but did not alter the proportion of membranes that were ultimately converted into smoothed forms ($>90\%$). The inhibitory activity of the fragment could not be attributed to contamination of the fragment preparation by actin or proteolytic enzymes. ATP-independent shape transformation was not inhibited. The band 3 fragment may compete with endogenous, intact band 3 for an association with the spectrin-actin network required for ATP-dependent smoothing of crenated membranes.

Key words: human erythrocyte, shape, band 3, ATP, membrane

Isolated erythrocyte membranes can undergo shape transformations that mimic those observed in intact red cells, where biconcave discs (discocytes) become reversibly crenated (echinocytes) when depleted of metabolic energy stores. Fresh white ghosts produced by hypotonic lysis crenate when the salt concentration is raised but can be induced to revert to a discoid shape in the presence of MgATP at 20–40°C [1,2]. This shape transformation from echinocyte to discocyte in Tris ghosts is dependent upon the hydrolysis of ATP, since nonhydrolyzable analogs of ATP do not support shape change [3–6]. Furthermore, the phosphorylation of spectrin or of phospholipid precursors appears to be neither necessary nor sufficient for shape change [7]. It has been suggested that an ATPase is involved [5,8–11], but the location and mechanism of such an enzyme remain unknown.

Methods for the purification of proteolytic fragments of several erythrocyte membrane proteins have been described, and these fragments have been used successfully to identify specific protein interactions in the membrane [12–14]. We are

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attempting to analyze the mechanism of erythrocyte shape change by observing the effect of such polypeptide fragments on this process. We show here that the progression of echinocyte to discocyte shape transformation is inhibited by the polypeptide portion of band 3 that is involved in the attachment of the submembrane reticulum to the bilayer [13]. These results implicate the bilayer-reticulum interaction in the maintenance of erythrocyte shape.

METHODS

Membrane Preparation

Human venous blood was withdrawn into a plastic syringe and heparinized (10 USP U/ml, final concentration). The blood was passed through a pad of a 1:1 (w/w) mixture of alpha-cellulose and Sigmacell (Sigma); for 3–5 ml blood, the pad was typically 1 cm thick, constructed between two pieces of 4.25-cm Whatman 541 filter paper in a 4.5-cm-diameter Buchner funnel. The red cells were eluted from the cellulose with Hank's balanced salt solution (calcium- and magnesium-free). This filtration depletes the blood of >95% of the white cells [15]. Tris and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) ghosts were produced as described [7] with the inclusion of 0.25 mM dithioerythritol (DTE) in all buffers.

For production of band 3 fragment, membranes were prepared from a unit of outdated human blood. The blood was filtered through cellulose as described above and the erythrocytes washed three times in 0.15 mM NaCl, 20 mM sodium phosphate (pH 8.0), 0.25 mM DTE. The packed cells were hemolysed by a rapid 1:40 dilution into 5 mM sodium phosphate (pH 8), 0.25 mM DTE containing 0.05 mg phenylmethylsulfonylfluoride (PMSF) per ml (added as 20 mg PMSF/ml in isopropanol) at 0°C. The membranes were washed free of cytoplasmic solutes in a Pellicon Cassette System using 0.5-μm Durapore filters (Millipore Corp) with 5mM sodium phosphate (pH 8.0) and 0.25 mM DTE containing 0.01 mg PMSF/ml. This 30-min washing procedure produces 200-ml packed ghosts without detectable structural or functional debilitation (data not shown; see [16]).

Preparation of Band 3 Fragment

Cytoplasmic fragments of Band 3 (CF3) were prepared as previously described [13] with the omission of the final gel chromatography step. CF3 preparations contained mainly (87–96%) three polypeptides of 40–45 kDa, with the larger polypeptide predominating (Fig. 1).

Treatment of membranes with Band 3 fragment. Packed Tris or HEPES ghosts diluted with one-fourth volume of 10 mM Tris or HEPES (pH 7.4) were mixed with an equal volume of CF3 in 0.15 M NaCl, 10 mM sodium phosphate (pH 7.4), or with buffer alone. Portions of the suspension were then placed on 25-mm discs of filter-type VSWP (Millipore Corp) and the salt was removed by floating the filters with stirring over 10 mM Tris (pH 7.4), 0.25 mM DTE, for 1–3 hr at 0°C. In one experiment, CF3 was replaced by an equivalent weight of either alpha-1-antitrypsin or alpha-1-acid glycoprotein (Sigma).

Shape-Change Assay

Ghost suspensions from the VSWP filters were diluted with a solute mixture at 0°C to yield (final concentration) 125 mM NaCl, 15 mM Tris (pH 7.4), 2 mM

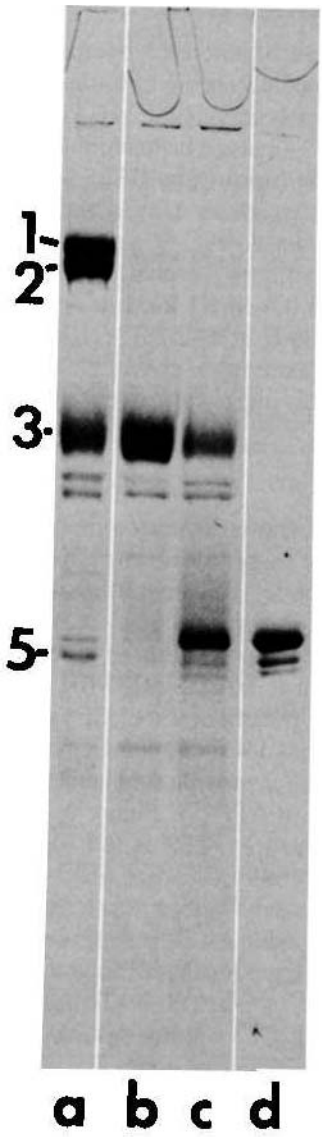

Fig. 1. Electrophoretic analysis of the preparation and purification of the band 3 cytoplasmic fragment (CF3): a), white ghosts; b), acid-stripped vesicles; c), vesicles after digestion with chymotrypsin; d), final CF3 preparation.

magnesium chloride, 0.2 mM ethylene glycol bis (beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.5 mM DTE, and with or without 2mM ATP. [In some samples, sodium orthovanadate (Fisher) was added at 0.01 mM final concentration.] After 3 min the tubes were plunged into a water bath at 30°C. Portions were removed at various times after the temperature elevation and mixed with 3 vol of 1.25% glutaraldehyde, 0.15 M NaCl, and 10 mM sodium phosphate (pH 7.0) at 0°C. The shapes of the fixed membranes were assayed by dark field microscopy as previously described [7].

Other Procedures

Tryptic peptide maps were generated by the technique of Elder et al [17]. Autoradiography was performed according to Laskey and Mills [18].

Sodium dodecyl sulfate-polyacrylamide gels [19] were cast as a 6–12% gradient of acrylamide and stained in Coomassie brilliant blue.

Competition of ankyrin binding by CF3. Membrane vesicles were prepared by low-tonicity extraction of Tris ghosts [13]; a high proportion (>50%) of vesicles prepared in this manner are inside-out. Vesicles were incubated with CF3 in the presence of 1 mM diisopropylfluorophosphate (DFP) in 0.15 M NaCl and 10 mM Tris (pH 7.4) with or without 0.5 M KI for 1 hr at room temperature. The vesicles were then sedimented at 114,000g in a 42.2 Ti rotor (Beckman Instr) for 30 min and the supernatants were electrophoresed as above, the stained gels scanned (Helena Lab Corp), and the ratio of the areas under the curve for bands 2.1 and 3 was determined.

RESULTS

The addition of CF3 to ghosts incubated in shape-change medium slowed the rate of echinocyte-to-discocyte shape transformation in a dose-dependent fashion (Fig. 2). The highest concentration of fragment (0.6 mg CF3/0.1 ml packed ghosts) increased the time of 50% conversion ($t_{1/2}$) by a factor of 2.2 ($t_{1/2}$ = 11 min with fragment, 5 min without), but the proportion of ghosts that ultimately changed shape (>99%) was not altered. Even at 0.075 mg CF3/0.1 ml packed ghosts (corresponding to 0.2 mg CF3/mg membrane protein or 2 mol CF3 per mole endogenous band 3) the $t_{1/2}$ of shape change (7 min) was 1.4 times greater than without CF3 (Fig. 2).

Hemolysis in HEPES buffer yields a subpopulation of ghosts that undergo shape transformation in the absence of ATP [7]. Figure 3 shows that this ATP-independent transformation was not inhibited by CF3 at 0.2 mg/0.1 ml packed ghosts (in this experiment a slight enhancement by CF3 was observed). In the presence of ATP an additional 40% of ghosts changed shape at a rate that was reduced by CF3. HEPES ghosts vary in the capacity to exhibit ATP-independent shape transformation, but the effect of CF3 was always selective for the increment in shape change requiring ATP.

Shape change dependent on MgATP is eliminated by 0.01 mM sodium orthovanadate [11]. Incubation with CF3 did not reverse vanadate inhibition and did not alter the basal level (<10%) of ghosts that are discoid after incubation in the presence of vanadate because they do not crenate or undergo shape change in the absence of MgATP.

The effect of the fragment was not mimicked by alpha-1-antitrypsin or alpha-1-acid glycoprotein, proteins that resemble the band 3 cytoplasmic fragment in acidic character. Electrophoretic analysis of ghosts incubated with CF3 did not reveal any increased cleavage of high molecular weight components. Furthermore, the ability of the fragment to slow shape transformation was not significantly affected by treatment with PMSF or DFP (Table I). Thus, the effect of the fragment on shape change cannot be attributed to contaminating chymotryptic activity.

One of the three CF3 components routinely obtained in the fragment preparations migrates electrophoretically with erythrocyte actin (Fig. 1). Because actin can inhibit shape transformation [20], the 43-kDa bands of erythrocyte actin and the CF3 preparation were radioiodinated and compared by high-resolution peptide mapping [17]. The maps did not appear to be related (patterns not shown). In order to estimate

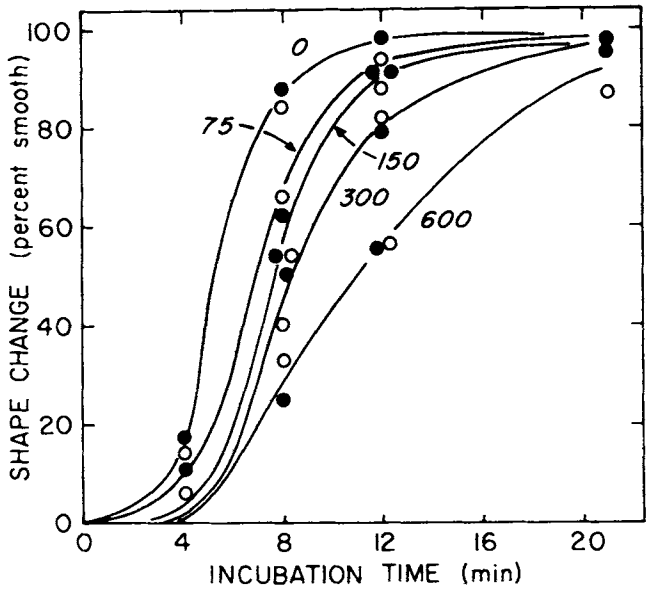

Fig. 2. Effect of CF3 on ATP-dependent shape transformation of Tris ghosts. The time course of shape change is plotted for incubation with CF3 at each of the concentrations indicated (μg CF3/0.1 ml packed ghosts). Open and filled circles distinguish the independent results of two observers.

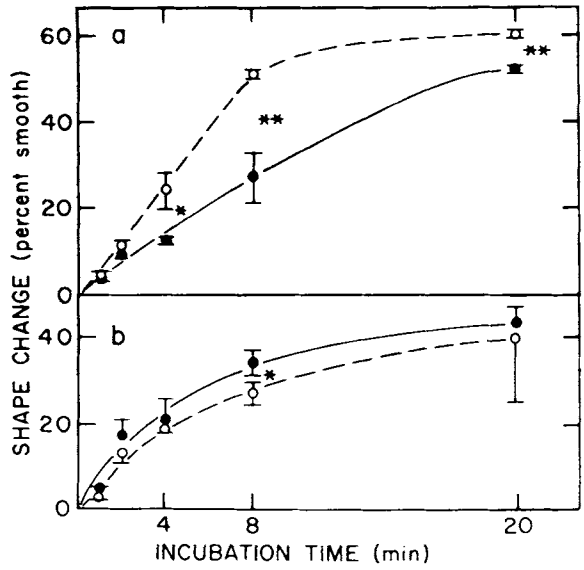

Fig. 3. Selective effect of CF3 on ATP-dependent shape transformation of HEPES ghosts. Ghosts were incubated with CF3 at 200 μg CF3/0.1 ml packed ghosts (filled circles) or without CF3 (open circles), and with ATP (a) or without ATP (b). Asterisks indicate differences in triplicate determinations found significant at $P < .05$ (*) or $P < .01$ (**) by the two-sample t-test.

TABLE I. Inhibition of Shape Transformation by CF3 in the Presence of Inhibitors of Proteolysis

Experiment	CF3	Inhibitor	Shape change (% smooth)
1[a]	–	–	62
	–	PMSF	72
	+	–	45
	+	PMSF	46
2[b]	–	–	60
	–	DFP	58
	+	–	23
	+	DFP	30

[a]Experiment 1: Tris ghosts were incubated with or without CF3 (0.3 mg/0.1 ml packed ghosts) and with or without PMSF (0.05 mg/ml). After removal of salt (see Methods), the ghosts were incubated for 30 min under standard shape-change conditions with additional PMSF as indicated.
[b]Experiment 2: CF3 at 2 mg/ml was incubated at room temperature with or without 1 mM DFP in 0.15 mM NaCl, 10 mM sodium phosphate (pH 7.4); three additions of DFP from a stock solution of 0.1 M DFP in propylene glycol or additions of solvent alone were made at intervals for 1 wk. The solutions were dialyzed against 0.15 M NaCl and 10 mM Tris (pH 7.4) before incubation with membranes (0.2 mg CF3/0.1 ml packed ghosts); shape change was assayed after 7 min under standard conditions.

the minimal level of the hypothetical contamination with actin, the positions of four characteristic actin peptides were located in lacunae on the autoradiographs of the CF3 map and each area was scanned with a microdensitometer. Comparison of the optical density at each position with the optical density at the corresponding spot on the map of a 1:1 mixture of actin and CF3 revealed that actin contamination could not have exceeded 0.1%. It is highly unlikely that traces of actin in CF3 could account for the observed inhibition of shape change.

The other major CF3 component (at 45 kDa; see Fig. 1) yielded a peptide map that was virtually identical to that of the 43-kDa component, and, as above, contamination by actin-related peptides was not detectable.

Electrophoretic analysis of the CF3 preparations revealed the presence of smaller polypeptides representing 4–13% of the staining profiles. Inhibitory activity was not correlated with the level of contamination by these fragments, and the preparation with the greatest enrichment (96%) in the 40–45-kDa components was fully active.

The cytoplasmic portion of band 3 is an attachment site of the erythrocyte submembrane reticulum to the hydrophobic domain of the membrane continuum. This attachment appears to be mediated through ankyrin (band 2.1) because the band 3 cytoplasmic fragment will compete for reassociation of solubilized ankyrin with ankyrin-depleted membrane vesicles [13]. The band 3 fragment will also remove some of the endogenous ankyrin from spectrin-depleted vesicles. As shown in Figure 4, the proportion of band 2.1 retained by vesicles incubated under shape-change conditions decreased as the level of added CF3 was increased; the effect of CF3 was enhanced when the incubation was performed with addition of 0.5 M KI, which destabilizes the ankyrin-membrane interaction.

This experiment was performed in the presence of DFP to prevent proteolysis of the easily degraded ankyrin (see Methods).

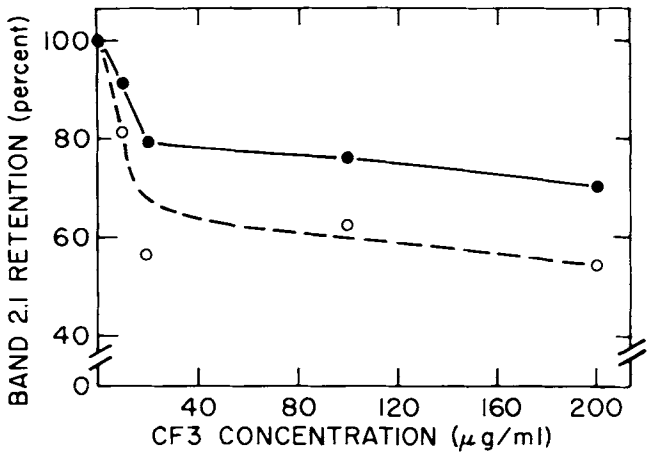

Fig. 4. Displacement of band 2.1 from membrane vesicles as a function of CF3 concentration. Band 2.1 retained on the membrane is expressed as percentage of control levels measured for incubation controls simultaneously incubated without CF3. Incubation was performed in Tris-buffered saline (see Methods) with 0.5 M KI (open circles) or without (closed circles).

DISCUSSION

Inhibition of shape change by CF3 is a reproducible phenomenon directed selectively at the process of active membrane smoothing involving the utilization of MgATP. Several less-interesting possibilities were examined and excluded by control experiments. The effect is not a proteolysis artifact, because the fragment did not promote membrane protein degradation, and its activity was not diminished by rigorous treatment with proteolysis inhibitors. The inhibition cannot be attributed to actin contamination, because the fragment was prepared from membranes stripped of most peripheral proteins and no actin-related peptides could be detected in peptide maps of CF3. It is also unlikely that the fragment acts nonspecifically (for example, by increasing the membrane's passive resistance to contour change) because other acidic polypeptides had no effect and ATP-independent shape change was not inhibited.

Tris ghosts remain permeable to ^{125}I-hemoglobin when held on ice. Thus, CF3 was introduced under conditions that allowed its penetration into ghosts and interaction with the cytoplasmic surface of the membrane. Resealing during shape change incubation is variable [21], but, in some experiments, ghosts washed after incubation retained traces of the fragment. We assume that the locus of action of CF3 is at the cytoplasmic surface, and we postulate, in general terms, that the inhibitory activity is related to the function of Band 3 in anchoring the spectrin-actin network through the association of the Band 3 cytoplasmic segment with ankyrin [13]. The displacement of ankyrin from membrane vesicles (Fig. 4) is a vestige of this function retained by the isolated cytoplasmic fragment.

Geometric constraints dictate that the conversion of a closed shell from crenated to smooth discoid form be accompanied by an overall reduction in positive curvature

and a slight expansion of the inner surface relative to the outer surface [22–24]. Which of the laminar elements of the red cell membrane plays an active role in MgATP-dependent membrane smoothing is not known. It has been conjectured that the spectrin-actin network and associated proteins possess mechanochemical activity [25,26]. However, although isolated membrane "skeletons" shrink dramatically when the ionic strength is elevated and this shrinkage can be reversed readily by reducing the ionic strength, no ATP-dependent volume changes of skeletons incubated at constant ionic strength have been observed [27]. The primary determinant of membrane contour might also reside in the bilayer domain [28]. We and others have suggested that ATP-dependent shape change might be driven by lipid phosphorylation reactions that increase the density of negative charge at the inner surface of the bilayer [6,29,30]. This possibility has been excluded, however, by recent results in which inhibitors and membrane extractions were used to manipulate shape change and phosphorylation independently [6,11,31].

There need not be a unitary mechanism, or single common path, for all of the modalities of red cell and ghost shape transformation. In the process of salt-induced crenation, both condensation of the submembrane reticulum [27] and changes in energetics of bilayer curvature [22,28,32] might contribute. As spectrin binding to the overlying membrane is prompted by increasing ionic strength [33], anchorage of the condensed reticulum could trap the membrane in the crenated state. We propose that ATP-dependent shape change involves an enzymatic apparatus (a putative "shape change ATPase" [10,11,31])-that utilizes MgATP to accelerate exchanges of spectrin-anchoring interactions. This would allow the network to reorganize to a slightly more extended configuration closer to that of the native membrane prior to its disturbance by hypotonic hemolysis. The transition to this state could be driven by an increase in the number of anchoring interactions allowed as relaxation of the spectrin-actin network reduces steric hindrance.

In this context the inhibitory effect of exogenous CF3 can be understood in terms of its competition with endogenous band 3 for binding to ankyrin. The model outlined above predicts that this competition would reduce the rate of the transition; but, because of the higher affinity of ankyrin binding to intact band 3 in situ [13], the endogenous interactions would eventually prevail to produce the smoothed contour of the membrane. The model predicts that ATP-dependent shape change will also be inhibited by ankyrin fragments bearing the band 3 binding site and by antibodies directed against CF3 and ankyrin. Preliminary observations indicate that affinity-purified antibodies to CF3 indeed mimic the effect of CF3 in retarding shape change [21]. The other possibilities are also under investigation.

It has been reported that antispectrin antibodies and G-actin can also inhibit ATP-dependent shape change [4,20]. This does not imply that the spectrin-actin network itself can perform the primary reactions that induce membrane smoothing, because these probes are known to engage in multivalent interactions within the network and may inhibit by introducing passive resistance to expansion of the inner surface. It is unlikely that the monovalent band 3 fragment interacts with the membrane in this fashion. Our observations thus direct attention to the possibility that in the isolated membrane, and perhaps in the intact red cell, surface contour is controlled metabolically through the dynamics of spectrin-anchoring interactions that define the overall composite structure.

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REFERENCES

1. Nakao M, Nakao T, Yamazoe S: Nature 187:945, 1960.
2. Weed RI, LaCelle PL, Merrill EW: J Clin Invest 48:795, 1969.
3. Hayashi S, Plishker GA, Vaughan L, Penniston JT: Biochim Biophys Acta 382:218, 1975.
4. Sheetz MP, Singer SJ: J Cell Biol 73:638, 1977.
5. Quist EE: Arch Biochem Biophys 203:203, 1980.
6. Fairbanks G, Patel V, Dino JD: Scand J Clin Lab Invest 41 (Suppl 156):135, 1981.
7. Patel VP, Fairbanks G: J Cell Biol 88:430, 1981.
8. Hayashi H, Jarrett HW, Penniston JT: J Cell Biol 76:105, 1978.
9. Schrier SL, Hardy B, Bensch KG: In Lux SE, Marchesi VT, Fox CF (eds): "Normal and Abnormal Red Cell Membranes," Vol 30. New York: Alan R. Liss, Inc., 1979, p 437.
10. Sheetz MP, Sawyer D, Jackowski S: In Brewer GJ (ed): "The Red Cell." New York: Alan R. Liss, Inc., 1978, p 431.
11. Fairbanks G, Patel V, Dino JD, Carter DP: Fed Proc 41: 513, 1982.
12. Bennett V: J Biol Chem 253:2292, 1978.
13. Bennett V, Stenbuck PJ: J Biol Chem 254:2533, 1979.
14. Morrow JS, Speicher DW, Knowles WJ, Hsu CJ, Marchesi VT: Proc Natl Acad Sci USA 77:6592, 1980.
15. Beutler E, West C, Blume K-G: J Lab Clin Med 88:328, 1976.
16. Rosenberry TL, Chen JF, Lee MML, Onigman P: J Biochem Biophys Methods 4:39, 1981.
17. Elder JH, Pickett RA, Hampton J, Lerner RA: J Biol Chem 252:6510, 1977.
18. Laskey RA, Mills AD: FEBS Lett 82:314, 1977.
19. Laemmli UK: Nature 227:680, 1970.
20. Birchmeier W, Singer SJ: Biochem Biophys Res Commun 77:1354, 1977.
21. Carter DP, Fairbanks G: (Unpublished observations.)
22. Sheetz MP, Singer SJ: Proc Natl Acad Sci USA 71:4457, 1974.
23. Beck JS: J Theor Biol 75:487, 1978.
24. Plishker GA, Vaughan L, Jarrett HW, Reid T, Roberts JD, Penniston JT: In Bolis L, Hoffman JF, Leaf A (eds): "Membranes and Disease." New York: Raven Press, 1976, p 19.
25. Kirkpatrick FH: Life Sci 19:1, 1976.
26. Sheetz MP, Painter RG, Singer SJ: In Pollard TD, Rosenbaum J, Goldman R (eds): "Cell Motility." New York: Cold Spring Harbor Laboratory, 1976, p 651.
27. Johnson RM, Taylor G, Meyer DB: J Cell Biol 86:371, 1980.
28. Lange Y, Gough A, Steck TL: J Membr Biol 69:113, 1982.
29. Allan D, Thomas P, Michell RH: Nature 276:289, 1978.
30. Quist EE, Reece KL: Biochem Biophys Res Commun 95:1023, 1980.
31. Patel V, Fairbanks G: (Manuscript in preparation.)
32. Johnson RM, Robinson J: Biochem Biophys Res Commun 70:925, 1976.
33. Bennett V, Branton D: J Biol Chem 252:2753, 1977.