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Ion Modulation of Membrane Permeability: Effect of Cations on Intact Cells and on Cells and Phospholipid Bilayers Treated with Pore-Forming Agents

C. Lindsay Bashford, Glenn M. Alder, John M. Graham, Gianfranco Menestrina,† and Charles A. Pasternak

Department of Biochemistry, St. George's Hospital Medical School, Cranmer Terrace, London SW17 ORE, United Kingdom and †Department of Physics, University of Trento, 380 50 Povo (TN), Italy

Summary. Leakage of ions (Na^+, K^+) and phosphorylated metabolites (phosphorylcholine, 2-deoxyglucose 6-phosphate) through membrane lesions in intact cells or in cells modified by 'pore-forming' agent has been studied. Leakage from intact cells is *induced* by protons and by divalent cations such as Cu^{2+}, Cd^{2+} or Zn^{2+}. Leakage from agent-modified cells—or across phospholipid bilayers modified by agent—is *prevented* by low concentrations of the same cations and by higher concentrations of Ca^{2+}, Mn^{2+} or Ba^{2+}; Mg^{2+}, dimethonium, spermine, or spermidine are virtually ineffective. The relative efficacy of a particular cation (e.g. Ca^{2+}) depends more on cell type than on the nature of the pore-forming agent. The predominant effect is on binding of cation to specific sites, not on surface charge. Surface charge, on the other hand, does affect leakage from agent-modified cells in that suspension in nonionic media reduces leakage, which can be restored by increasing the ionic strength: univalent (Na^+, K^+, Rb^+, NH_4^+) and divalent (Mg^{2+}, dimethonium) cations are equally effective; addition of protons or divalent cations such as Zn^{2+} to this system inhibits leakage. From this and other evidence here presented it is concluded that leakage across membranes is modulated by the presence of endogenous anionic components: when these are in the ionized state, leakage is favored; when unionized (as a result of protonation) or chelated (by binding to divalent cation), leakage is prevented. It is suggested that such groups are exposed at the extracellular face of the plasma membrane.

Key words divalent cations · membrane permeability · planar bilayers · plasma membrane · pore-formers · protons (pH) · toxins

Introduction

The actions on cells of agents as diverse as Sendai virus, *Staphylococcus aureus* α-toxin, melittin and other cationic proteins, activated complement, or detergents below their critical micellar concentration, have certain features in common (Esser et al., 1979; Bashford et al., 1984; Pasternak et al., 1985*a*,*b*; Esser, 1986). Pores with an average functional diameter of approximately 1 to 2 nm (Sims & Lauf, 1980; Wyke et al., 1980; Ramm, Whitlow & Mayer, 1985) are induced at low concentrations of agent, as a result of which intracellular ions and low molecular weight metabolic intermediates leak out (Pasternak & Micklem, 1973; 1974*a*), and extracellular ions like Na^+ (Poste & Pasternak, 1978) and Ca^{2+} (Volsky & Loyter, 1978; Impraim, Micklem & Pasternak, 1979; Campbell et al., 1981; Suttorp et al., 1985), or compounds such as α-methylglucoside (Pasternak & Micklem, 1974*b*; Impraim et al., 1980) and sucrose (Sims & Lauf, 1978; Ramm, Whitlow & Mayer, 1982) leak in (the word "pore" is used in a purely operational sense to imply some kind of membrane deformity that allows leakage of water-soluble compounds across the plasma membrane). Proteins do not leak in or out, except at high agent: cell ratio (Yamaizumi, Uchida & Okada, 1979; Bashford, Micklem & Pasternak, 1985): the agents are hemolytic because erythrocytes are less able to withstand the osmotic pressure induced by Na^+ and Cl^- entry than are nonerythroid cells (Knutton et al., 1976), and because erythrocytes do not possess mechanisms for recovery (Campbell et al., 1981; Ramm et al., 1983; Bashford et al., 1985) and membrane repair (Campbell & Morgan, 1985; Carney, Koski & Shin, 1985). Induction of leakage by each agent shows apparent positive cooperativity, and when added in pairs, agents act synergistically (Bashford et al., 1986). Leakage is inhibited by Ca^{2+} (Pasternak & Micklem, 1974*a*; Masuda & Goshima, 1980; Harshman & Sugg, 1985) and by other divalent cations (Impraim et al., 1979) such as Zn^{2+} (Gotze, Haupt & Fischer, 1968; Yamamoto & Takahashi, 1975; Avigad & Bernheimer, 1976; Boyle, Largone & Borsos, 1979; Pasternak et al., 1985*a*,*b*); suspending cells in media of low ionic strength also

reduces leakage, which can be restored by the addition of NaCl in a Ca^{2+}- or Zn^{2+}-sensitive manner (Pasternak et al., 1985*a*,*b*; Bashford et al., 1986).

Lesions created by detergents have been used to introduce 'nonpermeant' compounds into cells (e.g. Miller, Castellot & Pardee, 1978, 1979; Dunn & Holz, 1983; Wilson & Kirshner, 1983), and more recently Sendai virus and *S. aureus* α-toxin have been employed to bring Ca^{2+} chelates (Gomperts, Baldwin & Micklem, 1983; Ahnert-Hillger, Bhakdi & Gratzl, 1985), GTP analogues (Gray et al., 1983; Barrowman, Cockroft & Gomperts, 1986) or antibiotics (Cameron et al., 1986) into metabolically competent cells to study processes such as exocytosis. Pore-forming agents therefore have a role as biological tools, in addition to their relevance to diseases of viral (Pasternak, 1987*a*), microbial (McCartney & Arbuthnott, 1978; Arbuthnott, 1983), animal (e.g. Prince, Gunson & Scarpa, 1985) or immune (Schreiber, 1983; Morgan et al., 1984) origin.

The fact that cations modulate the extent of membrane damage induced by pore-forming agents, makes it desirable to understand the mechanism of their action. Because it is unlikely that cations interact with negatively charged (e.g. Sendai virus), positively charged (e.g. melittin) or neutral (e.g. Triton X-100) agents in a similar manner, at least part of their action is probably on the cell membrane itself. We have therefore compared the effects of cations on two types of cell that differ in the composition of their plasma membrane, namely erythrocytes and Lettre cells (an established line of malignant murine cells grown intraperitoneally), as well as on isolated phospholipid bilayers. At the same time we have examined the role of protons, since one explanation of the inhibitory action of low ionic strength could be in terms of a localized increase in H^+ concentration at the cell surface. In addition, we tried to answer two questions: do divalent cations affect leakage by binding to specific membrane components or by exerting a nonspecific screening effect on surface charge? Is the site inside or outside the cell? During this investigation we found that protons and certain divalent cations themselves induce leakage at sufficiently high concentration, and we have therefore examined the nature of this effect. Taken together, the results support a model to account for the various effects of different cations on the permeability characteristics of agent-modified cells. Our findings are compatible with the views that extracellular Ca^{2+} (Frankenhauser & Hodgkin, 1957; Wyn Jones & Lunt, 1967) and Zn^{2+} (Chvapil, 1976) play an important protective role on the integrity of cells *in vivo*.

Materials and Methods

Cation and Metabolite Leakage

Cells were washed in 150 mM NaCl, 5 mM KCl, 5 mM HEPES, 1 mM $MgSO_4$, pH adjusted to 7.4 at 22°C with NaOH (HEPES-buffered saline, HBS). 20% Vol/vol cell suspensions in HBS were incubated at 37°C for 30 to 45 min in the presence of [^{3}H]choline or [^{3}H]2-deoxyglucose to label the intracellular phosphorylcholine or 2-deoxyglucose 6-phosphate pools (Impraim et al., 1980). Washed cells were then incubated at approximately 5×10^6/ml with or without pore-forming agents with or without divalent cations in HEPES-buffered saline at 37°C. Cation leakage and leakage of phosphorylated metabolites was assessed after pelleting cells through oil as previously described (Impraim et al., 1980; Bashford et al., 1985). Hemolysis was measured by conventional means.

Electrophoretic Mobility of Cells

Free-flow electrophoresis was carried out using Bender and Hobein (Munich, W. Germany) Elphor vap 5 apparatus. The separation buffers contained 0.3 M mannitol, 1 mM $MgSO_4$, 5 mM HEPES, pH adjusted to 7.4 at 22°C with NaOH and additional salts as described below. The electrode medium contained 50 mM HEPES, pH adjusted to 7.4 at 22°C with NaOH. Cells were washed twice and finally resuspended at approx. 5% vol/vol in 2 ml of the separation buffer. The cells were applied to the electrophoresis apparatus at about 6 ml · hr^{-1}; the buffer flow rate was 600 ml · hr^{-1} and the temperature was 6.5°C. The distribution of cells in the effluent was assessed turbidometrically by monitoring absorbance at 500 nm.

Electrophoresis of single cells (microelectrophoresis, cytopherometry) was measured at 25°C using the apparatus described by Bangham et al. (1958) which was loaned to us by Dr. D. Gingell. Cells were washed twice in the appropriate medium and finally suspended at approximately 0.1% vol/vol prior to electrophoresis at an applied voltage of 50 V. The 'stationary level' of the cylindrical electrophoretic chamber was determined as described by Bangham et al. (1958). For $0.02 < I < 0.16$ the stationary level did not differ significantly from the theoretically expected location of $0.293r$ from the wall of the tube of radius r and in every case the mobility/level profile had the expected parabolic shape. In all experiments a minimum of five measurements were made of cells in the stationary level before and after reversal of the current flow on duplicate samples (Graham et al., 1986).

Conductivity Changes of Lipid Bilayer

Planar bilayer membranes were prepared by the apposition of two monolayers of egg phosphatidylcholine (Montal & Mueller, 1972; Menestrina, 1986). Bathing solutions (4 ml on each side of the membrane) contained salt solutions as indicated; Ca^{2+}, when present, was added to both sides. *S. aureus* α-toxin was added to one side (*cis* side) to give a final concentration in the range 5 to 30 μg/ml. The *cis* compartment was connected to the virtual ground and voltage signs are referred to it. Currents were recorded with Ag-AgCl electrodes monitored with a virtual grounded opera-

Table 1. Effect of cations on membrane permeability[a]

Cell type	Cation	Protection against leakage induced by pore-forming agent	Induction of leakage in absence of pore-forming agent	Stimulation of leakage induced by pore-forming agent at low ionic strength
Lettre cell; other nonerythroid cells	H^+	++++++	+++++	b
	Zn^{2+}, Cd^{2+}, Cu^{2+}	+++++	+++	b
	Ca^{2+}, Sr^{2+}, Mn^{2+}	+++	−	b
	Ba^{2+}, Mg^{2+}	+	−	+
	Dimethonium, NH_4^+, K^+, Na^+, Rb^+	−	−	+
Erythrocytes	H^+	++++++	+++++	b
	Zn^{2+}, Cd^{2+}, Cu^{2+}	+++++	+++	b
	Ca^{2+}, Sr^{2+}, Mn^{2+}	+	−	+
	Ba^{2+}, Mg^{2+}	+	−	+
	Dimethonium, NH_4^+, K^+, Na^+, Rb^+	−	−	+

++++++: active below 10^{-5} M (in HBS)
+++++: active below 10^{-4} M
+++: active at 10^{-3} M
+: active above 10^{-2} M
−: no activity below 5×10^{-2} M

[a] Membrane permeability measured by leakage of phosphoryl[^{3}H]choline (Lettre cells; other nonerythroid cells) or by hemolysis (erythrocytes). Data based on results presented in the Appendix and elsewhere in this paper, as well as on previously published (Impraim et al., 1979; Pasternak et al., 1985*a,b*; Bashford et al., 1986) and unpublished results.
b, Difficult to assess, as cation strongly *inhibits* leakage (*see* column 1).

tional amplifier with feed-back resistors in the range 10^7 to 10^9 ohms.

Pore-Forming Agents and Other Chemicals

Sendai virus was grown in embryonated eggs for three days (Impraim et al., 1980). *S. aureus* α-toxin was isolated (McNiven, Owen & Arbuthnott, 1972) from strain Wood 46 (NCTC 7121) and kindly donated by Dr. Joyce de Azavedo, Moyne Institute, Trinity College, Dublin; the *S. aureus* α-toxin used for lipid bilayer experiments was donated by Dr. K.D. Hungerer, Behringwerke, Marburg, W. Germany. Purified melittin (Dufton et al., 1984) was donated by Dr. R.C. Hider, University of Essex. Polylysine (Sigma Type IV, M_r 4000 to 1500), Triton X-100 (BDH) and divalent cations were commercial samples. Dimethonium (dibromide salt) was synthesized (McLaughlin et al., 1983) and donated by Dr. S.G. McLaughlin.

Cells

Lettre cells were grown intraperitoneally as an ascitic suspension in Swiss white mice, strain TO and harvested into heparinized HEPES-buffered saline. Blood from humans, dogs and rabbits was obtained by venepuncture and collected in heparinized tubes. Lettre cells and erythrocytes were washed in HEPES-buffered saline and stored as 20% vol/vol suspensions at room temperature for up to six hours prior to use.

Abbreviations

HAU: hemagglutination unit; HEPES: N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES: 2-[N-Morpholino]ethanesulfonic acid; TEA: Tetraethylammonium; PIPES: Piperazine-N,N′-bis-2-ethanesulfonic acid; MOPS: 3-[N-Morpholino]propanesulfonic acid; EPPS: N-[2-Hydroxyethyl]-piperazine-N′-3-propane sulfonic acid.

Results

Divalent Cations and Protons Prevent Agent-Induced Leakage

It has previously been shown that > 10 mM Ca^{2+} is required to inhibit leakage from erythrocytes affected by hemolytic viruses (Burnet, 1949), by activated complement (Kabat & Mayer, 1961), or by *S. aureus* α-toxin (Harshman & Sugg, 1985) whereas approx. 1 mM Ca^{2+} inhibits a similar amount of leakage from Lettre or Daudi cells (Bashford et al., 1984; Micklem et al., 1984). In contrast, Zn^{2+} is equally effective at inhibiting leakage from human erythrocytes or Lettre cells (Table 1). Rabbit, dog and sheep erythrocytes are as sensitive to Zn^{2+}, and as insensitive to Ca^{2+}, as are human erythrocytes.

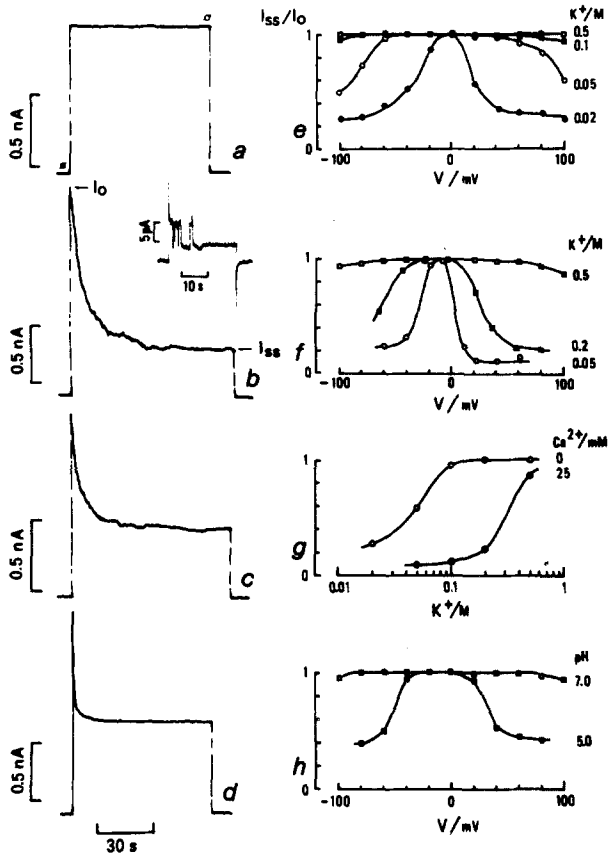

Fig. 1. Effects of Ca^{2+}, ionic strength and H^+ on *S. aureus* α-toxin-induced conductance in planar lipid bilayers. Panels *a-d* show the effect of applying a +60-mV voltage step from a 0-mV holding potential to membranes containing many channels. (*a*) 0.1 M KCl, pH 7.0; (*b*) 0.1 M KCl, 0.025 M $CaCl_2$, pH 7.0; inset shows a single-channel recording obtained under similar conditions; (*c*) 0.02 M KCl, pH 7.0, and (*d*) 0.1 M KCl, pH 5.0 (*e, f*) Voltage-dependence of the steady-state current (I_{SS}) to instantaneous current (I_0; *see* panel *b*) ratio at different KCl concentrations in the absence (*e*) or presence (*f*) of 25 mM Ca^{2+}. (*g*) K^+ dependence of I_{SS}/I_0 to a step change of 100 mV from zero in the absence (○) or presence (●) of 25 mM Ca^{2+}. (*h*) Voltage dependence of I_{SS}/I_0 ratio in 0.1 M KCl at pH 7 (□) or pH 5 (■)

This suggests that inhibition of leakage by Zn^{2+} is due to interaction with a different component(s) of the plasma membrane than is inhibition by Ca^{2+}. The interaction with Zn^{2+}, or with Ca^{2+} at physiological concentration, is likely to reflect specific binding rather than nonspecific screening of surface charge because dimethonium ($^+NH_3$-CH_2-NH_3^+), a compound that is as effective as Ca^{2+} or Mg^{2+} at screening surface charge (as judged by its ability to reduce electrophoretic mobility; *see* Fig. 6 below), but that does not bind to anionic sites (McLaughlin et al., 1983), has no effect on agent-induced leakage from Lettre cells or erythrocytes (Table 1).

Mn^{2+} and Sr^{2+} have an effect similar to that of Ca^{2+} on Sendai virus-induced leakage (Impraim et al., 1979). Of other divalent cations now tested, Cd^{2+} and Cu^{2+} resemble Zn^{2+} (Yamamoto & Takahashi, 1975; Boyle et al., 1979), whereas Ba^{2+} resembles Mg^{2+} (Table 1). It has to be stressed that although the *relative* potency of different cations for inhibiting leakage induced by every pore-forming agent tested is the same, namely $Zn^{2+} > Ca^{2+} > Mg^{2+}$, the *actual* potency varies from agent to agent: leakage induced by Sendai virus, for example, is more sensitive to inhibition by Zn^{2+} than is leakage induced by other agents, whereas leakage induced by *S. aureus* α-toxin is less sensitive to inhibition by Ca^{2+} than is leakage induced by other agents (*see* Appendix). The order of efficacy $Zn^{2+} > Ca^{2+} > Mg^{2+}$ also applies to α-toxin-induced leakage across phospholipid bilayers (Menestrina, 1986 and *unpublished observations*) and to the immobilization of eosin-labeled band 3 in erythrocyte ghosts (Clague & Cherry, 1986). It may be concluded that the predominant effect of ions like Mg^{2+}, dimethonium, spermine, spermidine or Co $(NH_3)_6^{3+}$ on Lettre cells, or the same ions plus Ca^{2+} on erythrocytes, is to screen surface charge, whereas the effect of divalent cations like Ca^{2+} (on Lettre cells) or Zn^{2+} (on Lettre cells or erythrocytes) is to bind to specific components.

H^+ also protects against leakage from Lettre cells and erythrocytes (Table 1). For experiments with Sendai virus the lesion was introduced at pH 7 at low ionic strength (where little leakage occurs), and its characteristics were subsequently assessed by transferring the cells to physiological saline in the absence of pore-former. For other agents, either this procedure or incubation directly with agent at different pH values was performed. Sendai virus-induced leakage was found to be the least sensitive, and Triton X-100 the most sensitive, to decreased pH (*see* Appendix); the residual leakage at low pH from Lettre cells induced by Sendai virus at ionic strength 0.15 is compatible with the previous observations of Patel and Pasternak (1985).

Conductivity across phosphatidylcholine bilayers is likewise reduced by H^+. Figure 1 shows this for α-toxin-induced channels: channels remain open indefinitely (> 1 min) (panel *a*), unless the bathing solutions contain Ca^{2+} (*b* and *f*), low ionic strength (*c* and *e*) or H^+ (pH 5; *d* and *h*). Note that Ca^{2+} closes channels better at low ionic strength (*f* and *g*). It is interesting to compare these effects with those of H^+ on the movement of toxins such as ricin and abrin across membranes: increasing H^+ protects cells against these toxins (Sandvig & Olsnes, 1982), an effect that has been suggested to be due to a change in local H^+ concentration at the surface of the membrane (Sandvig & Olsnes, 1985).

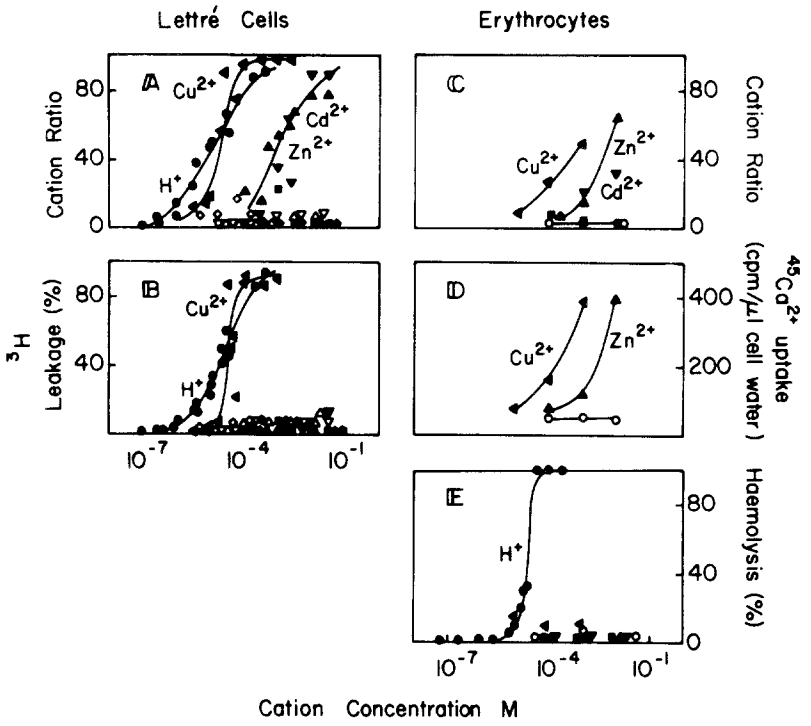

Fig. 2. Induction of permeability changes by cations. Lettre cells preincubated with [^{3}H]choline, or human erythrocytes, were incubated either in low pH buffers (●) or in HBS without or with $CuCl_2$ (◀), $ZnSO_4$ (▲), $CdSO_4$ (▼), $CaCl_2$ (■), $BaCl_2$ (♦), $MgCl_2$ (○), dimethonium Br_2 (□), spermine (△), spermidine (▽) or $Co(NH_3)_6\,Cl_3$ (◇) at the concentrations indicated. (*A*) Leakage of Na^+ and K^+ from Lettre cells. Cation ratio expressed as intracellular $(Na^+/Na^+ + K^+) \times 100$ and scaled such that the cation ratio of Lettre cells in HBS alone (approximately 30) is 0, and the cation ratio of the medium (97) is 100. (*B*) Leakage of phosphoryl[^{3}H]choline from Lettre cells, scaled such that 'background' leakage from cells not exposed to agent in the presence of the various cations (generally 10 to 30%) is 0. (*C*) Leakage of Na^+ and K^+ from erythrocytes expressed as in (*A*) (cation ratio of erythrocytes in HBS alone, approximately 20). (*D*) Uptake of ^{45}Ca by erythrocytes exposed to 0.1 mM $^{45}CaCl_2$ (10 mCi/mmol; 1.25×10^6 cpm/ml); intracellular ^{45}Ca was measured after spinning cells through oil (as for ^{3}H leakage). (*E*) Hemolysis measured by conventional means. Atomic symbols have been added to the panels to show which cations cause leakage

Cd^{2+}, Cu^{2+}, Zn^{2+} and Protons Induce Leakage in the Absence of Pore-Forming Agents

Incubation of Lettre cells with Cd^{2+}, Cu^{2+} or Zn^{2+} at concentrations higher than those necessary to inhibit leakage, i.e. approx. 0.1 to 1 mM, induces leakage of Na^+ and K^+ in the absence of pore-forming agent (Fig. 2*A*). Some of the data in Fig. 2 refer to cells incubated in the presence of 0.1% serum albumin; in the absence of albumin, lower concentrations of Cd^{2+}, Cu^{2+} or Zn^{2+} cause leakage. Protons likewise induce leakage in the absence of pore-forming agent (Fig. 2*A*); in other words the protective effect of Cd^{2+}, Cu^{2+}, Zn^{2+} or H^+ falls off as their concentration is increased, giving a biphasic response similar to that displayed by local anesthetics and other membrane-active compounds (Seeman, 1972).

In contrast to the effect of pore-forming agents, Cd^{2+} and Zn^{2+} do not induce leakage of α methyl glucoside (*results not shown*) or phosphorylated metabolic intermediates, whereas Cu^{2+} and H^+ do (Fig. 2*B*). It is to be noted that Zn^{2+} and Cd^{2+} also induce leakage of ions from erythrocytes (Fig. 2*C*), though not to the extent that they cause hemolysis (Fig. 2*E*). This observation of a decreased effect on erythrocytes compared with Lettre cells is compatible with the conclusion reached above, that Zn^{2+} binds to component(s) of the plasma membrane that are different in Lettre cells and erythrocytes.

Do Cd^{2+}, Cu^{2+}, Zn^{2+} and H^+ act not by inducing leakage but by inhibiting the Na^+ pump? The fact that the Na^+,K^+-ATPase of Lettre cell extracts is inhibited by Zn^{2+} suggests that they do (Table 2). However, three types of experimental observation make this possibility unlikely. First, an inhibitor of the Na^+ pump like ouabain does not cause a net change in intracellular K^+ of Lettre cells under conditions in which it clearly affects the Na^+ pump, as evidenced by an inhibition of the uptake of trace amounts of $^{86}Rb^+$ (Fig. 3). Second, under similar

Table 2. Inhibition of Na^+,K^+-ATPase of cell extracts by Ca^{2+} and Zn^{2+}[a]

Concentration of divalent cation	Enzyme activity in presence of	
	Ca^{2+}	Zn^{2+}
	(percent of control)	
10^{-6}	104	75
10^{-5}	104	52
10^{-4}	27	5
10^{-3}	25	<1
10^{-2}	13	<1

[a] Na^+,K^+-ATPase of sonicated Lettre cells was determined by assay of the ouabain-sensitive component according to the method of Avruch and Wallach (1971). The control value of the unpurified cell extract was 0.22 (±0.03, n = 5) μmoles ATP hydrolyzed per hour per mg protein.

Table 3. Effect of Zn^{2+} on K^+ and $^{86}Rb^+$ transport in erythrocytes[a]

	Cation content in cells after 20 min	
	High K^+ medium	Conventional medium
	(percent of control)	
K^+	142 ± 12	49 ± 5
$^{86}Rb^+$	ND	213 ± 43

[a] Erythrocytes (hematocrit 0.008) were incubated in 5 mM HEPES pH 7.4 (NaOH) for 20 min at 37°C in 155 mM KCl (high K^+ medium) or 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ (conventional medium) in the absence or presence of 10 mM Zn^{2+}; $^{86}Rb^+$ (0.1 μCi/ml) was present in some tubes. Cellular K^+ content and radioactivity were assayed after pelleting aliquots of the cell suspension through oil. The results are expressed as the percent of control values (absence of Zn^{2+}) ± SEM, n = 4; control values were 120 ± 3 mM K^+ in high K^+ medium or in conventional medium, and 18 ± 3 cpm $^{86}Rb^+$/μl cell water in conventional medium.

conditions in red cells, Zn^{2+} *stimulates* $^{86}Rb^+$ uptake rather than inhibiting it under conditions where net K^+ movement is outward (Table 3). In high K^+ medium, Zn^{2+} stimulates K^+ entry (Table 3), a result incompatible with inhibition of the Na^+ pump. Third, Zn^{2+} stimulates the uptake of an unrelated cation, namely $^{45}Ca^{2+}$ (Fig. 2*D*).

Univalent and Divalent Cations Induce Leakage from Agent-Affected Cells Suspended in Low Ionic Strength Medium

Pore-forming agents do not cause leakage from cells that are suspended in media of low ionic strength; leakage can be induced by addition of NaCl after

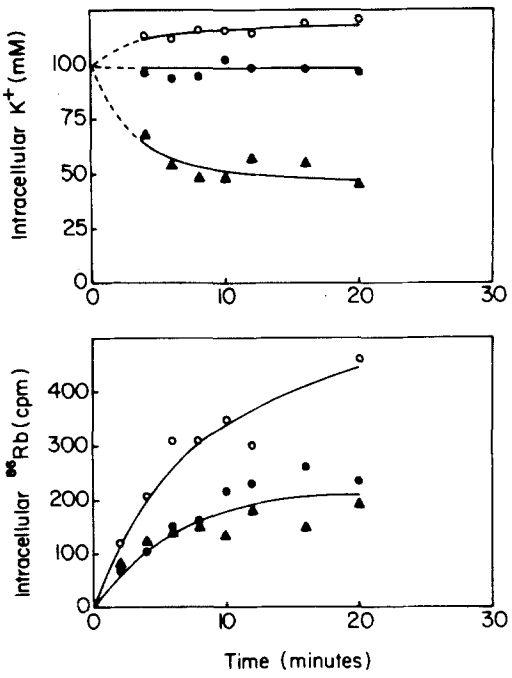

Fig. 3. Effect of Zn^{2+} on K^+ content and on $^{86}Rb^+$ uptake in Lettre cells. Lettre cells (10^7/ml) in HBS with 0.1% albumin alone (○) or with 2.5 mM $ZnSO_4$ (▲) or with 1.6 mM ouabain (●) were incubated at 37°C in the presence of $^{86}RbCl$ (82,000 cpm/ml), spun through oil and intracellular K^+ (upper panel) and ^{86}Rb (lower panel) measured

removal of excess agent (Bashford et al., 1986) This indicates that the inhibitory effect of low ionic strength is not due to altered binding of agent to cells. In order to examine this further, the effect of divalent cations was examined: Fig. 4 illustrates an experiment with Sendai virus. Dimethonium, which as mentioned above has little binding affinity for membranes, is as effective as NaCl or other salt, including $MgCl_2$ or $MgSO_4$, in stimulating leakage from Lettre cells (Fig. 4*A*). Other divalent cations are difficult to test, since their protective ability outweighs their possible "salt-like" effect. Because Ca^{2+} protects erythrocytes poorly, it *can* be tested for its "salt-like" activity: as seen from Fig. 4*B*, Ca^{2+} is effective at inducing hemolysis. Note that at high concentrations, Ca^{2+} becomes inhibitory, whereas Mg^{2+} does not. Zn^{2+} does not induce hemolysis at any concentration (*see also* Fig. 2). As the abscissae of Fig. 4 show, the extent of leakage is dependent on ionic strength, rather than on concentration. Other agents (melittin, complement, *S. aureus* α-toxin, Triton X-100), and other ions (KCl, LiCl, RbCl, CsCl, KSCN, choline Cl, NH_4Cl, ethylammonium Cl, diethylammonium Cl, triethylammonium Cl, Na_2SO_4, K_2SO_4, Na phosphate, K phosphate, K citrate), all affect Lettre cells and erythro-

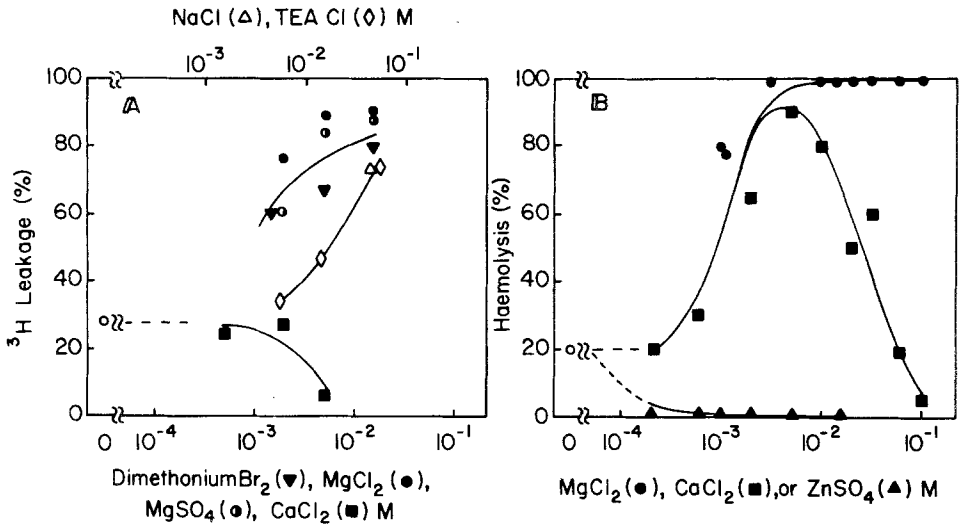

Fig. 4. Salt-induced permeability changes in Lettre cells and erythrocytes treated with Sendai virus at low ionic strength. Lettre cells preincubated with [^{3}H]choline (*A*), or human erythrocytes (*B*), were suspended in 0.3 M mannitol, 5 mM HEPES, pH adjusted to 7.4 at 22°C with NaOH and incubated with Sendai virus in the absence (○) or presence of NaCl (△), TEA Cl (◇), dimethonium Br_2 (▼), $MgCl_2$ (●), $MgSO_4$ (◑), $CaCl_2$ (■) or $ZnSO_4$ (▲) at the concentrations indicated, and leakage of phosphoryl[^{3}H]choline (*A*) or hemolysis (*B*) measured. Leakage of [^{3}H] has been scaled such that leakage from Lettre cells in the absence of Sendai virus (approximately 35% in salt-free medium) is 0

Table 4. Leakage of negatively charged, neutral and positively charged molecules from Sendai virus-treated cells at low ionic strength[a]

Condition	% leakage of:		
	[^{3}H]dGlc 6-P	α-methylglucoside	K^+
No salt	8 ± 3	14 ± 5	5 ± 9
No salt + 2 mM Ca^{2+}	< 1	< 1	ND
Salt (50 mM NaCl)	47 ± 7	52 ± 8	77 ± 1
Salt + 2 mM Ca^{2+}	6 ± 3	2 ± 2	12 ± 3

[a] 20% Lettre cells in Ham's f10 labeled with 5.3 μCi/ml [^{14}C]α-methylglucoside for 65 min and 6.7 μCi/ml [^{3}H]d-Glc for 30 min at 37°C. Cells were spun and resuspended to 20% in mannitol. They were then suspended at 1% in mannitol with and without 50 mM NaCl and 2 mM $CaCl_2$ as indicated and with and without 200 HAU/ml Sendai virus. Leakage of [^{3}H]choline and [^{14}C]α-methylglucoside from virus-treated cells (% leakage) was calculated after subtracting the leakage from control cells which was 54 ± 5% [^{3}H] and 73 ± 3% [^{14}C] (no salt, 57 ± 4% [^{3}H] and 74 ± 4% (2 mM $CaCl_2$), 56 ± 4% [^{3}H] and 79 ± 3% (50 mM NaCl) and 53 ± 4% [^{3}H] and 80 ± 2% (50 mM NaCl, 2 mM $CaCl_2$). Leakage of K^+ from Lettre cells is expressed relative to control cells which contained 15.3 ± 0.7 nmol K/10^6 cells (no salt), 14.3 ± 1.6 nmol K/10^6 cells (50 mM salt) and 15.1 ± 1.7 nmol K/10^6 cells (50 mM salt, 2 mM $CaCl_2$), respectively. Data are mean ± SEM (n = 6 [^{3}H] and [^{14}C], n = 3 K^+).

cytes similarly. It has been suggested that leakage of ions through a membrane may be controlled solely by a static charge at the mouth of the pore (Edmonds, 1981, 1984), and the effect of salt on leakage from cells suspended in low ionic strength might be due to such a cause. This, however, is unlikely, since salt induces leakage of negatively charged compounds such as dGlc-6-P, of uncharged compounds such as α-methylglucoside, and of positively charged compounds such as K^+ (Table 4).

Effect of Ions on Surface Charge

Media of low ionic strength may reduce leakage from cells treated with pore-forming agent for the following reason. At low ionic strength the local concentration of protons near the anionic groups, such as carboxyl, of the outer leaflet of the plasma membrane and of the glycocalyx (Levine et al., 1983) is increased relative to that in the presence of 150 mM Na^+ (or lower concentrations of divalent cations, which are not, of themselves, inhibiting). It is therefore postulated that dissociated, i.e. unprotonated, groups favor leakage, whereas protonation reduces leakage. Such a hypothesis implies that the surface charge of cells should increase as salt is added to cells suspended in media of low ionic strength. Yet increasing ionic strength *decreases* the electrophoretic mobility of cells, at neutral pH values (Furchgott & Ponder, 1941; Bateman & Zellner, 1956; Heard & Seaman, 1960; Wilkins, Otterwill & Bangham, 1962). On the other hand, theoretical descriptions of such decreases, generally measured at $I > 0.05$, are less satisfactory when $I <$ 0.02 (Levine et al., 1983; Donath & Voight, 1986).

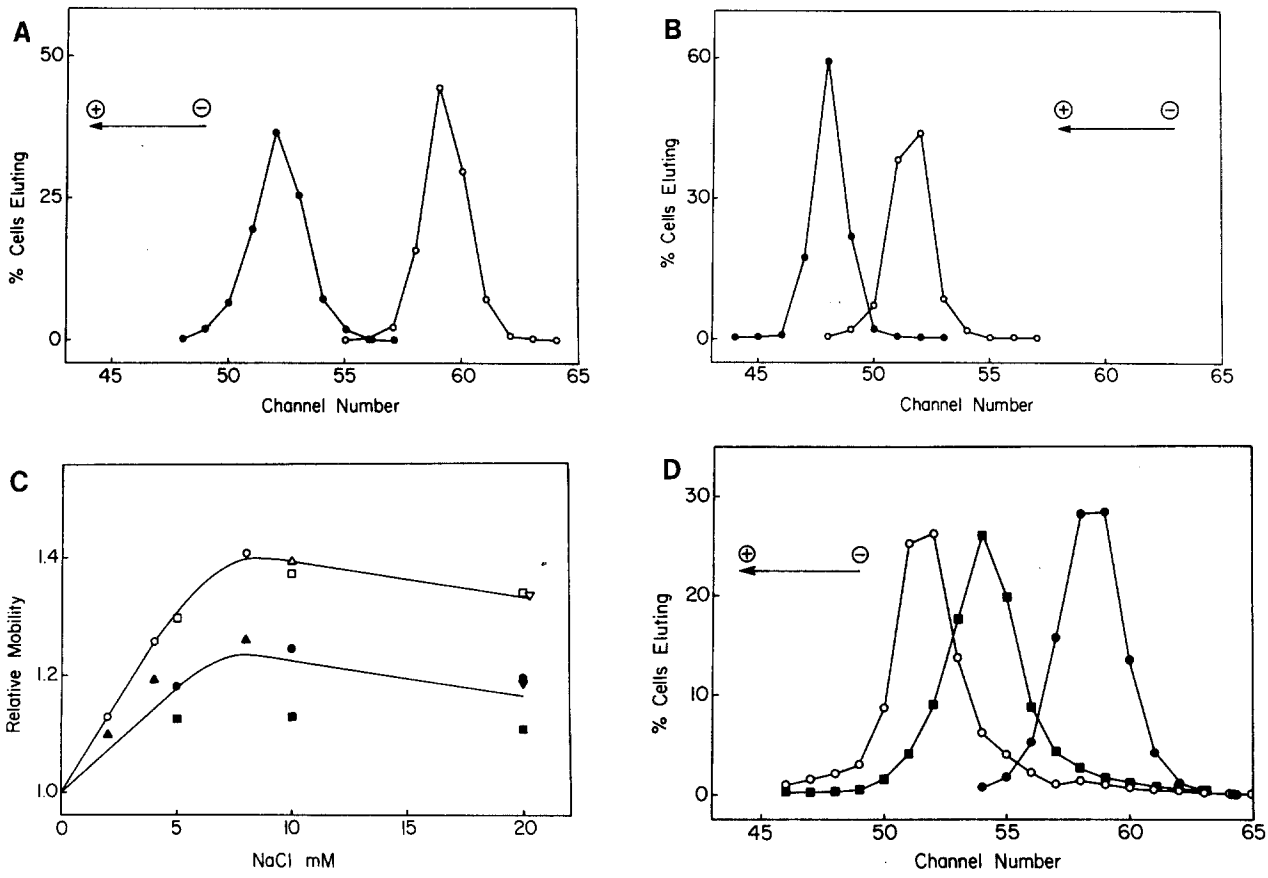

Fig. 5. Free-flow electrophoresis of Lettre cells and erythrocytes. Lettre cells or human erythrocytes were suspended in separation buffer medium and subjected to free-flow electrophoresis as described in Materials and Methods. (*A*) Lettre cells in the absence (○) or presence (●) of 20 mM NaCl. Chamber voltage 80 V · cm^{-1}. (*B*) Erythrocytes in the absence (○) or presence (●) of 20 mM NaCl. Chamber voltage 80 V · cm^{-1}. (*C*) Lettre cells (open symbols) or erythrocytes (filled symbols) in NaCl at the concentrations indicated. The mobilities are expressed relative to that of cells in the absence of NaCl (*see A* and *B*). Separate experiments are indicated by different symbols. Chamber voltage 80 to 105 V · cm^{-1}. (*D*) Lettre cells in the absence (○) or presence of 2.5 mM $CaCl_2$ (■) or 10 mM $MgSO_4$ (●). Note that 1 mM $MgSO_4$ is present throughout. Chamber voltage 105 V · cm^{-1}

In order to resolve this apparent paradox we have measured the electrophoretic mobility of Lettre cells and erythrocytes suspended in media of low ionic strength, in the presence or absence of added univalent and divalent cations.

Free-flow electrophoresis (Hannig & Heidrich, 1974; Graham et al., 1986) is a convenient technique for use at low ionic strength. At constant buffer flow and constant applied voltage, addition of salts of monovalent cations increases the electrophoretic mobility of Lettre cells (Fig. 5*A*) and erythrocytes (Fig. 5*B*) measured by this technique. The effect is maximal at an ionic strength of 0.014 (Fig. 5*C*) and is similar for the sulfates and chlorides of Na^+ and K^+. Salts of divalent cations such as Ca^{2+} or Mg^{2+} reduce the mobility of Lettre cells (Fig. 5*D*) and erythrocytes (*data not shown*).

We have repeated the experiments using a conventional microelectrophoresis apparatus in which the movement of single cells is measured (Bangham et al., 1958). At pH 6.2 addition of salt to Lettre cells (Fig. 6*A*) or erythrocytes (Fig. 6*B*) in mannitol medium increases electrophoretic mobility. The effect is maximal at $I \simeq 0.005$ for Lettre cells and at $I \simeq 0.015$ for erythrocytes. Our data for erythrocytes are in accord with those presented in Fig. 3 of the paper by Heard and Seaman (1960), in which erythrocyte mobility (at pH 6) was found to be greater at $I = 0.0145$ than at any other value of I. Equivalent concentrations of mono- and divalent salts of Na and K, judged by the current flowing during the measurement of electrophoretic mobility, gave identical erythrocyte mobilities, as indicated in Fig. 6*B*. At pH 7.4, divalent cations added to Lettre cells (Fig. 6*C*) or erythrocytes (Fig. 6*D*) suspended in mannitol medium reduce the electrophoretic mobility significantly. H^+ also reduces the mobility of Lettre cells (Fig. 6*E*) and erythrocytes

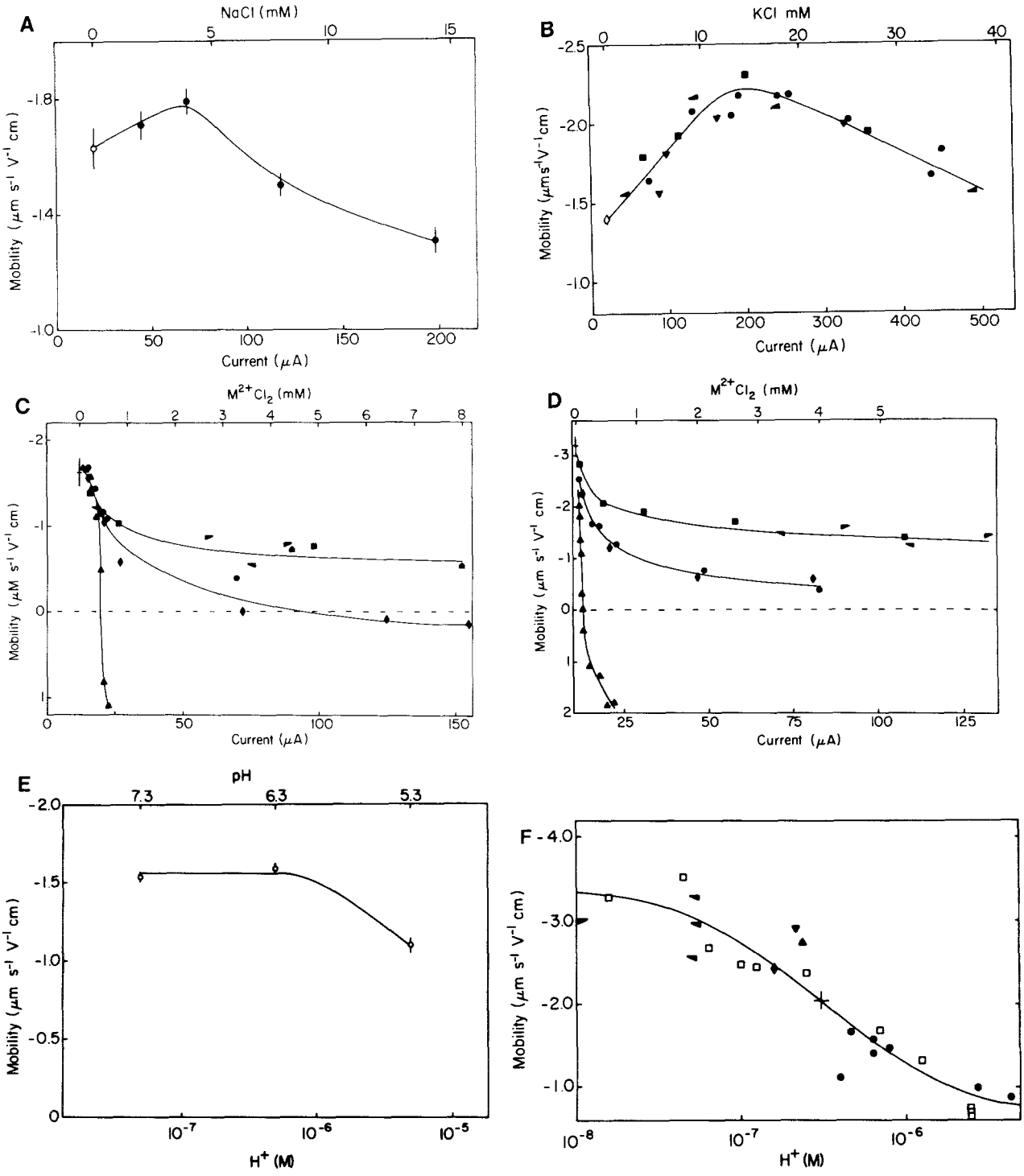

Fig. 6. Microelectrophoresis of Lettre cells and erythrocytes. Lettre cells (*A*, *C* and *E*) or human erythrocytes (*B*, *D* and *F*) were suspended in 0.3 M mannitol buffered as indicated below and subjected to electrophoresis at 25°C as described in Materials and Methods. (*A* and *B*) Effect of univalent cations. Lettre cells in the absence (○) or presence (●) of NaCl to give the resultant current indicated. Erythrocytes in the absence (◇) or presence of NaCl (▼), Na_2SO_4 (◀), KCl (●) or K_2SO_4 (■) to give the resultant current indicated. The buffer was 5 mM MES pH 6.2 in each case. The error bars in (*A*) indicate the SEM (n = 20). The concentration of NaCl (*A*) or KCl (*B*) giving the current measured is indicated at the top of each panel. (*C* and *D*) Effect of divalent cations. Lettre cells or erythrocytes in the presence of $CaCl_2$ (■), $MgCl_2$ (●), $MnCl_2$ (◀), dimethonium Br_2 (▶), $ZnSO_4$ (●), $CdSO_4$ (●) or $CuCl_2$ (▲) to give resultant current (and equivalent concentrations of M^{2+} as indicated at the top of each panel), as shown. The buffer was a mixture of 5 mM HEPES and 2.5 mM Tris, pH 7.4 in each case. The cross (+) indicates the mobility (±1 SD) of cells in the absence of added divalent cation. (*E* and *F*) Effect of protons. Lettre cells (*E*) in the presence of 5 mM MES or HEPES and NaOH to give the H^+ concentrations indicated. Erythrocytes (*F*) in the absence (□) or presence of 5 mM buffer (MES ●, imidazole ▲, phosphate ▼, PIPES ◆, MOPS ▶ or EPPS ◀) at the pH indicated. The current flow was 2 to 20 μA. The error bars in (*E*) indicate the SEM (n = 150); the cross in (*F*) indicates the pK_a value (6.52) for the best-fit Henderson-Hasselbach curve (solid line) to the data points

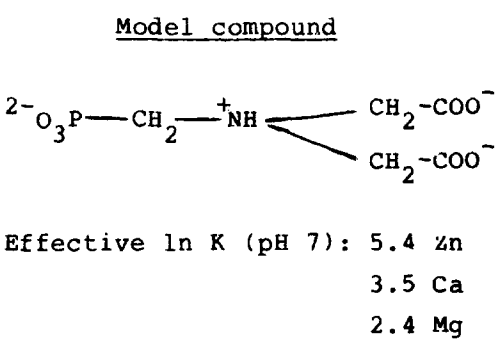

Fig. 7. Divalent cations binding by a PS head-group analogue. Stability constants of phosphonomethyliminodiacetate (Sillen & Martell, 1964) for the divalent cations indicated, calculated for pH 7 by Dr. R.J.P. Williams

(Fig. 6*F*) suspended in mannitol medium (*cf.* Fig. 3 of Heard and Seaman, 1960). At $I = 0.15$, the mobility of Lettre cells and erythrocytes was constant between pH 5 and 8 (*data not shown*). The marked effect of Cu^{2+}, which reverses the electrophoretic mobility of Lettre cells (Fig. 6*C*) and erythrocytes (Fig. 6*D*), may contribute to its effect on Lettre cell (Fig. 2*A*,*B*) and erythrocyte (Fig. 2*C*,*D*) permeability. In all cases the mobilities recorded were 'reversible': when the cells are replaced in isotonic saline they exhibit the same mobility as cells which had never been exposed to low ionic strength medium. The mobilities were neither time- nor cell concentration-dependent, provided that cells were not kept in low ionic strength medium of low pH for more than 45 min.

We confirmed the position of the stationary layer of the electrophoresis apparatus for $0.02 < I < 0.15$, using the experimental technique described by Bangham et al. (1958). This procedure also provided the expected parabolic dependency of mobility with distance from the edge of the tube. Henry's correction (Henry, 1938) is $0.01r$ in the apparatus we used (Bangham et al., 1958) and the coincidence of the theoretically predicted stationary level with that determined experimentally suggests that our observations are indeed from the stationary layer. We were unable to confirm the position of the layer experimentally for $I < 0.02$ because of the strong pH dependence of erythrocyte mobility at very low ionic strengths. The fact that the effects of salt on electrophoretic mobility could be observed by techniques as different as free-flow electrophoresis and microelectrophoresis make it unlikely that stimulation of mobility at low ionic strength is an experimental artifact. Furthermore, the effect of salt, at low ionic strength, on Lettre cell mobility (Fig. 6*A*) differs quantitatively from that on erythrocyte mobility (Fig. 6*B*), suggesting that the effect is related to events specific to the cell surface and not to the apparatus used for the measurement.

Discussion

The effects of the cations reported in this paper may be summarized as indicated in Table 1. The cations fall into three groups: (i) cations like H^+, Zn^{2+}, Cd^{2+} or Cu^{2+} that induce leakage at one concentration and protect against agent-induced leakage at a lower concentration; (ii) cations like Ca^{2+}, Sr^{2+}, Mn^{2+} (and to a lesser extent Ba^{2+} and Mg^{2+}) that protect against agent-induced leakage at concentrations 10 to 100 times greater than those required by the first group; (iii) cations like dimethonium, spermine, spermidine, $Co(NH_3)_6^{3+}$, and univalent cations (Na^+, K^+, Rb^+, NH_4^+) that neither induce nor protect. There is some overlap between groups (ii) and (iii) in that for some agents, spermine and $Co(NH_3)_6^{3+}$ protect as much as Mg^{2+}. The relative efficacy of different cations at preventing leakage correlates approximately with the stability constants for divalent metals bound to the compound illustrated in Fig. 7 (Sillen & Martell, 1964), and therefore suggests that a membrane constituent such as phosphatidylserine may act as a ligand. Certainly (a) the inhibitory action of Ca^{2+} on *S. aureus* α-toxin-induced leakage through planar phosphatidylcholine bilayers is increased tenfold by the addition of phosphatidylserine (Menestrina, 1986) and (b) it is known that erythrocytes, which are less sensitive to inhibition by Ca^{2+} than are other cells (Micklem et al., 1984; Table 1; Fig. 4; Appendix) have little phosphatidylserine exposed at their extracellular surface (Op den Kamp, 1979). However, other membrane constituents are likely to be involved as well, since Zn^{2+} protects erythrocytes to the same extent as other cells. The protective effect of Zn^{2+} (10^{-4} to 10^{-5} M) clearly implies predominantly a binding action, whereas any effect of Mg^{2+} ($> 10^{-2}$ M) is compatible with a screening effect alone (e.g. McLaughin, Szabo & Eisenman, 1971).

The electrophoresis data suggest that the ionization and/or chelation of the groups conferring surface charge on the cells may have a role in modulating membrane permeability. This would explain (i) the stimulation of leakage from agent-modified cells in low ionic strength media by monovalent cations (and by noninhibitory divalent cations such as Mg^{2+}); (ii) the greater efficacy of Ca^{2+} and Zn^{2+} compared with divalent cations such as Mg^{2+} or dimethonium at blocking leakage in physiological saline; (although we found no difference between Ca^{2+} and Mg^{2+} on the electrophoretic mobility of Lettre cells, this is not unexpected: Gambale,

Menini and Rauch (1987) have reported that Ca^{2+} and Mg^{2+} affect surface potential to the same extent, but that Ca^{2+} is approximately twice as effective as Mg^{2+} at reducing the single-channel conductance of channels formed by gramicidin A) and (iii) the inhibition of leakage by protons, especially at low ionic strengths. Recent theoretical considerations (Levine et al., 1983; Donath & Voight, 1986; Pasquale et al., 1986) suggest that the groups responsible for electrophoretic mobility (predominantly sialic acid residues) lie at some distances from the membrane interface. Furthermore, in low ionic strength the groups appear to be further away from the membrane than in isotonic medium (Wolf & Gingell, 1983), presumably due to a charge-charge repulsion mechanism. Such changes in the architecture of the cell surface may have a role in modulating the lesions induced by pore-forming agents at a site closer to the lipid bilayer.

The fact that divalent cations and protons have similar effects on leakage and on electrophoretic mobility, is compatible with the view that the action of divalent cations is from the outside, not from the cytoplasmic side, of the plasma membrane (Pasternak et al., 1985*a*). Certainly an increase in intracellular Ca^{2+} *induces* leakage of ions (Meech, 1976; Schulz & Heil, 1979), including opening of K^+ channels (Miller, 1983; Schwartz & Passow, 1983; Wolff et al., 1986), whereas extracellular Ca^{2+} has the opposite effect (Frankenhauser & Hodgkin, 1957; Woodhull, 1973; Taylor, Armstrong & Bezanilla, 1976; Schulz & Heil, 1979; Yamamoto, Yeh & Narahashi, 1984; French, Krueger & Worley, 1986); note that an increase in intramitochondrial Ca^{2+} makes them leaky to sucrose (Al-Nasser & Crompton, 1986).

How do divalent cations and protons act to inhibit leakage? It is premature to speculate on anything other than a general model, since the nature of the lesions induced by pore-forming agents are not known. The present experiments on (a) the induction, by ions such as dimethonium, of leakage from cells in low ionic strength (Fig. 4) and (b) the protective effect of low pH (Appendix) show that the extent to which lesions are 'open' or 'shut' depends on the surface charge at that part of the membrane. Ionization of negatively charged membrane components that repel each other induces leakage; reducing the charge by metal binding or by protonation inhibits leakage. Whether, at the higher concentrations of Zn^{2+}, Cd^{2+}, Cu^{2+} or H^+ that themselves induce leakage (Fig. 2), the opposite effect occurs, or whether these cations induce leakage by another mechanism—perhaps from inside cells—is at present unclear.

Functionally the lesions resemble the channels of communicating junctions (Loewenstein, 1981) as regards approximate pore size (1 to 2 nm diam.) and closure by Ca^{2+} or H^+. Structurally they do not: agents as diverse as Sendai virus, melittin or triton neither contain, nor are they likely to induce, protein channels such as those of communicating junctions (Unwin & Ennis, 1984); although at high concentration of C9 or *S. aureus* α-toxin, annular protein structures are seen by electron microscopy (Bhakdi & Tranum-Jensen, 1984), their relevance to leakage of compounds across the membrane is debatable (Dankert & Esser, 1985). Moreover the lesions created by the agents under discussion are not fixed in size (Bashford et al., 1985) and at high concentration of agent allow proteins to leak across them (Yamaizumi et al., 1979; Bashford et al., 1986). The kind of membrane deformation induced by such agents is as likely to be generated by an adverse lipid-protein interaction as by one between protein and protein, or between lipid and lipid. In regard to the latter, it may be noted that phase separation of particular lipids in membranes is *stimulated* by divalent cations (Cullis & de Kruijff, 1979) and their relative potency does not match that indicated in Table 1. The observation that Ca^{2+} (Hauser, Finer & Darke, 1977) and other divalent cations (Hauser & Shipley, 1984) cause phospholipids such as phosphatidylserine to crystalline without most of the water molecules normally associated with the polar head group may be relevant to their action in preventing leakage of water-soluble molecules, in that it implies a less polar milieu at the surface of the bilayer.

The protective action of zinc (Table 1) may find an application in therapeutics in order to limit cytopathic effects induced by certain viruses, animal and bacterial toxins, and by inopportune activation of complement (Pasternak, 1987*b*). Unlike Cd^{2+} or Cu^{2+}, Zn^{2+} is rather nontoxic in experimental animals or humans (Underwood, 1977; Prasad, 1978); even at relatively high concentration, its effect on cells is not serious (Fig. 2). On the contrary, it has been reported to protect animals against certain bacterial infections (Snyder & Walker, 1976; Tocco-Bradley & Kluger, 1984), and zinc lozenges have been recommended as a remedy against the common cold (Eby, Davis & Halcombe, 1984). Speculation that some of the beneficial actions of zinc are exerted not through stimulation of zinc-containing enzymes, but by an effect on the plasma membrane of cells (Chvapil, 1976; Bettger & O'Dell, 1981), is supported by the experiments here described.

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References

Ahnert-Hillger, G., Bhakdi, S., Gratzl, M. 1985. Minimal requirements for exocytosis. A study using PC12 cells permeabilized with staphylococcal α-toxin. *J. Biol. Chem.* **260:**12730–12734

Al-Nasser, I., Crompton, M. 1986. The reversible Ca^{2+}-induced permeabilization of rat liver mitochondria. *Biochem. J.* **239:**19–29

Arbuthnott, J.P. 1983. Host damage from bacterial toxins. *Philos. Trans. R. Soc. London B* **303:**149–165

Avigad, L.S., Bernheimer, A.W. 1976. Inhibition by zinc of hemolysis induced by bacterial and other cytolytic agents. *Infect. Immun.* **13:**1378–1381

Avruch, J., Wallach, D.F.H. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim. Biophys. Acta* **233:**334–347

Bangham, A.D., Flemans, R., Heard, D.H., Seaman, G.V.F. 1958. An apparatus for microelectrophoresis of small particles. *Nature (London)* **182:**642–644

Barrowman, M.M., Cockroft, S., Gomperts, B.D. 1986. Two roles for guanine nucleotides in the stimulus-secretion sequence of neutrophils. *Nature (London)* **319:**504–507

Bashford, C.L., Alder, G.M., Menestrina, G., Micklem, K.J., Murphy, J.J., Pasternak, C.A. 1986. Membrane damage by haemolytic viruses, toxins, complement and other cytotoxic agents: A common mechanism blocked by divalent cations. *J. Biol. Chem.* **261:**9300–9308

Bashford, C.L., Alder, G.M., Patel, K., Pasternak, C.A. 1984. Common action of certain viruses, toxins, and activated complement: Pore formation and its prevention by extracellular Ca^{2+}. *Biosci. Rep.* **4:**797–805

Bashford, C.L., Micklem, K.J., Pasternak, C.A. 1985. Sequential onset of permeability changes in mouse ascites cells induced by Sendai virus. *Biochim. Biophys. Acta* **814:**247–255

Bateman, J.B., Zellner, A. 1956. The electrophoretic properties of red blood cells: The effect of changing pH and ionic strength. *Arch. Biochem. Biophys.* **60:**44–51

Bettger, W.J., O'Dell, B.L. 1981. A critical physiological role of zinc in the structure and function of biomembranes. *Life Sci.* **28:**1425–1438

Bhakdi, S., Tranum-Jensen, J. 1984. Mechanism of complement cytolysis and the concept of channel-forming proteins. *Philos. Trans. R. Soc. London B* **306:**311–324

Boyle, M.D.P., Largone, J.J., Borsos, T. 1979. Studies on the terminal stages of immune hemolysis. IV. Effect of metal salts. *J. Immunol.* **122:**1209–1213

Burnet, F.M. 1949. Haemolysis by Newcastle disease virus. *Nature (London)* **164:**1008

Cameron, J.M., Clemens, M.J., Gray, M.A., Menzies, D.E., Mills, B.J., Warren, A.P., Pasternak, C.A. 1986. Increased sensitivity of virus-infected cells to inhibition of protein synthesis does not correlate with changes in plasma membrane permeability. *Virology* **155:**534–544

Campbell, A.K., Daw, R.A., Hallett, M.B., Luzio, J.P. 1981. Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement. *Biochem. J.* **194:**551–560

Campbell, A.K., Morgan, B.P. 1985. Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. *Nature (London)* **317:**164–166

Carney, D.F., Koski, C.L., Shin, M.L. 1985. Elimination of terminal complement intermediates from the plasma membrane of nucleated cells: The rate of disappearance differs for cells carrying C5b-7 or C5b-8 or a mixture of C5b-8 with a limited number of C5b-9. *J. Immunol.* **134:**1804–1809

Chvapil, M. 1976. Effect of zinc on cells and biomembranes. *Med. Clin. N. Am.* **60:**799–812

Clague, M.J., Cherry, R.J. 1986. Immobilization of band 3 protein and inhibition of melittin action by divalent cations. *Biochem. Soc. Trans.* **14:**883–884

Cullis, P.R., Kruijff, B. de 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* **599:**399–420

Dankert, J.R., Esser, A.F. 1985. Proteolytic modification of human complement protein C9: Loss of poly (C9) and circular lesion formation without impairment of function. *Proc. Natl. Acad. Sci. USA* **82:**2128–2132

Dawson, R.M.C., Elliott, D.C., Elliott, W.H., Jones, J.M. 1969. Data for Biochemical Research, 2nd Ed., pp. 484–485. Oxford University Press, Oxford

Donath, E., Voight, A. 1986. Electrophoretic mobility of human erythrocytes. On the applicability of the charged layer model. *Biophys. J.* **49:**493–499

Dufton, M.J., Cherry, R.J., Coleman, J.W., Stanworth, D.R. 1984. The capacity of basic peptides to trigger exocytosis from mast cells correlates with their capacity to immobilize band 3 proteins in erythrocyte membranes. *Biochem. J.* **223:**67–71

Dunn, L.A., Holz, R.W. 1983. Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J. Biol. Chem.* **258:**4989–4993

Eby, G.A., Davis, D.R., Halcombe, W.W. 1984. Reduction in duration of common colds by zinc gluconate lozenges in a double-blind study. *Antimicrob. Agents Chemother.* **25:**20–24

Edmonds, D.T. 1981. A calculation of the current-voltage characteristic of a voltage-controlled model membrane ion channel. *Proc. R. Soc. London B.* **214:**125–136

Edmonds, D.T. 1984. Electrostatic models for ion channels and pumps. *In:* The Molecular Basis of Movement Through Membranes. C.A. Pasternak and P.J. Quinn, editors. *Biochem. Soc. Symp.* **50:**247–264

Esser, A.F. 1986. C9-mediated cytotoxicity and the function of poly(C9). *UCLA Symp. New Ser.* **46**

Esser, A.F., Bartholomew, R.M., Jensen, F.C., Muller-Eberhard, H.J. 1979. Disassembly of viral membranes by complement independent of channel formation. *Proc. Natl. Acad. Sci. USA* **76:**5843

Frankenhauser, B., Hodgkin, A.L. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (London)* **137:**218–244

French, R.J., Krueger, B.K., Worley, J.F. 1986. From brain to bilayer: Sodium channels from rat neurons incorporated into planar lipid membranes. *In:* Ionic Channels in Cells and Model Systems. R. Latorre, editor. pp. 273–290. Plenum, New York

Furchgott, R.F., Ponder, E. 1941. Electrophoretic studies on human red blood cells. *J. Gen. Physiol.* **24:**447–457

Gambale, F., Menini, A., Rauch, G. 1987. Effects of calcium on the gramicidin A single channel in phosphatidylserine membranes. Screening and blocking. *Eur. Biophys. J.* **14:**369–374

Gomperts, B.D., Baldwin, J.M., Micklem, K.J. 1983. Rat mast cells permeabilized with Sendai virus secrete histamine in

response to Ca^{2+} buffered in the micromolar range. *Biochem. J.* **210:**737–745

Gotze, O., Haupt, I., Fischer, H. 1968. Immune haemolysis: reaction of the terminal complement component. *Nature (London)* **217:**1165–1167

Graham, J.M., Pasternak, C.A., Wilson, R.B.J., Alder, G.M., Bashford, C.L. 1986. Measurement of the effect of cations on the electrophoretic mobility of Lettre cells and human erythrocytes using free flow electrophoresis. *Electrophoresis* **7:**Proc. Int. Electrophoresis Soc., pp. 77–85

Gray, M.A., Austin, S.A., Clemens, M.J., Rodrigues, L., Pasternak, C.A. 1983. Protein synthesis in Semliki Forest virus-infected cells is not controlled by permeability changes. *J. Gen. Virol.* **64:**2631–2640

Hannig, K., Heidrich, H.G. 1974. The use of continuous preparative free flow electrophoresis for dissociating cell fractions and isolation of membranous components. *Meth. Enzymol.* **31:**746–761

Harshman, S., Sugg, N. 1985. Effect of calcium ions on staphylococcal alpha toxin-induced hemolysis of rabbit erythrocytes. *Infect. Immun.* **47:**37–40

Hauser, H., Finer, E.G., Darke, A. 1977. Crystalline anhydrous Ca-phosphatidylserine bilayers. *Biochem. Biophys. Res. Commun.* **76:**267–274

Hauser, H., Shipley, G.G. 1984. Interactions of divalent cations with phosphatidylserine bilayer membranes. *Biochemistry* **23:**34–41

Heard, D.H., Seaman, G.V.F. 1960. The influence of pH and ionic strength on the electrokinetic stability of the human erythrocyte membrane. *J. Gen. Physiol.* **43:**635–654

Henry, D.C. 1938. A source of error in micro-cataphoretic measurements with a cylindrical bore cell. *J. Chem. Soc.* 997–999

Impraim, C.C., Foster, K.A., Micklem, K.J., Pasternak, C.A. 1980. Nature of virally-mediated changes in membrane permeability to small molecules. *Biochem. J.* **186:**847–860

Impraim, C.C., Micklem, K.J., Pasternak, C.A. 1979. Calcium, cells and virus: Alterations caused by paramyxoviruses. *Biochem. Pharmacol.* **28:**1963–1969

Kabat, E.A., Mayer, M.M. 1961. Experimental Immunochemistry. pp. 133–240. Charles Thomas, Springfield, Illinois

Knutton, S., Jackson, D., Graham, J.M., Micklem, K.J., Pasternak, C.A. 1976. Microvilli and cell swelling. *Nature (London)* **262:**52–54

Levine, S., Levine, M., Sharp, K.A., Brooks, D.E. 1983. Theory of the electrokinetic behavior of human erythrocytes. *Biophys. J.* **42:**127–135

Loewenstein, W.R. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* **61:**829–913

McCartney, C., Arbuthnott, J.P. 1978. Mode of action of membrane-damaging toxins produced by Staphylococci. *In:* Bacterial Toxins and Cell Membranes. J. Jeljaszeiwicz, and T. Wadstrom, editors. pp. 89–127. Academic, New York

McLaughlin, S.G., Szabo, G., Eisenman, G. 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J. Gen. Physiol.* **58:**667–687

McLaughlin, A., Eng, W.K., Vaio, G., Wilson, T., McLaughlin, S. 1983. Dimethonium, a divalent cation that exerts only a screening effect on the electrostatic potential adjacent to negatively charged phospholipid bilayer membranes. *J. Membrane Biol.* **76:**183–193

McNiven, A.C., Owen, P., Arbuthnott, J.P. 1972. Multiple forms of staphylococcal alpha-toxin. *J. Med. Microbiol.* **5:**113–122

Masuda, A., Goshima, K. 1980. The role of extracellular calcium ions in HVJ (sendai virus)-induced cell fusion. *Biochim. Biophys. Acta* **599:**596–609

Meech, R.W. 1976. Intracellular calcium and the control of membrane permeability. *In:* Calcium in Biological Systems. C.J. Duncan, editor. pp. 161–191. Cambridge University Press, Cambridge

Menestrina, G. 1986. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: Voltage-dependent inhibition by divalent and trivalent cations. *J. Membrane Biol.* **90:**177–190

Micklem, K.J., Nyaruwe, A., Alder, G.M., Pasternak, C.A. 1984. The effect of Ca^{2+} on virus-cell fusion and permeability changes. *Cell Calcium* **5:**537–550

Miller, C. 1983. Integral membrane channels: Studies in model membranes. *Physiol. Rev.* **63:**1209–1242

Miller, M.R., Castellot, J.J., Pardee, A.B. 1978. A permeable animal cell preparation for studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S phase initiation. *Biochemistry* **17:**1073–1080

Miller, M.R., Castellot, J.J., Pardee, A.B. 1979. A general method for permeabilizing monolayer and suspension culture cells. *Exp. Cell Res.* **120:**421–425

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69:**3561–3566

Morgan, B.P., Sewry, C.A., Siddle, K., Luzio, J.P., Campbell, A.K. 1984. Immunolocalization of complement component C9 on necrotic and non-necrotic muscle fibres in myositis using monoclonal antibodies: A primary role for complement in autoimmune cell damage. *Immunology* **52:**181–188

Op den Kamp, J.A.F. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* **48:**47–71

Pasquale, L., Winiski, A., Oliva, C., Vaio, G., McLaughlin, S. 1986. An experimental test of new theoretical models for the electrokinetic properties of biological membranes. The effect of UO_2^{++} and tetracaine on the electrophoretic mobility of bilayer membranes and human erythocytes. *J. Gen. Physiol.* **88:**697–718

Pasternak, C.A., 1987*a*. Viruses as toxins-with special reference to paramyxoviruses. *Arch. Virol.* **93:**169–184

Pasternak, C.A., 1987*b*. A novel form of host defence: Membrane protection by Ca^{2+} and Zn^{2+}. *Biosci. Rep.* **7:**81–91

Pasternak, C.A., Alder, G.M., Bashford, C.L., Buckley, C.L., Micklem, K.J., Patel, K. 1985*a*. Cell damage by viruses, toxins and complement: Common features of pore-formation and its inhibition by Ca^{2+}. *In:* The Molecular Basis of Movement Through Membranes C.A. Pasternak and P.J. Quinn, editors. *Biochem. Soc. Symp.* **50:**247–264

Pasternak, C.A., Bashford, C.L., Micklem, K.J. 1985*b*. Ca^{2+} and the interaction of pore-formers with membranes. *Proc. Int. Symp. Struct. Interactions, Suppl. J. Biosci.* **8:**273–291

Pasternak, C.A., Micklem, K.J. 1973. Permeability changes during cell fusion. *J. Membrane Biol.* **14:**293–303

Pasternak, C.A., Micklem, K.J. 1974*a*. The biochemistry of virus-induced cell fusion. Changes in membrane integrity. *Biochem. J.* **140:**405–411

Pasternak, C.A., Micklem, K.J. 1974*b*. Virally-mediated membrane changes: Inverse effects on transport and diffusion. *Biochem. J.* **144:**593–595

Patel, K., Pasternak, C.A. 1985. Permeability changes elicited by influenza and Sendai virus: Separation of fusion and leakage by pH jump experiments. *J. Gen. Virol.* **66:**767–775

Poste, G., Pasternak, C.A. 1978. Mechanisms of virus-induced cell fusion. *In:* Cell Surface Reviews 5. G. Poste and G.L. Nicolson, editors. pp. 306–349. North Holland Publishing, Amsterdam, New York, Oxford

Prasad, A.S. 1978. Trace elements and iron in human metabolism. pp. 251–346. John Wiley & Sons, Chichester

Prince, R.C., Gunson, D.E., Scarpa, A. 1985. Sting like a bee! The ionophoric properties of melittin. *TIBS* **10:**99

Ramm, L.E., Whitlow, M.B., Mayer, M.M. 1982. Transmembrane channel formation by complement: Functional analysis of the number of C5b6, C7, C8 and C9 molecules required for a single channel. *Proc. Natl. Acad. Sci. USA* **79:**4751–4755

Ramm, L.E., Whitlow, M.B., Koski, C.L., Shin, M.L., Mayer, M.M. 1983. Elimination of complement channels from the plasma membranes of U937, a nucleated mammalian cell line: Temperature dependence of the elimination rate. *J. Immunol.* **131:**1411–1415

Ramm, L.E., Whitlow, M.B., Mayer, M.M. 1985. The relationship between channel size and the number of C9 molecules in the C5b-9 complex. *J. Immunol.* **134:**2594–2599

Sandvig, K., Olsnes, S. 1982. Entry of the toxic proteins abrin, modeccin, ricin and diphtheria toxins into cells. I. Requirement for calcium. *J. Biol. Chem.* **257:**7495–7503

Sandvig, K., Olsnes, S. 1985. Effect of the chaotropic ions thiocyanate and perchlorate on the entry of ricin into Vero cells. *Biochem. J.* **228:**521–523

Schreiber, A.D. 1983. Paraoxysmal nocturnal hemoglobinuria revisited. *N. Engl. J. Med.* **309:**723–725

Schulz, I., Heil, K. 1979. Ca^{2+} control of electrolyte permeability in plasma membrane vesicles from cat pancreas. *J. Membrane Biol.* **46:**41–70

Schwartz, W., Passow, H. 1983. Ca^{2+}-activated K^+ channels in erythrocytes and excitable cells. *Annu. Rev. Physiol.* **45:**359–374

Seeman, P. 1972. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24:**583–655

Sillen, L.G., Martell, A.E., editors. 1964. Stability Constants of Metal Ion Complexes. *Chem. Soc. Publ.* **17,** London

Sims, P.J., Lauf, P.K. 1978. Steady-state analysis of tracer exchange across the C5b-9 complement lesion in a biological membrane. *Proc. Natl. Acad. Sci. USA* **75:**5669–5673

Sims, P.J., Lauf, P.K. 1980. Analysis of solute diffusion across the C5b-9 membrane lesion of complement: Evidence that individual C5b-9 complexes do not function as discrete uniform pores. *J. Immunol.* **125:**2617–2625

Snyder, S.L., Walker, R.I. 1976. Inhibition of lethality in endotoxin-challenged mice treated with zinc chloride. *Infect. Immun.* **13:**998–1000

Taylor, R.E., Armstrong, C.M., Bezanilla, F. 1976. Block of sodium channels by external calcium ions. *Biophys. J.* **16:**27a

Tocco-Bradley, R., Kluger, M. 1984. Zinc concentration and survival in rats infected with *S. typhimurium*. *Infect. Immun.* **45:**332–338

Underwood, E.J. 1977. Trace Elements in Human and Animal Nutrition. 4th Edition, pp. 196–242. Academic, New York

Unwin, P.N.T., Ennis, P.D. 1984. Two configurations of a channel-forming membrane protein. *Nature (London)* **307:**609–613

Volsky, D.J., Loyter, A. 1978. Role of Ca^{2+} in virus-induced membrane fusion. Ca^{2+} accumulation and ultrastructural changes induced by Sendai virus in chicken erythrocytes. *J. Cell Biol.* **78:**465–477

Wilkins, D.J., Otterwill, R.H., Bangham, A.D. 1962. On the flocculation of sheep leukocytes: 1. Electrophoretic studies. *J. Theor. Biol.* **2:**165–175

Wilson, S.P., Kirshner, N. 1983. Calcium-evoked secretion from digitonin-permeabilized adrenal medullary chromaffin cells. *J. Biol. Chem.* **258:**4994–5000

Wolf, H., Gingell, D. 1983. Conformational response of the glycocalyx to ionic strength and interaction with modified glass surfaces: Study of live red cells by interferometry. *J. Cell Sci.* **63:**101–112

Wolff, D., Vergara, C., Cecchi, X., Latorre, R. 1986. Characterization of large-unitary-conductance calcium-activated potassium channels in planar lipid bilayers. *In:* Ionic Channels in Cells and Model Systems. editor. R. Latorre, p. 307–322. Plenum, New York

Woodhull, A.M. 1973. Ionic block of sodium channels in nerve. *J. Gen. Physiol.* **61:**687–708

Wyke, A.M., Impraim, C.C., Knutton, S., Pasternak, C.A. 1980. Components involved in virally-mediated membrane fusion and permeability changes. *Biochem. J.* **190:**625–638

Wyn Jones, R.G., Lunt, O.R. 1967. The function of calcium in plants. *Bot. Rev.* **33:**407–426

Yamaizumi, M., Uchida, T., Okada, Y. 1979. Macromolecules can penetrate the host cell membrane during the early period of incubation with HVJ (Sendai virus). *Virology* **95:**218–221

Yamamoto, D., Yeh, J.Z., Narahashi, T. 1984. Voltage-dependent calcium block of normal and tetramethrin-modified sodium channels. *Biophys. J.* **45:**337–344

Yamamoto, K., Takahashi, M. 1975. Inhibition of the terminal stage of complement-mediated lysis (reactive lysis) by zinc and copper ions. *Int. Arch. Allergy Appl. Immunol.* **48:**653–663

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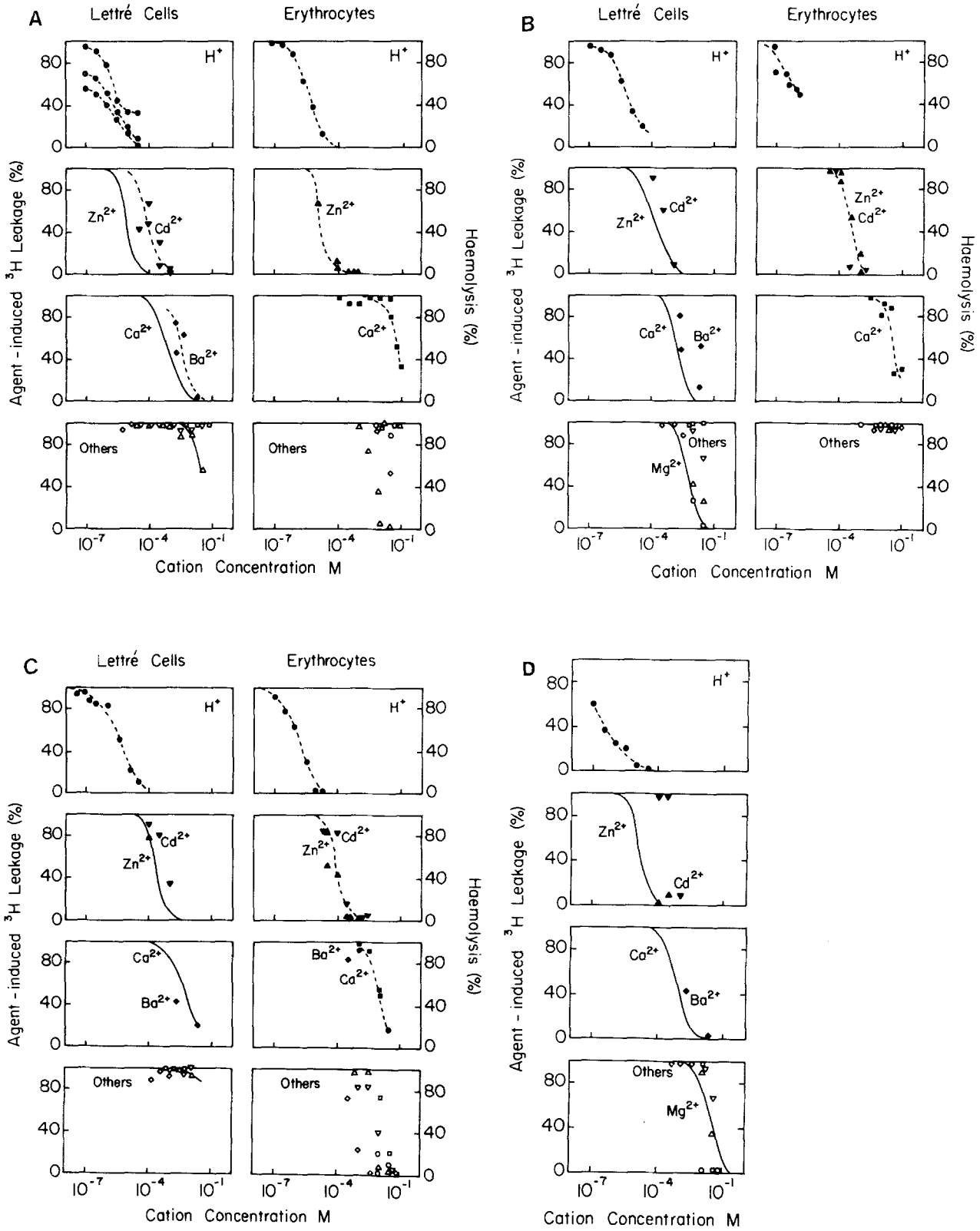
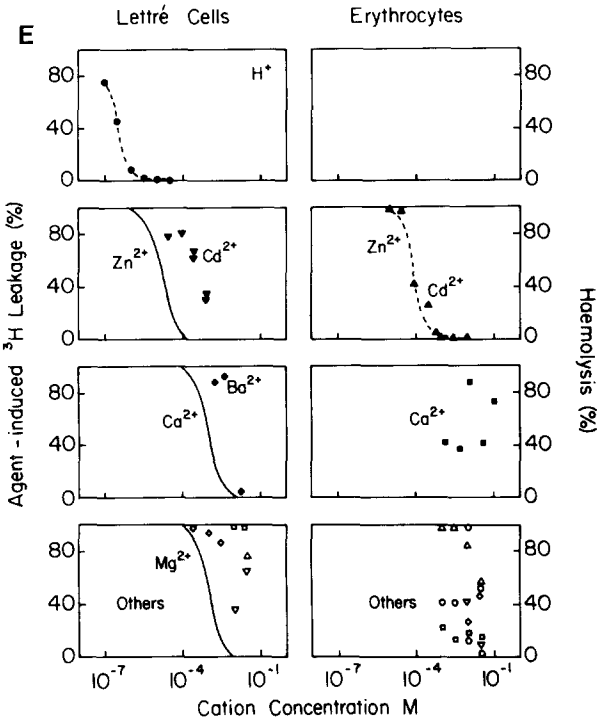
Appendix

Data for Protection Against Leakage Induced by Pore-Forming Agents

Lettre cells preincubated with [^{3}H]choline (left-hand panel), or erythrocytes (right-hand panel), suspended in HBS were exposed to Sendai virus (panels *A*), melittin (panels *B*), *S. aureus* α-toxin (panels *C*), polylysine (panel *D*) or Triton X-100 (panels *E*) under conditions such that approximately 50% of phosphoryl [^{3}H]choline leaked from Lettre cells and approximately 50% of erythrocytes were hemolyzed during 10-min incubation at 37°C. Human erythrocytes were used for Sendai virus, melittin and triton; rabbit erythrocytes were used for α-toxin; polylysine does not induce significant hemolysis in either type of cell, and results are shown for Lettre cells only.

Cations at the concentrations indicated were present during the incubation as follows:


A
Lettré Cells
Erythrocytes
B
C
D
H+
Zn2+
Cd2+
Ca2+
Ba2+
Mg2+
Others
Agent - induced 3H Leakage (%)
Haemolysis (%)
Cation Concentration M
10-7
10-4
10-1
0
40
80



Top pair of figures: H^+ (pH set with citrate-phosphate buffers according to Dawson et al. (1969) and diluted tenfold in HBS lacking HEPES). In the case of Sendai virus (panel *A*), leakage is shown for cells suspended in the above media (●), or similar media diluted with 0.3 M mannitol to give an ionic strength of 0.025 (◐) and 0.075 (◑), respectively.

Next pair of figures: $ZnSO_4$ (solid line for Lettre cells; ▲ for erythrocytes) or $CdSO_4$ (▼).

Next pair of figures: $CaCl_2$ (solid line for Lettre cells; ■ for erythrocytes) or $BaCl_2$ (◆).

Bottom pair of figures: $MgCl_2$ (solid line for Lettre cells; ○ for erythrocytes), dimethonium Br_2 (□), spermine (△), spermidine (▽) or $Co(NH_3)_6\ Cl_3$ (◇).

Broken lines (drawn by eye) join the various data points. The solid lines, constructed from previously published data (Pasternak et al., 1985*a,b;* Bashford et al., 1986), are shown for comparison. In order to clarify any differences between Zn^{2+} and Cd^{2+}, between Ca^{2+} and Ba^{2+}, and between Mg^{2+} and other divalent cations, the appropriate atomic symbols have been added to the panels. All data for ^3H leakage from Lettre cells are scaled such that 'background' leakage from cells not exposed to agent in the presence of the various cations (generally 10 to 30%) is 0. Erythrocytes not exposed to agent showed no hemolysis under any conditions.