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Erythrocyte stability under imposed fields

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Abstract. Lysis of erythrocytes offers an unique opportunity to probe the fine structure of the bilayer as a function of its state of energization. Critical monitoring of the volumes, ion fluxes and related measures in erythrocytes exposed to a variety of milieu and treatments showed that one can critically distinguish the nature of the prelytic perturbations and the proximate forces actually responsible for the disruption of the membranes among surface charge density, elastic energy etc.

Keywords. Osmolysis; erythrocytes; detergents; pores; polyols; ionophores; ionic strength.

Introduction

The bimolecular leaflet structure of the biological membranes is an equilibrium structure in the strict thermodynamic sense (Israelachvili *et al.,* 1980). This planar, smectic mesophase offers the expedient solution to pack amphiphilic lipid molecules which are characterized by bulky acyl chain moieties compared to their relatively small head groups. As a consequence, any imposed field of adequate magnitude could affect this equilibrium state, the end result being a disruption of the membrane (Sitaramam, 1981). Two thoughts of considerable importance need to be entertained: firstly, a shift in the equilibrium state must necessarily be identified with enhanced internal energy of the system since,

$$\Delta G = -RT \ln K_{eq},$$

such that disruption of membranes represents a most conclusive evidence for a change in the internal energy of the system ($dU \neq 0$); secondly, disruption not only entails flux of molecules across the bilayer barrier, but also entails and is preceded by a flux of lipid molecules within the bilayer (Sitaramam 1988a; Mathai and Sitaramam, 1989; Sitaramam *et al*., 1989). The central question is whether such a flux of lipid molecules in the prelytic domain is continuous or catastrophic. If catastrophic, the membrane would exhibit an abrupt enhancement of conductance at a critical field intensity without any evidence for prelytic disturbances in conductance. If continuous, two consequences arise: firstly, the prelytic domain would be characterized by enhanced porosity of the membrane; secondly, and most importantly, this enhanced porosity necessarily accompanies and is a forerunner of disruption in any situation of imposed fields, including even the physiological energy transduction mechanisms (Mathai and Sitaramam, 1989; Sitaramam *et al*., 1989).

In recent years, it has been demonstrated that disruption is but an extreme case of variable porosity in membranes with several kinds of imposed fields, *e.g*., gravitational fields (Sitaramam and Sarma, 1981; Sarma and Sitaramam, 1982), respiration (Sambasivarao and Sitaramam, 1985), ATP hydrolysis (Sambasivarao *et al*., 1988), electrostatic fields created by the unscreened fixed charges on the RBC membranes when acutely suspended in media of low ionic strength (Sambasivarao

Abbreviations used: CTAB. Cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate.

et al., 1986; Sitaramam *et al.*, 1988). Comparable evidence exists in the case of pulses of electrical fields (Teissie and Tsong, 1977), action potentials (Villegas *et al.*, 1966) etc. The erythrocyte offered an excellent tool since its lysis can be monitored readily. A systematic investigation led to a number of insights into the nature of disruption and the proximate fields responsible for it, often at variance with the generally held beliefs. The results on osmotic lysis, 'hypertonic lysis' in non-electrolyte media, lysis by detergents and lysis by ionophores are briefly discussed.

Materials and methods

These are as indicated in the legends to figures and in the corresponding cross references.

Results and discussion

Hypotonic lysis of erythrocytes

Erythrocytes, when suspended in hypotonic media, would swell with consequent increase in the elastic energy of the membrane. The elastic energy of the membrane would be parabolically related to the change in the average interfacial area of the head groups of the lipids (Israelachvili *et al.*, 1980); when the elastic energy exceeds the intermolecular cohesive forces that define the bilayer geometry, the cell disrupts. The specific conclusions borne out by the experiment were (i) an increase in the (elastic) free energy of the membrane would affect the equilibria of the component protein molecules of the membrane, as demonstrated by a prelytic increase in the conductance of the potassium *via* calcium-independent, potassium channels (Sambasivarao *et al.*, 1986); (ii) the onset of lysis would correspond to the maximal volume of the cells to lysis, as seen by Coulter profiles of cell radii (figure 1A); (iii) treatment with certain ionophores would lead to loss of internal potassium as well as a corresponding shift in the onset of lysis to lower tonicities; whereas enhanced permeation to the external NaCl by other ionophores would lead to a shift of the onset to greater tonicities (figure 1B); (iv) this would be due to preferential loss of internal potassium or gain in sodium from without, as can be demonstrated by light scatter studies on volume changes (figure 1C,D); (v) the light scatter studies exhibit a precise relationship between potassium loss (= volume change) and turbidity changes, whose limits can be determined critically in a simultaneous measurement of potassium loss and turbidity (figure 1E). Thus, the hypotonic lysis could be precisely defined in terms of the' component physical changes; the field responsible could be identified unequivocally as the elastic energy profile as a slave variable tracking the osmotic gradient. Variations in elastic energy were seen to have their own consequence of affecting the structural/dynamic K_{eq} of the potassium channel towards greater conductance (Sambasivarao *et al.*, 1986). The missing link was the fact that progressive lysis occurs even in saline media albeit to a small degree; and what could be the reason for it?

Lysis in non-electrolyte media

The phenomenon of hypertonic disruption of erythrocytes in non-electrolyte media

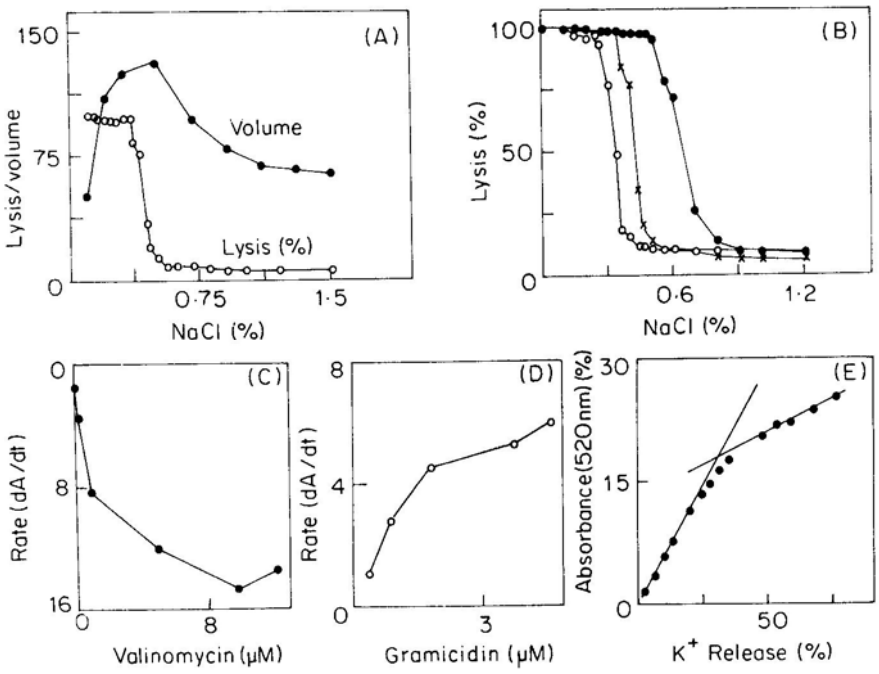

Figure 1 (**A**) The mode of the frequency distributions of the volume profiles (in femtolitres) of rat erythrocytes obtained with Coulter Counter ZM with Channelyzer C256 at varying NaCl concentrations was plotted along with per cent lysis profiles (hemoglobin released into cellfree supernatents). Note that the maximal volume corresponds to the onset of lysis, but the completion of lysis does not correspond to the least volume. (**B**) Per cent lysis profiles in control (×), 1 μM valinomycin-treated (O) and 10 μM gramicidin A-treated (●) rat erythrocytes. Note the concordant shift in the onset and completion of lysis corresponding to the efflux and influx of the solute with each ionophore. (**C**) Rate of change in absorbance of constantly stirred samples of rat erythrocytes at 520 nm as a function of the varying concentrations of valinomycin. The direction was reversed in this and the next (**D**) plots to keep it parallel to the volume changes. Note the saturation kinetics. (**D**) Rate of change of absorbance in rat erythrocytes with gramicidin, plotted as in (**C**). (**E**) The efflux of potassium was measured with an Orion potassium electrode and an Orion 901 Ionalyzer. The change in absorbance was simultaneously monitored in a parallel sample. Both measurements were plotted as a function of time after addition of 1 uM valinomycin to the stirred suspensions. The lines were obtained by the least square linear regression analysis to show that up to 15% change in turbidity reliably measures volume changes due to solute fluxes.

has been characterized as one of disaffected equilibrium state of the erythrocyte membranes due to decreased screening of the external fixed negative charges (Sambasivarao *et al.*, 1986; Sitaramam *et al*., 1988). The consequent increase in self-potential of the charged 'sphere' would account for a variety of dynamic fluxes of ions and solutes including an instability in the bilayer (Sitaramam *et al*., 1988). Figure 2A shows the phenomenon of hypertonic disruption in an osmometric profile of erythrocyte lysis (=hemoglobin release) in sucrose media. Figure 2B also shows a parallel increase in lysis in NaCl media with time and this increase was inversely proportional to the ionic strength of the media. Thus, lysis during prolonged periods in NaCl media was shown to be entirely due to the exposure of

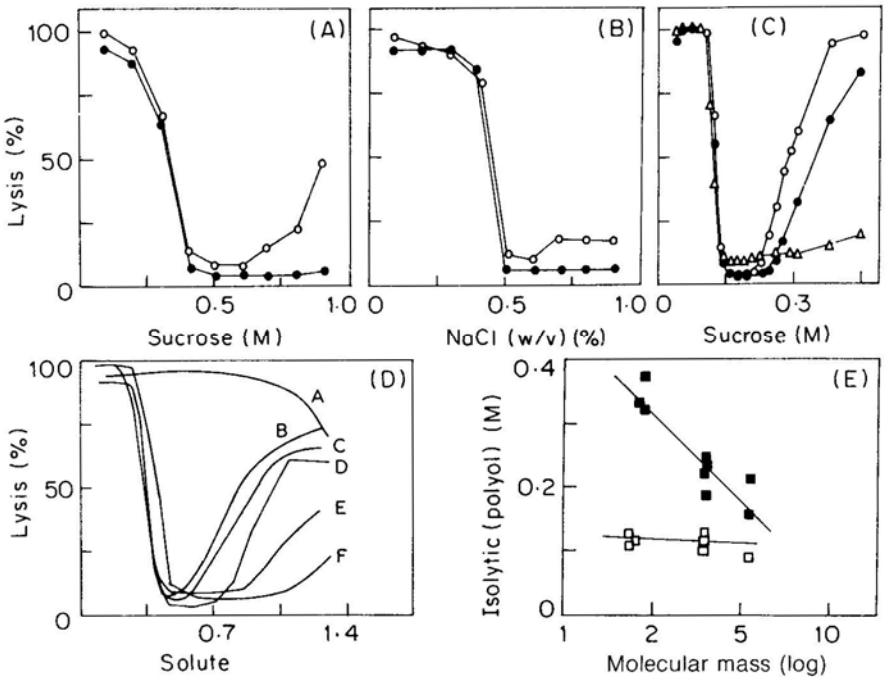

Figure 2. (**A**) Per cent lysis (as in figure 1A) at 15 min and 4 h (●)and 4 h (O) in sucrose media of varying tonicity. Note that the enhanced lysis at 4 h also enhances with osmolality of the media, hence the name 'hypertonic disruption'. (**B**) Per cent lysis in NaCl media, as in (**A**). Note that NaCl media also exhibit finite lysis at hypertonicities similar to sucross media. (●), 15 min; (O), 4 h incubations. (**C**) Effect of ionic detergents on hypertonic disruption profiles in sucrose media at 4h (as in A). (●) Control; (○), 34·7 μM SDS (Δ), 2·74μM CTAB. Note that nonionic detergents had no effect and that these pronounced stimulatory and inhibitory effects of these detergents of opposing charge were without any influence in the hypotonic domain or the basal lysis in the same osmotic profiles. (**D**) Hypertonic disruption profiles in polyols of different molecular mass. A, Erythritol; B, lactose; C, maltose; D, sucrose; E, mannitol and F, glucose. Note that there is no distinction in the extent of lysis in the hypotonic domain among the polyols with the marginal exception of glucose. Per cent lysis was plotted against the molar equivalents of NaCl % w/v for each polyol (Sitaramam *et al.,* 1988). (**E**) Effect of molecular mass on per cent lysis in the hypertonic and hypotonic domains. (■), Polyol concentration corresponding to 20% lysis in the hypertonic domain; (□), polyol concentration corresponding to 50% lysis in the hypotonic domain. The lines represent linear regression by least squares. Data from an extended version of (**D**).

fixed charges on the surface of erythrocytes and these charges are most likely to be proteinaceous in origin and not from the glycocalyx. Figure 2C shows that this hypertonic disruption is due to externally faced negative charges since it was inhibited by cetyltrimethylammonium bromide (CTAB), a cationic detergent, whereas it was accentuated by sodium dodecyl sulfate (SDS), an anionic detergent. Erythrocyte lysis offers an exquisite example of the consequences of enhanced free energy of the membrane in terms of the strict molecular weight dependence of the inferred permeability to polyols. Figure 2D shows the effect of various polyol media on the osmometric profiles of lysis in these media of low ionic strength. Figure 2E shows the derived plot of per cent lysis in hypo- and hyper-tonic domains of fixed

lysis *vis-a-vis* the molecular weight of the external polyol showing the behaviour of the erythrocyte membrane as a perfect molecular sieve under these conditions of disaffected equilibrium state.

Disruption of erythrocytes in the presence of detergents

Figure 3A, B shows the effect of CT AB and SDS on lysis of erythrocytes in sucrose and NaCl media. These data on the minimal lytic concentrations for these ionic detergents (since non-ionic detergents did not exhibit dependence on the ionic strength of the medium) can be critically accounted for in terms of charge repulsion/attraction and charge screening. A negatively charged membrane exhibits greater interfacial concentration of the detergent of opposite charge in the unscreened state, i.e., in non-electrolyte media, rather than in electrolyte media. Figure 3C shows that the effect of CTAB is 2-fold in nonelectrolyte media: it causes inhibition of lysis at lower concentrations and enhances lysis at higher concentrations by its detergent action. Preliminary studies indicate that the lysis by different ionic detergents in RBC is not comparable; the anionic detergents cause lysis primarily by a charge-mediated instability in the membrane rather than by detergency, whereas the cationic detergents appear to cause lysis only by detergency. The osmotic dependence of such lysis appears to suggest that the prelytic hole produced by anionic detergents is much smaller than that by the cationic detergents as evidenced by the lysis profiles in polyols of varying magnitude (data not given). These studies indicate that the proximate cause of lysis is different for different detergents, depending on their charge as well as on the net charge density on the membrane.

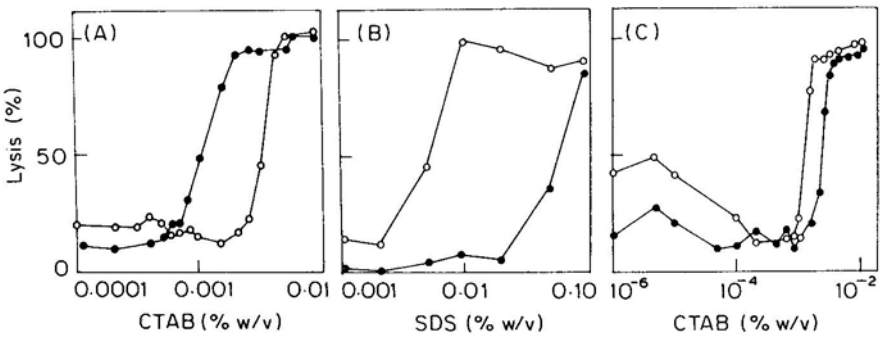

Figure 3. (**A**) Per cent lysis (release of hemoglobin into cell-free supernatants) in rat erythrocytes suspended in 0·31 M sucrose (●) and 0·155 M NaCl (O) media in the presence of various concentrations of CTAB. (**B**) Per cent lysis in rat erythrocytes suspended in 0·31 M sucrose (●) and 0·155 M NaCl (O) media in the presence of various concentrations of SDS. (**C**) Effect of CTAB on per cent lysis of rat erythrocytes in sucrose media at 15 min (as in **A** and **B**) (●) and at 4 h (O), both at room temperature. Note that the 4 h incubation refers to hypertonic disruption demonstrated in figure 2, which increases with time and sucrose concentration. CTAB has a marked biphasic effect at 4 h due to its dual role as a charged species (protective) and a detergent (lytic).

Lysis of erythrocytes in the presence of ionophorous antibiotics

Primarily, any antibiotic that enhances the net permeability of the membrane to the

external osmolyte will cause lysis equivalent to hypotonic lysis. This can be critically investigated by a combination of osmolysis profiles and Coulter profiles of volume/diameter of these cell populations. Our very first attempts at the demonstration of the action of valinomycin and gramicidin D on such profiles exhibited a major contradiction worthy of note. The gramioidin-treated cells exhibited loss of internal potassium, increase in cell volume indicating larger uptake of sodium ions from the medium such that the onset of lysis still corresponds to the maximal volume of the cells, i.e., hematocrit. The same was not true of valinomycin-treated cells. The cells contracted due to loss of potassium from within. However, the onset of hypotonic lysis was not accompanied by volume expansion, indicating the most odd situation of hypotonic lysis without elastic stretch (figure 4). Thus, the cause of this hypotonic lysis cannot be due to volume-dependent elastic stretch. Studies on the ionic dependence as well as studies with all other known monovalent-selective cationophores indicated that the cause of lysis was the expression of the internal charges on the inner leaflet of the cell membrane and the mechanism was once again a charge-mediated instability in the membrane.

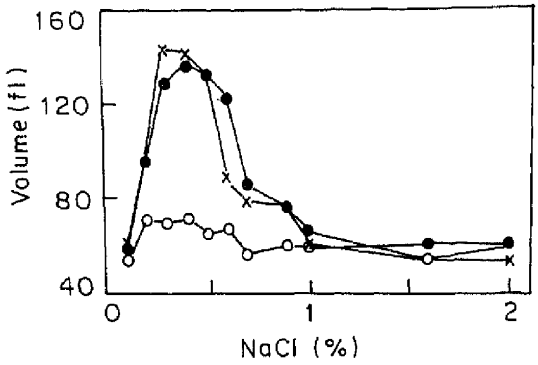

Figure 4. Mode of the frequency distributions of the volume profiles (in femtolitres) of rat erythrocytes obtained with Coulter Counter ZM with Channelyzer C256 at varying NaCl concentrations. (×), Control; (●), 10 μM gramicidin-treated; (O), 1 μM valinomycin-treated. The cells were washed thrice in 0·9% NaCl, pre-treated with the ionophores in small aliquots and diluted into NaCl media of varying tonicity for Coulter measurements. Lysis results for these erythrocytes were as in figure 1B.

Lysis is a result of major deviation of the resting membrane from its equilibrium structure, with defined and measurable prelytic events. Most current literature attempts to view the bilayer and its components as of invariant interactions within themselves and with each other in the structural, kinetic as well as dynamic senses. Each subsystem in the membrane relaxes, when challenged with an increase in free energy, with its own characteristic relaxation time. This gives rise to complex dynamics whose causality is however entirely definable and tractable. The central question ultimately relates to whether we wish to view the bilayer as isoentropic and planar, or, negentropic capable of structural evolution, since the latter view would violate the traditional notions in chemical thermodynamics. These notions relate to the definition of the chemical potential of a species on the assumption of invariant or negligible changes in entropy, internal energy and volume work. Our

studies on erythrocytes and mitochondria have given lie to this generally held assumption, requiring an active investigation in the identification of the proximate cause-effect relationship of the same event such as lysis under diverse conditions.

Acknowledgement

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References

Israelachvili, J. N., Marcelja, S. and Horn, R. G. (1980) *Q. Rev. Biophys.,* **13,** 121.
Mathai, J. C. and Sitaramam, V. (1989) *Biochim. Biophys. Acta,* **976,** 214.
Sambasivarao, D. and Sitaramam, V. (1985) *Biochim. Biophys. Acta,* **806,** 195.
Sambasivarao, D., Rao, N. M. and Sitaramam, V. (1986) *Biochim. Biophys. Acat,* **857,** 48.
Sambasivarao, D., Kramer, R., Rao, N. M. and Sitaramam, V. (1988) *Biochim. Biophys. Acta,* **933,** 200.
Sarma, M. K. J. and Sitaramam, V. (1982) *Biochem. Biophys. Res. Commun.,* **105,** 362.
Sitaramam, V. (1981) *Indian J. Biochem. Biophys.,* **18,** 96.
Sitaramam, V. (1988a) *J. Sci. Ind. Res. India,* **47,** 395.
Sitaramam, V. (1988b) *Indian J. Biochem. Biophys.,* **25,** 172.
Sitaramam, V. and Sarma M. K. J. (1981) *Proc. Natl. Acad. Sci. USA,* **78,** 3441.
Sitaramam, V., Sambasivarao, D. and Rao, N. M. (1988) *Indian J. Biochem. Biophys.,* **25,** 590.
Sitaramam, V., Sambasivarao, D. and Mathai, J. C. (1989) *Biochim. Biophys. Acta,* **975,** 252.
Teissie, J. and Tsong, T. Y. (1977) *Proc. Natl. Acad. Sci. USA,* **74,** 1923.
Villegas, R., Villegas, G. M., Blei, M., Herrera, F. C. and Villegas, J. (1966) *J. Gen. Physiol.,* **50,** 43.