Biochimica et Biophysica Acta 857 (1986) 48–60
Elsevier

BBA 73045

Anomalous permeability and stability characteristics of erythrocytes in non-electrolyte media

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(Received October 8th, 1985)

Key words: Hypertonic disruption; Osmolysis; Membrane surface charge; Ionic detergent; (Rat erythrocyte)

The permeability characteristics of the erythrocyte membrane were critically evaluated in electrolyte and non-electrolyte (sucrose) media by ion-selective electrodes and radioactive polyol fluxes as well as by the novel technique of osmometry. K^+ efflux demonstrated a linear osmotic susceptibility distinct from Na^+ influx upon incubation in NaCl media of various tonicities. In non-electrolyte media, acidification of the medium, large fluxes of K^+, sucrose and even haemoglobin (as manifest by hypertonic disruption) were consistent with enhanced porosity of the bilayer due to the field created by surface charge density leading to density fluctuations in the bilayer.

Introduction

Despite major efforts over half a century, several ambiguities persist in the understanding of the behaviour and stability of erythrocytes in electrolyte and non-electrolyte media [1–6]. The stability/lysis of erythrocytes can be categorized broadly into osmotic and non-osmotic mechanisms. The osmotic mechanism of lysis, which includes hypotonic and colloidal lysis, is primarily mechanical and is characterized by influx of water due either to low osmolality of the external medium or to a decrease in the reflection coefficients to the external osmolytes due to a change in membrane porosity. Non-osmotic mechanisms include physical disruption due to detergency, toxins, action of lipases, etc., and involve a perturbation in the bilayer structure rather than mere influx of water.

A testable consequence of such a distinction lies in the retarding influence of hypertonicity only on the osmotic mechanism of lysis. The absence of such a clear distinction, compounded by lack of adequate osmotic methodology, has resulted in much ambiguity in the past. For instance, suspension of erythrocytes from a variety of species in non-electrolyte media was known to result not only in ion and solute fluxes but also in lysis [1–3,5–8]. Changes in the volume (cf. Ref. 9) of a perfect osmometer cannot exist without concomitant osmolyte fluxes (cf. Refs. 2 and 3). Similarly osmolyte fluxes are inconsistent with the notion of invariant isotonicity. Neglect of these logical contradictions has led to considerable confusion with regard to the mechanism of lysis in hypertonic electrolyte and non-electrolyte media, on resuspension of the cells into so-called isotonic media (cf. Refs. 2 and 3). An explanation for electrolyte fluxes, on suspension of erythrocytes in non-electrolyte media, was thus far restricted to reversal of Donnan potentials across a membrane of invariant semipermeability (cf. Ref. 1), which would be untenable if the bulk porosity of the membrane changed even to non-electrolytes such as polyols.

Since the primary mechanistic distinction lies in the influence of external osmotic pressure on lysis, we investigated, in depth, the influence of

osmotic pressure due to electrolyte and non-electrolyte media on ion and solute fluxes as well as lysis. We have developed in recent years the novel technique of osmometry in which the critical external osmotic pressure corresponding to lysis is evaluated by break-point analysis, which yields valuable information on the internal solute content of cells and organelles as well as changes in the reflection coefficients to internal and external osmolytes [10–13]. In this paper, we show that: (i) there exists a causal relationship between stability and permeability characteristics of erythrocyte membranes in electrolyte and non-electrolyte media; (ii) the anomalous permeability and stability characteristics of the erythrocytes in non-electrolyte media arise due to a non-osmotic mechanism of dynamic perturbations in the lipid bilayer, owing to the field created by unscreened fixed negative surface charges; (iii) the paths of diffusion of ions such as K^+ in electrolyte and non-electrolyte media as well as the physical principles governing such diffusion are essentially different.

Materials and Methods

Materials

Sucrose, Tris, valinomycin, gramicidin S, were obtained from Sigma, U.S.A. Sodium dodecyl sulphate was obtained from Bio-Rad Laboratories. All other reagents were of analytical grade. Uniformly labelled [^{14}C]sucrose was obtained from the Bhabha Atomic Research Centre, Bombay.

Methods

Isolation of erythrocytes. Erythrocytes were obtained from heparinized blood from non-anaesthetized rats and were washed twice in 0.9% NaCl at room temperature, as described earlier [12]. The packed cell volume (PCV) of the stock suspension was determined by the capillary micro-hematocrit method.

Acute osmolysis of rat erythrocytes. Erythrocytes equivalent to 20 μl of whole blood were suspended in 3.0 ml of various concentrations of NaCl or sucrose (as specified) containing 3.3 mM Tris-HCl (pH 7.4), for 15 min at room temperature, unless specified otherwise. After incubation, the cells were centrifuged at $2000 \times g$ for 10 min and the absorbance at 540 nm was measured in the supernatants. Lysis of erythrocytes was calculated taking the absorbance of haemoglobin of erythrocytes suspended in 3.3 mM Tris-HCl buffer as equivalent to 100% lysis. Osmolytic profiles were obtained by plotting percent lysis against external solute concentration.

In experiments in which NaCl was substituted with other solutes for osmolytic experiments, external solute concentration was generally expressed as percentage equivalent of NaCl to facilitate comparison. For instance, 0.31 M sucrose is expressed as 0.9% (w/v) NaCl equivalent (i.e., 0.155 M NaCl). The osmolality of solutions was monitored using a Wescor vapour pressure osmometer (model 5100C).

Gradual haemolysis. 1.0-ml aliquots of a suspension of twice washed erythrocytes in 0.9% NaCl (w/v) were loaded into a number of dialysis bags and were separately dialysed at room temperature for 2 h in 200 ml each of varying concentrations of NaCl solutions (0.1% to 0.9%). The dialysing medium also contained 3.3 mM of Tris-HCl (pH 7.4). At the end of dialysis, the contents of each bag were collected individually and percentage lysis was monitored as A_{540nm} of cell-free supernatants, after correction for incidental changes in the volume of the dialysate. The percentage lysis, thus obtained, was plotted as a function of external NaCl concentration employed during dialysis (cf. Ref. 14).

Release of K^+ from erythrocytes. Loss of K^+ from erythrocytes into the medium was measured, under constant, gentle magnetic stirring, with an Orion 93-19 K^+-selective double-junction membrane electrode assembly as a function of time, at 30°C in unbuffered media. 100 μl of stock erythrocyte suspension was added to 30 ml of the specified medium. K^+ release was monitored consequent to acute suspension of erythrocytes in the medium as also on gradual exposure of the cells to the medium, which was obtained by dialysing the cells against varying external NaCl concentrations. K^+ concentration in the dialysing medium was also monitored with K^+ electrode. Total internal K^+ was measured upon addition of 0.04% (v/v) Triton X-100. K^+ concentrations were obtained from detailed standard curves obtained individually in specified NaCl and sucrose media at the same time.

Drifts in electrode potentials with time were minimal upto 3 h of measurement and were corrected by detailed external standards run in parallel. The K^+ electrode was further calibrated against K^+ estimations in erythrocyte lysates by flame photometry (Toshniwal, India) as well as by atomic absorption spectrometry (Varian).

Release of H^+ from erythrocytes. pH was measured with a double-junction Ross pH electrode (using a Orion 901 ionalyzer), as a function of time in varying concentrations of sucrose and NaCl media of 30 ml each, without any buffer in the medium. The pH of the sucrose/NaCl media at the start of the experiment was brought to 7.0 by adding minute amounts of 0.01 M NaOH. 100 μl of stock erythrocytes were added to the specified medium at time, $t = 0$, under constant gentle magnetic stirring.

Equilibration of [^{14}C]sucrose across erythrocyte membrane. Stock erythrocytes (packed cell volume 70%) were diluted with 0.206 M sucrose medium with 3.3 mM Tris-HCl (pH 7.4), containing 0.2 μCi per ml of radioactive sucrose, such that the NaCl contamination from the stock red cells was approx. 0.5 mM in the incubation medium. 1.0 ml aliquots of cell suspension at each specified time point were layered on 10 ml of 0.6 M sucrose in 1.0 cm diameter test-tubes and were pelleted under 2 min at 3000 rpm at 30°C. The pellets, after thorough cleaning of the centrifuge tube wall, were extracted with 1.0 ml of 0.1% NaCl at 100°C for 2 min and the supernatants were counted for radioactivity in Bray's mixture, in a Packard Tri-Carb liquid scintillation spectrometer (model 3255).

In several combinations of incubation conditions attempted, this procedure yielded the lowest blank (corresponding to 0.1% of counts loaded) and the highest signal-to-noise ratio (less than 4% of counts as would be expected from 100% equilibration across cells assumed to have a constant packed cell volume), due to an effective combination of gradient as well as unlabelled sucrose wash externally.

Osmometric analysis. Fig. 1 illustrates the osmolytic profiles in control, valinomycin (10^{-6} M)-, and gramicidin S (10^{-5} M)-treated erythrocytes. Loss of the internal osmolyte, K^+, in valinomycin-treated cells, confers osmotic protection in hypotonic NaCl media as judged by the leftward shift in the onset of lysis, whereas enhanced permeability to ions and, in particular, to external Na^+, by gramicidin S (unpublished observations), enhances the osmotic susceptibility, i.e., a rightward shift [15,16]. These shifts can be evaluated by an analysis of break-points, corresponding to the onset or completion of lysis, the points of intersection of independent regression lines [10–13]. The data presented in Fig. 1 yielded break-points corresponding to the onset of lysis (expressed as % (w/v) external NaCl): control, 0.53%; valinomycin-treated, 0.39%; and gramicidin-treated, > 0.9%. The break-point analysis, which obeys an F distribution [17], measures a material constant of the population of cells and these shifts were significant at $P < 0.001$. A detailed account of statistical methodology for break-point analysis will be published elsewhere, though it may be briefly summarized here that as little as 2–5% changes in internal osmolality can be reliably detected in such studies at the $P < 0.05$ level of significance.

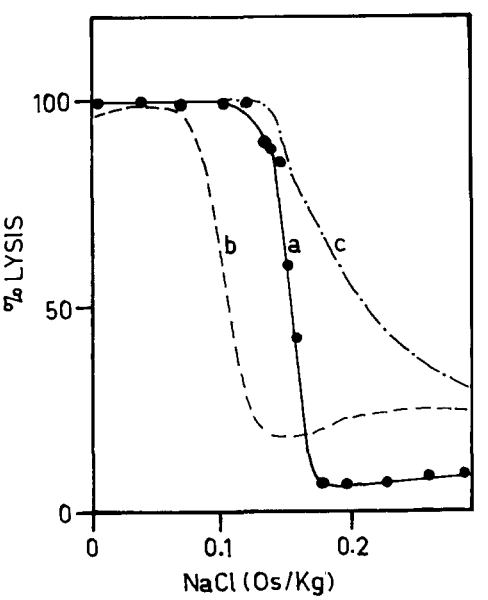

Fig. 1. Effect of valinomycin and gramicidin S on the osmolysis profiles of rat erythrocytes in NaCl media. Osmolytic profiles were obtained as described earlier [12]. (a) Control, (b) with 10^{-6} M valinomycin and (c) 10^{-5} M gramicidin S in the incubation media. Data points for the control alone are shown here for clarity. The osmolality of the incubation media was determined using a Wescor vapour pressure osmometer.

Two important corollaries arise in the determination of break-points: the high statistical sensitivity precludes comparison of data from different days and requires comparison of various treatments on the same day to exclude systematic errors; when high variation in the magnitude of lysis occurs day to day, as in osmometric profiles in the hypertonic domain, the data may not permit least-squares regression fitting, due to non-linearities in the lysis profiles. The critical pressures corresponding to onset of lysis in such situations can be assessed by very detailed osmometric analyses with large number of data points to permit exact visualization of these critical pressures. Therefore, all the data presented in these studies represent highly reproducible patterns in at least three independent experiments, though each reported experiment represents data from a single preparation. The validity and the versatility of the technique of osmometry has been discussed at greater length [10–13].

Results

Traditional methodology of measurement of fluxes has two major limitations: (i) centrifugal phase separation in radioactive experiments could lead to equilibration of small molecular weight substances due to dramatic changes in the porosity of the membrane [10–13,18,19]; and (ii) the osmolality of the external medium (and, therefore, the physical state of membrane) was not examined for its contribution to the quantum of fluxes. Use of ion-selective electrodes and osmometric evaluation of solute fluxes were therefore combined to facilitate unambiguous conclusions. We initially examined the nature of K^+ efflux in NaCl media of varying tonicity as a reference for the subsequent studies on ion fluxes in non-electrolyte media.

K^+ efflux from erythrocytes in NaCl media

Evidence for progressive K^+ efflux from human erythrocytes in hypotonic media was three-fold: (i) a leftward shift in osmolytic profiles in gradual hemolysis (by dialysis of erythrocytes against NaCl media of varying tonicity) as opposed to acute hemolysis (Refs. 14 and 20, cf. Fig. 2); (ii) a prelytic increase in K^+ conductance, as suggested by Seeman et al. [20] based on the observed slow release of K^+ in hypotonic media (even though the centrifugal methodology was used for phase separation); and (iii) changes in cell volume in hypotonic media less than those theoretically anticipated [21], compatible with changes in internal osmolality [22]. Is the efflux of K^+ merely prelytic, or does any specific relationship exist between K^+ efflux and the volume of the cell? For instance, detergents also produce a prelytic increase in permeability [23] and even single-channel conductance behaviour in detergent-doped artificial membranes [24], indicative of non-proteinaceous 'flaws' in the membrane.

K^+ efflux from rat erythrocytes was monitored directly by a K^+-selective electrode in NaCl media of varying tonicity. K^+ efflux upon dialysis of erythrocytes exhibited marked non-linear dynamics (Fig. 3C) and quantitation was difficult due to Donnan effects leading to sequestration of K^+ partially within the dialysis bags. On the other hand, K^+ efflux exhibited a linear activation with increasing hypotonicity of the medium on acute suspension of erythrocytes in NaCl media of varying tonicity such that the rate of K^+ efflux

$$J_{K^+} = J_{K^+}(\max) - \tilde{K}_{K^+}\Pi \qquad (1)$$

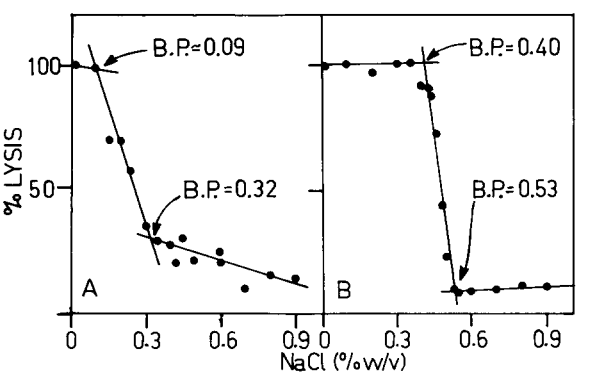

Fig. 2. Break-point analysis of erythrocyte osmolysis profiles. (A) Osmolysis profiles obtained by gradual hemolysis method. (B) Osmolysis profile of erythrocytes on acute suspension of cells to varying NaCl concentrations (refer to Materials and Methods). Percentage lysis of erythrocytes is plotted as a function of NaCl concentration. B.P., break-point, indicated for the onset and completion of lysis expressed as external osmolyte concentration. Each break-point was obtained from individual regression lines $Y = m_1x + c_1$ and $Y = m_2x + c_2$ such that B.P. $= (c_2 - c_1)/(m_1 - m_2)$. The differences in break-points for the onset as well as completion of lysis were significant $P < 0.001$.

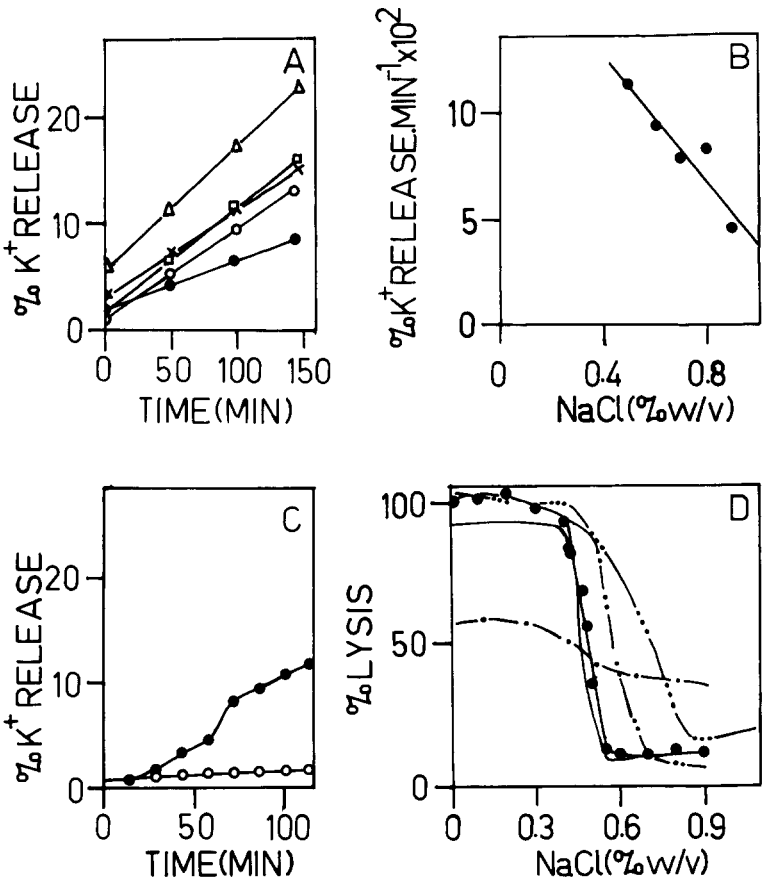

Fig. 3. Effect of external osmolarity on ion fluxes from erythrocytes: K^+ release was monitored with a K^+ electrode at various concentrations of NaCl, as described in Materials and Methods and expressed as percentage of total internal K^+, obtained on addition of 0.04% (v/v) Triton X-100. (A) Rate of release of K^+ obtained from regression analysis of % K^+ release as a function of time on acute suspension of the cells at various NaCl concentrations: (△) 0.5; (□) 0.6; (×) 0.7; (○) 0.8; (●) 0.9% (w/v) NaCl. (B) Rate of K^+ efflux plotted as a function of external NaCl concentration ($r = 0.9$, $P < 0.01$). (C) K^+ efflux on dialysis of erythrocytes against 0.9% NaCl (○) and 0.4% NaCl (●). Lysis was less than 4%, on dialysis in 0.4% NaCl over 2 h. Note the marked non-linear profile of K^+ release on dialysis against 0.4% NaCl. (D) Effect of prior dialysis of erythrocytes against NaCl media of varying tonicity, on subsequent osmolysis profiles (cf. Fig. 2A). Stock suspension of erythrocytes (50% packed cell volume in 0.9% NaCl) were dialyzed against 200 vols. of NaCl media of varying tonicity. 0.35% (·–·–·); 0.5% (————); 0.9% (●); 1.5% (– · · –) and 2.5% (– · · · –). Data points omitted (except for 0.9%) for clarity. At the end of dialysis, aliquots of erythrocyte suspension were directly added to NaCl media of varying tonicity as in Fig. 2B, to obtain erythrocyte osmolysis profiles. Note that dialysis in 0.35% led to considerable lysis on dialysis, though resuspension even in water did not result in complete lysis of cells.

where Π is the external osmotic pressure and $\tilde{K}_{K^+}$ an empirical elastic constant of the osmotic susceptibility of K^+ efflux (Figs. 3A and B). These data conclusively show that K^+ efflux is not merely prelytic and is comparable to similar volume-dependent fluxes for K^+, Cl^- and Rb^+ from lymphocytes [25,26]. Hypotonic activation of the K^+ channel offered an attractive explanation for $\tilde{K}_{K^+}$. Since volume expansion would actually lower the chemical potential of K^+ within, the experimental data would actually underestimate $\tilde{K}_{K^+}$ (vide infra).

Does the internal osmoticum vary with incubation of erythrocytes in hypertonic NaCl media? In a variety of complex experimental situations, exposure of cells to hypertonic media and subsequent return to presumed 'isotonic' media was shown to result in lysis [3], cell death [27], etc. We exploited the technique of osmometry to evaluate the internal osmolyte content of erythrocytes dialysed against NaCl media of varying tonicity. Fig. 3D shows that the onset of lysis in subsequent osmolytic profiles shifted to right with increasing concentration of NaCl in the prior dialysing medium. Since K^+ efflux would be actually prevented at higher tonicities, the rightward shift of the osmolytic profiles could be accounted for only in terms of enhanced Na^+ permeation inwards, with consequent enhanced requirement for isotonicity.

Zade-Oppen reported such 'post hypertonic disruption' of erythrocytes even when erythrocytes were incubated in non-electrolyte media [2]. Instability of erythrocytes from a variety of species on

incubation in non-electrolyte media was known [2,5,7], though the underlying mechanism remained obscure.

Behaviour of erythrocytes on suspension in non-electrolyte media

The explanation for K^+ efflux, acidification of medium and lysis of erythrocytes suspended in non-electrolyte media has been fragmentary in the sense that ion fluxes were, so far, accounted for within the confines of Donnan theory, whereas lysis was largely unaccounted for [1,5,6]. The central assumption was that the permeability/conductance of the bilayer was unmodified for ions as well as non-electrolytes, while the driving force was considered to be the chemical potential gradient of the ions. Since anions exhibit higher conductance than cations, anion exchange (say, Cl^-/OH^- exchange via band 3 protein) was thought to lead to acidification of the medium. An explanation for K^+ loss was sought in terms of a reversal of the Donnan potential, though it was known that the large K^+ efflux could not be mediated via saturable transporters, nor was the cation efflux specific to any cation nor even related to the ionic radii of the hydrated or unhydrated species [8]. Though a colloidal mechanism of lysis was speculated [2], permeability to non-electrocytes (e.g., sucrose, which is foreign and inert) was considered invariant, even though K^+ efflux was considered to occur through the membrane matrix per se [8]. The unresolved key issues focus on: (i) whether permeability to non-electrolytes was enhanced to account for lysis by a colloidal swelling mechanism, in which case, changes in membrane potential or ΔpH would be either marginal or secondary to a more fundamental, unidentified driving force for these fluxes; (ii) whether the phenomenon of lysis is promoted or inhibited by hypertonicity of the medium to distinguish between colloidal lysis and a primary destabilization of the membrane by a non-osmotic mechanism and (iii) whether the role of anion exchange was causal or incidental to an electroneutral efflux of cations.

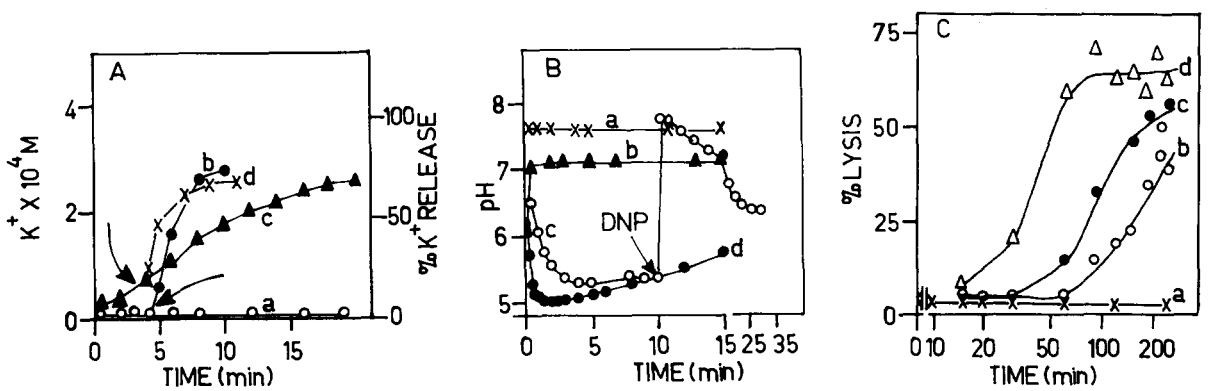

Fig. 4. (A) Loss of K^+ from erythrocytes. K^+ release into the medium was followed as a function of time as described in Materials and Methods. (a) 0.9% (w/v) NaCl medium; (b) valinomycin added to NaCl medium; (c) 0.31 M sucrose medium and (d) valinomycin added to sucrose medium. Arrows indicate time of addition of valinomycin (10^{-6} M). External K^+ was not detectable in NaCl at zero time, unlike in the case of sucrose medium. The presence of 3.3 mM Tris-HCl (pH 7.4) did not affect the dynamics of K^+ release (data not shown). (B) Release of H^+ from erythrocytes: pH of the medium was followed as a function of time, on addition of 100 μl of stock suspension of erythrocytes. (a) Sucrose with 3.3 mM Tris-HCl (pH 7.4) at 30°C; (b) unbuffered NaCl at 30°C; (c) unbuffered sucrose at 3°C; (d) unbuffered sucrose at 30°C. Arrow indicates addition of neutralized 2,4-dinitrophenol (DNP), 50 μM. Addition of erythrocytes pretreated with 50 μM DNP yielded a profile similar to that of curve c, DNP alone contributing to the observed shift in pH in unbuffered media without cells (data not given). There was no measurable lysis upto 15 min in these experiments. Na^+ contamination from stock suspension of erythrocytes was less than 0.3 mM in the assay medium (Figs. 4A and B). (C) Lysis of erythrocytes suspended in buffered sucrose media of 0.206 M (a), 0.31 M (b), 0.38 M (c) and 0.45 M (d) at 30°C as a function of time. 20 μl of stock suspension of erythrocytes were added to 3.0 ml of sucrose solutions of specified concentrations with 3.3 mM Tris-HCl buffer (pH 7.4). After incubation at 30°C for various time intervals, absorbance was measured at 540 nm in cell-free supernatants (cf. Fig. 1). In NaCl media of corresponding osmolarity, lysis was not observed upto 4 h of incubation (data not given).

K^+ efflux and acidification of the medium in sucrose media

K^+ release from rat erythrocytes in non-electrolyte media was reinvestigated to confirm the human erythrocyte data [1,5,6] (cf. Fig. 4A): (i) up to 60% of internal K^+ was released in isotonic 0.31 M sucrose media by 15 min at 30°C, while the release was barely measurable in isotonic NaCl media (cf. (Fig. 3a); (ii) presence of minimal buffer to arrest acidification of the medium had no significant effect on the release of K^+; (iii) K^+ release was largely electroneutral, since Cl^- was also extruded (data not given, cf. Refs. 4 and 6). We could further show that K^+ movement across the membrane in sucrose media was restricted, since addition of valinomycin enhanced its release both in sucrose and in NaCl media.

Acidification of the medium by rat erythrocytes was observed only in unbuffered non-electrolyte (sucrose) media, which also exhibited comparable properties to human erythrocytes [1,5,6] (Fig. 4B). (i) Acidification of the medium was initially faster than K^+ release in sucrose media and, at 30°C, the medium became progressively less acidic in a time scale comparable to K^+ release. (ii) Acidification was marginally slower at 3°C, without a biphasic profile. We also observed that addition of the protonophore, 2,4-dinitrophenol (DNP), prior to or during incubation of erythrocytes in sucrose media, had no effect on acidification; the observed pH changes on addition of 2,4-dinitrophenol were entirely due to the neutralized 2,4-dinitrophenol per se. Thus, if acidification was due to H^+ efflux across the bilayer, it appeared to be unrestricted, unlike K^+ movement. Release of K^+ from erythrocytes in non-electrolyte media exhibited two major differences. (1) The magnitude of release was about 20-times larger than that in NaCl media. (2) The rate of K^+ release was independent of external osmolality in a range of 0.35 to 0.9 osmol/kg of external sucrose, unlike in NaCl media wherein K^+ release was inhibited by 65–70% linearly in an osmolality range of 0.16–0.3 osmol/kg (cf. Fig. 3B).

Lysis of erythrocytes in sucrose media

If the mechanism of lysis of erythrocytes in non-electrolyte media were due to colloidal swelling, it would imply not only enhanced porosity to the non-electrolyte (i.e., sucrose), but also inhibition of lysis at higher sucrose tonicities. The release of haemoglobin in rat erythrocytes showed interesting features (Fig. 4C): (i) the onset of lysis showed a variable time delay, which decreased with increasing tonicity of the medium, and (ii) the magnitude and rate of lysis actually increased with increasing tonicity of the medium!

Since both K^+ efflux and acidification can also be measured as rates, it could be shown that these ion fluxes as well as lysis exhibited a dramatic and parallel inhibition on addition of NaCl to the sucrose medium, such that as little as 20 mM NaCl resulted in nearly total inhibition (data not shown, cf. Refs. 6–8). The rate of acidification of the medium reached maximum by 0.32 M sucrose, beyond which both acidification and K^+ efflux were nearly insensitive to external osmolality of sucrose (data not given). The volume of human erythrocytes in sucrose media was recently demonstrated to be less than in matching NaCl media above isotonicity, as evidenced by direct Coulter counter methodology [21], consistent with a large prelytic efflux of K^+.

Osmolytic profiles of rat erythrocytes in sucrose media and the effect of temperature

Enhanced lysis with increasing osmolality of sucrose media would imply that, at a convenient period of incubation, the onset of lysis should be clearly distinguishable in the hypo- and hypertonic domains, separated by a well-defined, intermediate non-lytic domain. This was seen to be the case for sucrose media at 4 h incubation at 30°C. (Fig. 5). In 20 such experiments, the break-point of onset of lysis was 0.4 ± 0.016, whereas the onset of hypertonic disruption * was at 0.63 ± 0.041 of external sucrose (expressed as % (w/v) NaCl equivalent, mean $\pm$ S.D.). The superiority of the use of the genetically inbred rats as opposed to less standardized species (including human) deserves mention (cf. Ref. 7; comparable human

* Throughout the text, we use the term 'hypertonic' disruption in sucrose media, although the onset of lysis begins at approx. 0.63% equivalent sucrose. Since the notion of isotonicity is irrelevant in a situation marked by rapid solute fluxes, the usage of terms 'hypotonic' and 'hypertonic' disruption only indicate the directional relation of lysis vis-a-vis external osmotic pressure and not the absolute range.

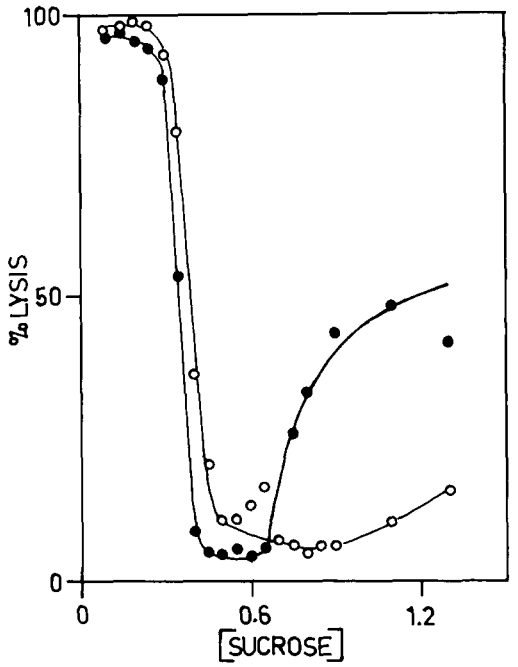

Fig. 5. Effect of temperature on hypertonic disruption of rat erythrocytes: % release of haemoglobin was plotted against ambient sucrose concentration (% NaCl (w/v) equivalent) on incubation for 4 h at 30°C (●) 3°C (○). The break-point for the onset of lysis was significantly to the right at 3°C compared to that at 30°C ($P < 0.01$, corresponding to a shift of approx. 30 mosmol/kg) and was also seen at 15 min incubation (data not given).

erythrocyte data not given). This high phenomenological reproducibility permitted an evaluation of shifts in break-points of onset of lysis in either domain to unravel the underlying mechanism, provided comparisons were made within the same preparation and experiment, since the magnitude of lysis still varied from 40 to 90% in the hypertonic domain (cf. Materials and Methods).

Incubation of erythrocytes at 3°C showed a large shift in the onset of hypertonic disruption to the right, rather than its abolition (Fig. 5). Taken together with the observation of a small, but reproducibly measurable (in three independent experiments), shift of the break-point for the onset of hypotonic lysis, the data were suggestive of enhanced permeability to sucrose at lower temperatures, such that a much larger osmolality of sucrose was required to exert comparable hypertonicity under cold conditions. The shifts in break-points were statistically significant in hypo- as well as hypertonic domains ($P < 0.05$ and $P < 0.01$, respectively, based on an F distribution for the confidence interval for the break-point [17]).

A paradox of critical importance emerged in that the external sucrose appeared more permeable in the cold, though the mechanism of lysis could not be due to colloidal swelling. More direct evidence was required to establish the nature of sucrose permeation.

Permeation of sucrose into rat erythrocytes

The use of centrifugal methodology for phase separation in experiments on the passive uptake of radioactive sucrose into erythrocytes was unavoidable due to the marked fragility of erythrocytes during Millipore filtration. The exact magnitudes of such an uptake would be definitely suspect, since (i) centrifugation was known to affect the permeability of biological membranes [10–12,18, 19] and (ii) the percentage equilibration of labelled sucrose based on independent packed cell volume measurements would not be exact due to dynamic changes in erythrocyte volume and morphology on exposure to non-electrolyte media [9]. Though the assay protocol incorporated a gradient wash of erythrocytes in hypertonic sucrose to achieve low blanks and high signal-to-noise ratio, the magnitudes varied in different experiments from 25–100% equilibration, at 0–3°C. A time study of uptake up to 4 h was possible in 0.206 M sucrose media, wherein coincident lysis was less than 4% (cf. Fig. 4C). Data from different experiments could not be pooled due to the presence of a biphasic profile of uptake, with a peak varying from 5 to 20 min in three different experiments. The existence of a biphasic profile as seen in a representative experiment as in Fig. 6, further confirmed that the absolute magnitudes of equilibration were unreliable due to coincident volume changes (cf. Ref. 9). The radioactive uptake experiments could only suggest (i) consistently greater uptake of sucrose from sucrose media in the cold, and (ii) absence of measurable uptake of labelled sucrose on incubation in the presence of 140 mM NaCl and 20 mM sucrose.

The severe methodological limitations inherent to an apparently simple problem of measurement of labelled sucrose required additional confirmation that external sucrose does equilibrate with the

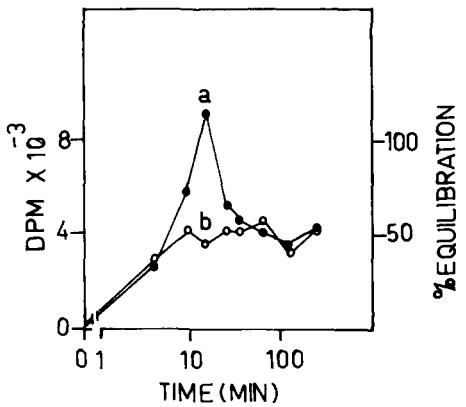

Fig. 6. Equilibration of (U-^{14}C]-labelled sucrose across erythrocyte membrane at 3°C (a) and 30°C (b) in 0.206 M sucrose media with Tris-HCl buffer (pH 7.4), as a function of time. Earliest time interval measured was 4 min. Zero-time value represents the blank without cells. Percentage equilibration was calculated from packed cell volume. dpm were calculated based on both internal and external standards. Data points were mean values of duplicate measurements in a single experiment. Parallel incubation in 140 mM NaCl and 20 mM sucrose with equal amount of radioactive sucrose yielded incorporation 80% of the blank only.

interior, based on more robust methodology. Since more than 60% of internal K^+ was lost within 15 min of suspension of erythrocytes in sucrose media and since addition of NaCl (above 20 mM) at any time point to such sucrose media arrests further efflux of K^+ [6], an osmotically uncompensated K^+ efflux should be detectable in the osmometric analysis of lysis profiles as in Fig. 1. Detailed osmometric analysis of profiles of lysis in NaCl media, after prior incubation in isotonic NaCl and sucrose media exhibited identical break-points both for the onset and completion of lysis without measurable shifts, indicating a relatively invariant internal tonicity despite the large efflux of internal K^+ at 15 min (Fig. 7). The only external osmolyte being sucrose, the osmometric data unambiguously demonstrated permeation of sucrose inwards. The apparent iso-osmotic exchange of K^+ and sucrose, though intriguing, has also been well documented in mitochondria [28,29]. The possibility of cytoskeletal support regulating erythrocyte volume changes contributory to such apparent iso-osmotic exchange needs to be further explored (cf. Ref. 21).

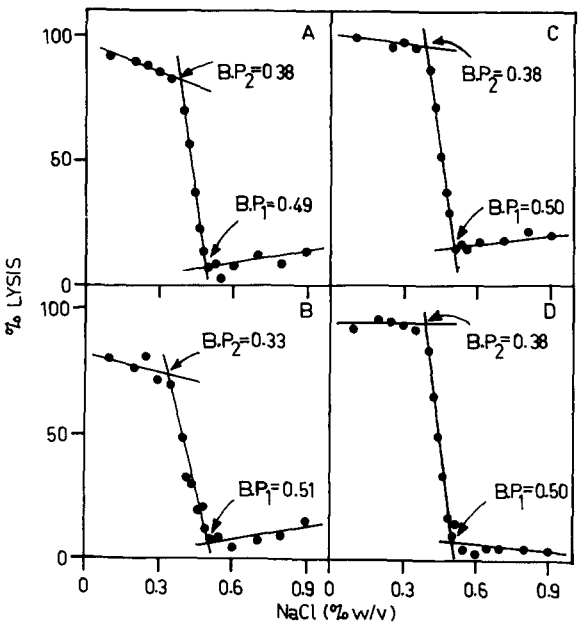

Fig. 7. Erythrocytes were incubated in the presence of buffer (Tris-HCl (pH 7.4)) in 0.31 M sucrose at 3°C, 30°C and in 0.9% NaCl (w/v) at 3°C and 30°C. At the end of incubation, 20-μl aliquots of incubated cells were added to 3.0 ml each of various NaCl solutions to obtain osmolytic profiles of erythrocytes (cf. Fig. 1B) for each treatment, correspondingly labelled (A–D); 0.31 M sucrose at 3°C (A); 30°C (B); 0.9% NaCl at 3°C (C) and 30°C (D). Break-points of the onset of lysis (B.P.$_1$) and completion of lysis (B.P.$_2$), expressed as the external NaCl concentration, were indicated by arrows. Each break-point was obtained from individual regression lines as in the legend to Fig. 2.

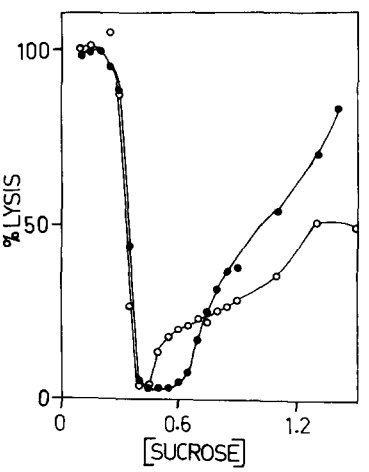

Fig. 8. Effect of valinomycin (10^{-6} M, final assay concentration) on hypertonic disruption of rat erythrocytes: % release of haemoglobin was plotted against ambient sucrose (% NaCl (w/v) equivalent). Control (●); with valinomycin (○).

Factors governing the critical external osmolality corresponding to the onset of hypertonic disruption

The external sucrose concentration corresponding to the onset of hypertonic disruption decreased with temperature (cf. Fig. 5), time of incubation and on addition of valinomycin (which lowers internal osmolality due to loss of K^+) in rat erythrocytes (Fig. 8) as well as in human erythrocytes (data not given). The phenomenon was independent of $(Na^+ + K^+)$-ATPase (i.e., on addition of 10^{-4} M ouabain), external pH in the range of 6–9, presence of hormones such as insulin, or uncouplers such as 2,4-dinitrophenol (data not given). This phenomenon of hypertonic disruption and attendant ion and solute fluxes should not be confused with the 'Gardos' effect [30], since no attempt was made to deplete endogenous ATP levels and lysis was present in fresh as well as aged (about 12 h) preparations.

Rôle of surface charge density in the anomalous behaviour of erythrocytes in non-electrolyte media

Permeation of sucrose across the erythrocyte membrane in media of low conductivity indicated a dramatic change in the permeability characteristics of the erythrocyte membrane prior to lysis [10,11]. A similar phenomenon was demonstrated in mitochondria due to the combined influence of respiration and unscreened fixed anionic charges on the surface, such that the instability of the membrane was but an extreme case of drastic changes in bulk porosity of the membrane [13]. Mitochondria also exhibit the phenomenon of hypertonic disruption in sucrose media [12]. The influence of metabolism was unlikely in the case of erythrocytes. The mechanism of lysis appeared to be primarily physical in nature. Erythrocytes were known to possess $(1–10) \cdot 10^4$ e^-/nm^2 (e^-, the electron charge) on the exterior, which would contribute to a considerable field upon exposure to media of low ionic strength [31]. The situation would be comparable to enhanced permeability and instability observed on addition of Ca^{2+} to negatively charged liposomes, wherein the differential surface charge density across the bilayer would be the driving force for changes in permeability and instability of the bilayer [32]. Since the exterior charges could be augmented or neutralized selectively by anionic and cationic detergents due to their interfacial preference, we investigated

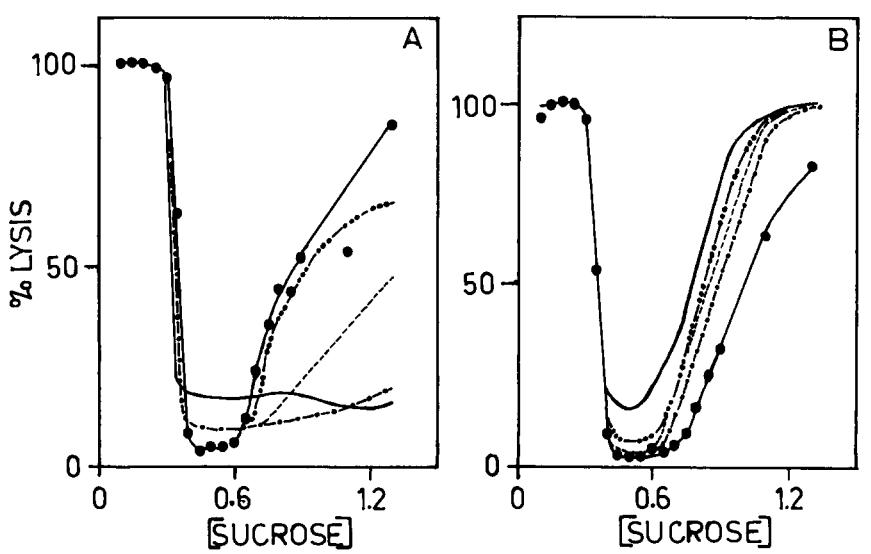

Fig. 9. Effect of varying concentrations of ionic detergents on hypertonic disruption of erythrocytes: Osmolytic profiles of erythrocytes were obtained as in Fig. 5 in sucrose media of varying tonicity (expressed as NaCl equivalents) in the presence varying concentrations of detergents. (A) Effect of CTAB % (w/v): ●, without CTAB; –···–, 10^{-5}%; — — —, $5 \cdot 10^{-5}$%; ·–·–·, 10^{-4}% and ————, $5 \cdot 10^{-4}$%, (B) Effect of SDS % (w/v): ●, without SDS; ·–·–·, $5 \cdot 10^{-4}$%; — — —, 10^{-3}%; –···–, $2 \cdot 10^{-3}$%; ————, $5 \cdot 10^{-3}$%. Data points ($n = 19$ for each curve) except controls, were omitted for clarity.

the effect of sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) on lysis profiles in sucrose media (Fig. 9A and B). (i) SDS promoted hypertonic disruption; (ii) CTAB inhibited hypertonic disruption; (iii) the effects of detergents were proportional to their concentrations; (iv) the effects were manifest well below their lytic range; (v) hypotonic lysis was relatively unaffected and (vi) the break-point corresponding to the onset of hypotonic lysis was relatively unaffected, whereas the magnitude of hypertonic lysis was selectively modified. Triton X-100, a nonionic detergent was totally without effect in its prelytic range, indicating the specificity of charges on the detergent. The inhibitory effect of low concentrations of NaCl could be readily explained since screening of surface charges would be proportional to $\log[Na^+]$ in the bulk phase. Since screening would not be absolute, one would expect a small but measurable lysis even in NaCl media in the hypertonic range on prolonged incubation. Incubation of erythrocytes at 30°C for 4 h resulted in reproducible lysis upto 10–15% in NaCl media above 0.6% (w/v) (data not given, cf. Ref. 3).

Discussion

Behaviour of erythrocytes in electrolyte media

The instantaneous osmotic pressure, Π, required for constant tonicity by a permeable external solute, S, at time $t = 1$, is

$$\Pi^S(t=1) = \Pi^S(t=0) + RT\,\mathrm{d}s \int_{t=0}^{t=1} \mathrm{d}t \tag{2}$$

where R is the gas constant and T, the temperature. The shifts in break-points (ΔB.P.) therefore obey the relationship

$$\Delta\text{B.P.} = RT\int_0^t J_{S_o} - RT\int_0^t J_{S_i} \tag{3}$$

where S_o and S_i represent the external and internal osmolytes. In the case of incubation in NaCl media, these represent Na^+ and K^+, given an electroneutral exchange (the minus sign refers to efflux). Since it was observed that

$$J_{K^+} = J_{K^+}(\max) - \tilde{K}\Pi \tag{1}$$

the observed shifts in break-points as a function of external osmolality of NaCl media (Fig. 3D), as the basis of post-hypertonic disruption in electrolyte media [3], could be accounted for only when $\tilde{K}_{K^+} \gg \tilde{K}_{Na^+}$, since the flux of an ion

$$J_i = g_i \cdot \Delta\mu_i \tag{4}$$

where g_i is the conductance and $\Delta\mu_i$ the ion's chemical potential difference.

A difference in $\tilde{K}_{K^+}$ and $\tilde{K}_{Na^+}$ would be consistent with permeation of these ions through separate channels. In the case of a channel mechanism mediating electroneutral efflux:

$$J_{K^+} = n \cdot f \cdot \bar{g}_{K^+} \cdot \Delta\mu_{K^+} \tag{5}$$

where n is the number of channels, f, the fraction of open channels (which subsumes $\bar{\tau}$, the mean channel open time) $\bar{g}_{K^+}$, mean channel conductance for K^+ across the membrane. The empirical elastic constant, $\tilde{K}_{K^+}$, can be rewritten as

$$\tilde{K}_{K^+} = \mathrm{d}(n \cdot f \cdot \bar{g}_{K^+} \cdot \Delta\mu_{K^+})/\mathrm{d}\Pi \tag{6}$$

Since $\Delta\mu_{K^+}$ decreases with an increasing volume of the erythrocyte, there would be an actual underestimation of $\tilde{K}_{K^+}$ as in Fig. 3B.

A major justification for the postulation of the elastic constant, $\tilde{K}$, arises from an empirical demonstration of the osmotic dependence of a large number of membrane-bound enzyme systems [12,13,33–35], transporters [12,36] etc., whose activity was shown to obey the general relationship [13]

$$A = A_{\max} - \tilde{K}\Pi \tag{7}$$

Since the elastic energy contribution by osmotic stretch per se would be merely $1 \cdot 10^2$ cal or less, while the activation energy for a protein would be approx. 1.0 kcal, such osmotic activation was considerred forbidden on a priori thermodynamic grounds [37] and awaits a proper thermodynamic explanation.

Behaviour of erythrocytes in non-electrolyte media

The primary observation of importance was the rapid influx of sucrose, as evidenced by (i) osmotic analysis in NaCl media subsequent to incubation in sucrose media (Fig. 7), (ii) the rightward shifts

of the onset of lysis at 3°C, both in the hypo- and hypertonic domains (Fig. 5); and (iii) the corroborative radioactive experiments, despite methodological uncertainties (Fig. 6). We have shown elsewhere [10–13,38] that sucrose permeation, being thermodynamically forbidden, would be consistent with simultaneous lowering of interfacial and diffusional barriers to the solute, (i.e., induced pores), consistent with density fluctuations in the bilayer characterized by areas of lipid condensation and rarefaction. Enhanced permeation of sucrose in the cold further argues against any diffusion-based model, including the electrostatic and the proteinaceous mechanisms (i.e., transporters or channels) and would be accounted for only by a perturbation in the bilayer. Similar phenomena were also critically demonstrated during centrifugation [10–12] and concomitant to oxidative phosphorylation [13,39].

Given the sucrose permeation across the bilayer, the assumption of constant leak conductance for ions, a prerequisite for models based on Donnan potentials (cf. Ref. 40), would be untenable. The inferred changes in membrane potential would be the effect rather than the cause of ion fluxes across a porous membrane. On the contrary, the negative surface charge density on the exterior would also drive the cations outwards across the porous bilayer, which equilibrate with the external bulk phase due to their poor interfacial affinity [41]. The observed inhibition of lysis by inhibitors of the anion transporter [7] could be via an inhibition of electroneutral efflux of Cl^-, though further work is needed to define the marginal and secondary role of this transporter.

The injurious effect of hypertonic non-electrolyte media, reversible within short time intervals was also seen with other cells, e.g., viability of lymphocytes [27], contractility of cardiac muscle [42], the hormonal responsitivity of the skeletal muscle [43] and swelling phenomena associated with mitochondria [13], etc. A general mechanism based on surface charge density difference across the membrane can only be a working hypothesis for these diverse phenomena unless some of its major consequences are carefully demonstrated. Of particular importance are the effect of ionic detergents and divalent ions such as Ca^{2+} on the membrane and the nature, dynamics and the molecular sieving properties of the induced pores by such detergents and on exposure to non-electrolyte media.

Acknowledgements

We are grateful to Dr. A.V. Subramanyam for helpful discussions. The technical assistance of K. Yashodarani and Amulya Augustine is gratefully acknowledged.

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