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HUMAN ERYTHROCYTE GHOSTS: RELATIONSHIP BETWEEN MEMBRANE PERMEABILITY AND BINDING KINETICS OF THE FLUORESCENT PROBE 1-ANILINONAPHTHALENE-8-SULPHONATE

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SUMMARY

1. The time dependence of the fluorescence enhancement of 1-anilinonaphthalene-8-sulphonate after addition to haemoglobin-free human erythrocyte ghosts was studied in 150 mM NaCl, 10 mM sodium phosphate (pH 7.0) at 25 °C.

2. Enhancements were analysed in terms of "fast", "medium" and "slow" phases.

3. Ghost samples shown to be at least partly resealed towards Na^+ and/or osmotically responsive display a slow phase (half-time about 80 or 120 s depending on conditions at haemolysis). The medium phase (half-time about 14 s) is characteristic of physically intact membranes in a non-resealed state. The fast phase (half-time less than instrument response time) always comprises at least 50% of the total enhancement.

4. 1-Anilinonaphthalene-8-sulphonate fluorescence parameters suggest that most probe binding sites are similar, and that they are not involved in membrane changes leading to resealing.

5. It is proposed that 1-anilinonaphthalene-8-sulphonate sites are only distinguished by location on the extracellular or intracellular side of a membrane 1-anilinonaphthalene-8-sulphonate permeability barrier. Diffusion of 1-anilinonaphthalene-8-sulphonate across the barrier produces the observed medium or slow phases, depending on the state of the membrane.

INTRODUCTION

Freedman and Radda[1] observed that the increase of fluorescence following addition of the probe ANS (1-anilinonaphthalene-8-sulphonate) to a suspension of erythrocyte ghosts was biphasic, with immediate and time-dependent components. These were attributed to presence of "fast" and "slow" sets of binding sites. Further investigation[2] established that the two sets of sites could not be distinguished in terms

Abbreviation: ANS, 1-anilinonaphthalene-8-sulphonate.

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of the fluorescence enhancement or lifetime of bound ANS, or the dissociation constant for probe–membrane binding, and that variation of ionic strength did not affect their relative proportions.

Erythrocyte membranes are able to "reseal" by regaining most of the impermeability to cations which is lost immediately after hypotonic haemolysis. Incubation at 37 °C in isotonic media favours resealing[3,4] though detailed behaviour depends on the conditions of haemolysis, for example presence or absence of alkaline earth cations[5–8]. Hoffman[4] has discriminated between ghosts which reseal before incubation, those which become resealed upon incubation, and those which remain "leaky" towards cations even after incubation.

This report describes a test of the proposition that the sets of sites revealed by ANS enhancement kinetics are similar at the molecular level, but are separated by a permeability barrier such that only one set is directly exposed to the extracellular medium. The correlation between ghost membrane integrity as judged by cation impermeability and the accessibility of the ANS binding sites giving time-dependent kinetics was examined.

This work formed part of the D.Phil. thesis of D.S.Smith[9].

EXPERIMENTAL

Materials

Out-dated human blood was supplied by the Transfusion Service of the Oxford Regional Hospitals Board. ANS, ammonium salt, from K and K Laboratories Inc., Plainview, N.Y., U.S.A. was purified as previously described[10]. Crystalline bovine serum albumin was from Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex, Great Britain.

Ghost preparations

Haemoglobin-free ghosts were prepared by the method of Dodge *et al.*[11], with all operations carried out at 4 °C. Blood was washed three times by centrifuging in isotonic buffer and the packed cells thus obtained were haemolysed by rapid addition with stirring to 10 vol. of hypotonic buffer. The ghosts were spun down (Sorvall RC2-B centrifuge, GSA head, 13000 rev./min, 15 min), the supernatant discarded, and the ghost pellet resuspended. This cycle was repeated until the supernatant was colourless (three or four times). Ghosts were stored in hypotonic buffer at 4 °C and used within ten days of preparation.

"Phosphate-haemolysed" ghost were prepared from cells washed in 310 imosM sodium phosphate (pH 7.4) and haemolysed in 20 imosM sodium phosphate (pH 7.4)[11]. "Mg^{2+}- haemolysed" ghosts were from cells washed in 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), and haemolysed in 1 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4).

Ghost resealing

This was tested by the method of Hoffman *et al.*[3] Ghosts were washed into isotonic NaCl (150 mM NaCl, 10 mM sodium phosphate, pH 7.0) to give a suspension 1.2 mM in phospholipid phosphate (about 2 mg/ml protein). Samples were incubated at 37 °C for 3 h, control samples being left at 4 °C. Aliquots were then spun down (Spinco ultracentrifuge, 40 rotor, 20000 rev./min, 5 min) and the ghosts resuspended

in isotonic KCl (a medium analogous to isotonic NaCl, with potassium replacing sodium) to their original volume, at room temperature. After 5 min, the process was repeated and after a given number of washes each resuspended ghost aliquot was assayed for sodium on an EEL flame photometer.

Fluorescence experiments

A Hitachi Perkin–Elmer MPF 2A fluorimeter was used. All ANS kinetics experiments were performed at 25 °C in the following manner. The ghost sample under investigation was washed into isotonic NaCl to give a final concentration of 60 μM in phospholipid phosphate. A 2-ml aliquot was placed in a cell in the fluorimeter sample compartment, and a small volume of an ANS solution in the same buffer was added with rapid mixing to give a final probe concentration of 10 μM.

With excitation wavelength set at 380 nm and emission at 500 nm, and slit widths of 10 nm, the time-course of fluorescence enhancement was followed on the chart recorder. Results were analysed by semilogarithmic plots of log $(F_\infty - F_0)/(F_\infty - F_t)]$ against time, where F_0 and F_t are fluorescence signals at times zero and t after ANS addition, and F_∞ is the final fluorescence signal. This method is known to give good fits to kinetic data from experiments with ghosts[2] or submitochondrial particles[12]. Kinetic runs showing two time-dependent phases were analysed by subtracting the contribution of the slower phase (extrapolated back to zero time). The residual data were then treated in the normal way to give the parameters of the faster time-dependent phase.

Binding parameters for ANS–ghost systems which had reached equilibrium in isotonic NaCl were obtained from fluorescence measurements analysed by standard methods[12]. Thus the limiting ANS enhancement (ratio of signals from completely bound or completely free probe) was found from a double reciprocal plot, and the dissociation constant and number of the ANS sites from a Scatchard plot[13]. Scatchard data were obtained at membrane concentrations of 60 μM phosphate.

Analytical methods

Protein was estimated by the method of Lowry *et al.*[14] with crystalline bovine serum albumin as standard. Ghost phospholipid phosphate was assayed by digestion according to the King[15] procedure followed by phosphate estimation on a Technicon Autoanalyser (method N-4c).

RESULTS

Phosphate-haemolysed ghosts

Immediate examination of enhancement kinetics of stored ghosts washed into isotonic NaCl showed a fast phase (half-time less than mixing and recorder response time of 2 to 3 s) and a time-dependent phase of half-time less than 7 s. The latter was occasionally so rapid as to give in effect an overall fast enhancement. After equilibration in the isotonic medium for 3 h at either 4 °C or 25 °C, the following kinetic behaviour was found. ANS enhancement comprised a fast phase, and a time-dependent phase of half-time 14.2 ± 2.2 s. (All results in this section are expressed as mean $\pm$S.D. from at least three of five different ghost preparations.) The fast phase comprised $57.5 \pm 2.1\%$ of the overall enhancement. Fig. 1A shows the result of an experiment of this type.

When ghosts in isotonic NaCl were incubated for 3 h at 37 °C, a new time-dependent kinetic phase of half-time 82±20 s appeared. Fig. 1B shows the result of incubation of the ghost sample of Fig. 1A, analysed in terms of one fast and two time-dependent phases. It becomes convenient to separate three different types of ANS enhancement behaviour in phosphate-haemolysed ghosts. These are "fast", "medium" (half-time about 14 s) and "slow" (half-time about 80 s). Of the total final enhancement of incubated ghosts the fast phase comprised 57.3±2.5%, the medium phase 27.3±4.6% and the slow phase 15.3±5.5%. The medium phase corresponds in rate to the "slow" phase of Freedman[2] measured with unincubated ghosts, or the time-dependent phase found in frozen–thawed ghosts by Fortes and Hoffman[16].

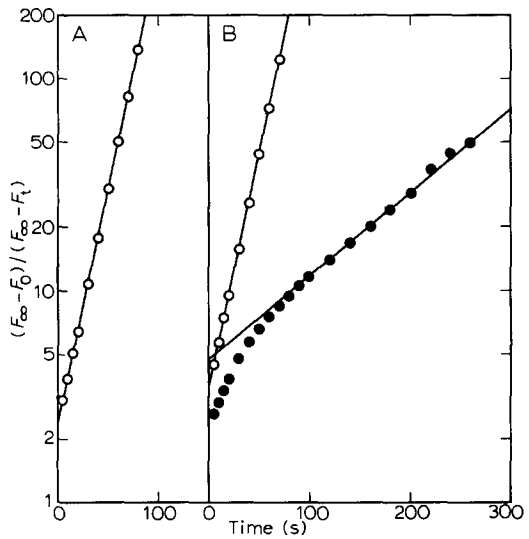

Fig. 1. ANS kinetics of phosphate-haemolysed ghosts. Experimental conditions were as described in the text. A, unincubated ghosts. This sample shows a medium phase of half-time 13.9 s comprising 42% of the overall enhancement (F_∞), the remainder being fast phase. B, the same ghost sample after incubation. ●—●, experimental data showing a slow phase of half-time 77 s contributing 21% of F_∞. ○—○, analysis of the data after subtraction of the slow phase, revealing a medium phase of half-time 14.0 s, contributing 22% of F_∞.

Incubation of any ghost sample did not change the size of the overall ANS enhancement, or the size of the fast phase, or the rate constant of the medium phase enhancement. Double reciprocal and Scatchard binding plots performed with one ghost preparation before and after incubation could be fitted in terms of a single set of binding sites and revealed no change in ANS limiting fluorescence enhancement ($2.2 \cdot 10^2$ with emission observed at 500 nm), dissociation constant ($2.0 \cdot 10^{-4}$ M) or number of membrane sites (0.17 moles/mole membrane phospholipid).

Thus the only effect of incubation detected by ANS fluorescence is the conversion of a fraction of ANS medium sites to slow type. Under the phase contrast microscope, incubation was found to have converted a fraction of the ghosts into spherical forms as distinct from the original biconcave disc shape. The effect was rather variable. Small membrane buds formed by exocytosis were occasionally observed after incubation. Their relationship to the small vesicles produced in presence of low trypsin

levels[17] is not clear but in any case the buds observed under our incubation conditions comprised a negligible amount of the total membrane.

Unincubated ghosts in an isotonic KSCN medium (150 mM KSCN, 10 mM potassium phosphate, pH 7.0) displayed an overall ANS fluorescence enhancement only 55% of that found in isotonic KCl. However, the relative contributions of medium and fast phases were unaltered. Double reciprocal and Scatchard plots revealed that the effect of SCN^- was merely to increase the apparent dissociation constant to $3.9 \cdot 10^{-4}$ M, without affecting the limiting fluorescence enhancement or the number of membrane sites available to ANS.

Mg^{2+}-haemolysed ghosts

Packed Mg^{2+}-haemolysed ghosts were faint pink in colour as distinct from the white of phosphate-haemolysed ghosts. This retention of haemoglobin after haemolysis in Mg^{2+}-containing media has been noted by Bramley and Coleman[8]. The amount of trapped haemoglobin was too small to interfere with fluorescence measurements. Under phase contrast, stored Mg^{2+}-haemolysed ghosts appeared as exclusively spherical forms. When an aliquot of stored Mg^{2+}-haemolysed ghosts was placed in an isotonic buffer and examined under the microscope, the ghosts were observed to have collapsed. This osmotic responsiveness was not observed with stored phosphate-haemolysed ghosts.

Fig. 2 shows results of kinetic runs with an Mg^{2+}-haemolysed ghost preparation before and after incubation in isotonic NaCl. Both samples showed fast and slow phases only, of approximately equal proportions. The different haemolysis conditions did not affect ANS fluorescence or binding parameters as measured in the standard isotonic NaCl medium, nor did the subsequent 37 °C incubation. No structural changes upon incubation could be seen under the microscope.

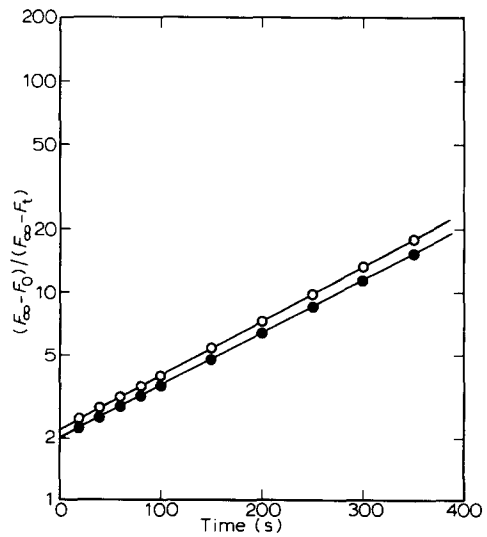

Fig. 2. ANS kinetics of Mg^{2+}-haemolysed ghosts. Experimental conditions were as described in the text. ○—○, unincubated ghosts. The slow phase has half-time 116 s and comprises 45% of the overall enhancement, the remainder being fast phase; ●—●, the same sample after incubation. The slow phase has half-time 120 s and comprises 50% of the overall enhancement.

Resealing of ghost preparations

The ghost preparations used in fluorescence studies were checked for resealing. Table I shows that 37 °C incubation does result in ghost populations which have regained some degree of cation (specifically Na^+) impermeability as compared with control samples. These latter show minimal resealing. For comparison with the results of Hoffman *et al.*[3] who used three washes before cation assay, the average sodium concentration inside incubated Mg^{2+}-haemolysed ghosts after three washes was at at least 34 mM since packed ghosts never occupied more than 10% of the suspension volume.

TABLE I

RESEALING OF GHOST PREPARATIONS

Experiments were carried out as described in the text. The table shows the sodium concentration (mM) in the ghost suspension after given numbers of washing steps. The fifth row of the table shows expected readings for completely leaky cells occupying 10% of the suspension volume. The flame photometer gave concentrations to within 0.05 mM.

Ghost sample and treatment	*Sodium concentration (mM)*		
	3 washes	*4 washes*	*5 washes*
Phosphate-haemolysed, control	0.35	0.05	0.0
Phosphate-haemolysed, incubated	1.6	0.3	0.1
Mg^{2+}-haemolysed, control	0.4	0.2	0.0
Mg^{2+}-haemolysed, incubated	3.4	1.85	1.6
Expected for leaky cells	0.15	0.0	0.0

DISCUSSION

These studies have made use of ghosts washed essentially free of their original cell contents[8,11] such as have been used in most physicochemical investigations of membrane structure[1,2,12,16,18,19,20]. In effect they are produced by haemolysis at a series of reducing tonicities, and are different from the ghosts commonly used by investigators of the membrane resealing phenomenon[3–6] which are produced by a single haemolysis step and retain of the order of 10% of their original cell contents (haemoglobin *etc.*). Nevertheless, we have shown that at least a fraction of the ghosts of haemoglobin-free preparations do still possess the capacity to reseal their membranes as judged by restoration of a degree of impermeability to Na^+.

Our studies show that the kinetics of the ANS fluorescence enhancement following addition to ghosts reflect the integrity of the membranes. We conclude that the membrane reorganisation which occurs during resealing involves a change in structure at the permeability barrier separating fast from medium or slow probe sites.

Ghost preparations shown to be at least partly resealed exhibit the slow phase. The small degree of resealing found with unincubated Mg^{2+}-haemolysed ghosts (Table I) which show the slow phase does not appear consistent with their haemoglobin retention and osmotic responsiveness, properties not expected of "leaky" membranes[7]. Two alternative possibilities might account for this behaviour. Firstly, the stored ghosts

in the hypotonic buffer may be already resealed with respect to Na^+ and therefore unable to incorporate significant amounts of cations when placed in isotonic buffer for 3 h at 4 °C (though this process must be sufficiently rapid at 37 °C). However, examination of ghosts thus treated showed that their original cell volumes had been regained after the initial fast fall, suggesting that isoosmolarity with the exteral medium had been achieved by diffusion of ions across the membranes. The effect has been noted by Teorell[21]. The second possibility is that, as suggested by Bodemann and Passow[7] the regain of the permeability barrier to any given permeant might occur at a different stage in a gradual membrane reorganisation process. For example, ghosts impermeable to sucrose but permeable to Rb^+ have been shown to exist[7].

The best interpretation seems to be that the unincubated Mg^{2+}-haemolysed ghosts have regained their membrane cation impermeability to an intermediate extent sufficient to display osmotic response, but not enough to be detected over the time scale (about a half hour) of the resealing test. (The membrane ANS barrier is regained to the fullest extent, however.) The medium ANS phase observed with phosphate-haemolysed preparations may reflect a similar situation with respect to the ANS permeability. The general conclusion is that slow ANS phases are shown by any membrane preparation which is resealed and/or osmotically responsive.

The fluorescence parameters of ANS do not reveal any difference between the sites responsible for the fast, medium or slow ANS responses (this work and ref.2). We propose that this reflects binding of ANS at sites distributed on the extracellular and intracellular sides (where these terms are applied with respect to an ANS permeability barrier) of the ghost membrane. The sites must be similar at the molecular level, but those on the outer side of the membrane will always give a fast ANS response, whereas the response from sites within the permeability barrier may be fast, medium or slow depending on the state of the membrane. The Mg^{2+}-haemolysed ghosts which display no medium phase must comprise membranes which have regained their ANS impermeability to the fullest extent possible under our conditions. A fraction of permanently "leaky" ghosts[4,7] might be present, however, contributing solely to the fast phase. According to this picture, the incubated phosphate-haemolysed samples, which show all three kinetic phases, must comprise a mixed population of ghosts with different permeability characteristics.

The above scheme seems to fit the known facts better than a distinction between sites either exposed on the outside of, or buried within, the membrane matrix. These would be expected to show distinct fluorescence properties. (On the other hand it is reported that frozen–thawed ghosts[16] display a time-dependent ANS phase of medium rate even though all permeability barriers are thought to be destroyed.)

Our ghost preparations show no redistribution of ANS amongst the membrane sites in presence of the membrane-binding thiocyanate ion. The ratio of fast to medium phases in a phosphate-haemolysed preparation is not altered. In this respect our preparations differ from frozen–thawed ghosts with which thiocyanate is reported[16] to reduce the ANS time-dependent phase. However, we do confirm that the effect of added anion is to compete for ANS sites, since its only effect is to increase the measured apparent value of K_D.

This work shows that ANS enhancement kinetics may be used to follow the membrane structure change which results in ghost resealing. A step towards location of the ANS permeability barrier within the membrane would be made if the positions

of the two sets of ANS sites lying on either side of the barrier were known. However, no report has appeared in which these sites have been distinguished at the molecular level. Our results also add support to the belief[12,20,22] that certain time-dependent phases of arylaminonaphthalene sulphonate probe responses in mitochondrial systems may be explained in terms of diffusion across permeability barriers.

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