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Is an intact cytoskeleton required for red cell urea and water transport?

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In order to determine the membrane protein(s) responsible for urea and water transport across the human red cell membrane, we planned to reconstitute purified membrane proteins into phosphatidylcholine vesicles. In preparatory experiments, we reconstituted a mixture of all of the red cell integral membrane proteins into phosphatidylcholine vesicles, but found that *p*-chloromercuribenzenesulfonate (pCMBS), which normally inhibits osmotic water permeability by approximately 90%, has no effect on this preparation. The preparation was also unable to transport urea at the high rates found in red cells, though glucose transport was normal. White ghosts, washed free of hemoglobin and resealed, also did not preserve normal urea and pCMBS-inhibitable water transport. One-step ghosts, prepared in Hepes buffer in a single-step procedure, without washing, retained normal urea and pCMBS-inhibitable water transport. Perturbations of the cytoskeleton in one-step ghosts, by removal of tropomyosin, or by severing the ankyrin link which binds band 3 to spectrin, caused the loss of urea and pCMBS-inhibitable water transport. These experiments suggest that an unperturbed cytoskeleton may be required for normal urea and pCMBS-inhibitable water transport. They also show that the pCMBS inhibition of water transport is dissociable from the water transport process and suggest a linkage between the pCMBS water transport inhibition site and the urea transport protein.

Introduction

The suggestion that the human red cell anion exchange protein, band 3, is the locus for the channel through which water enters the cell [1,2] is based on indirect experimental evidence. In order to subject this suggestion to a critical examination, we proposed to reconstitute purified band 3 into phosphatidylcholine vesicles whose water permeability could be measured by light-scattering methods. To discriminate between protein and lipid mediated water transport, we planned to inhibit the transport with the mercurial sulfhydryl reagent, *p*-chloromercuribenzenesulfonate (pCMBS), which Macey and Farmer [3] had found to inhibit osmotic water transport by approx. 90%. Toon and Solomon [4] showed that pCMBS inhibited water transport by binding to a single saturable site, whose K_i they determined. Thus, we expected to determine whether pCMBS-inhibitable osmotic water transport could be attributed unequivocally to band 3. In preparatory experiments, we reconstituted a mixture of all of the integral red cell membrane proteins into vesicles, according to the method of Carruthers and Melchior [5,6]. Although incorporation of the membrane proteins increased water transport in

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the vesicles, and glucose transport in this preparation was normal, we were unable to find any pCMBS inhibition by the stopped-flow light scattering method.

We then turned to white ghosts, in which the topology of the membrane proteins and the interactions among them and the membrane lipids are much closer to those of the native state. To our surprise, there was no pCMBS inhibition of water flux in resealed white ghosts, which had also lost the ability to transport urea at the very high rates observed in red cells. Osmotic water flux in ghosts had previously been measured by the light-scattering method and found to be approximately equal to the flux in red cells (Levin et al. [7], Colombe and Macey [8]), but these observations had been made in ghosts that had not been washed to remove the hemoglobin (Hb). We found that ghosts prepared in a single-step procedure (called 'one-step' or 'pink' ghosts) retained normal urea transport and pCMBS-inhibitable water transport; we have learned that small modifications in the process of osmotic lysis can have large effects on red cell permeability. In general, procedures which affect the integrity of the cytoskeleton also impair normal urea transport and pCMBS inhibition of water transport.

Materials and Methods

Materials

pCMBS, *N*-ethylmaleimide (NEM), 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid (Hepes), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), trypsin, soybean trypsin inhibitor (type II-s), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Mercaptoethanol was from Eastman (Rochester, NY). Tris(hydroxymethyl)aminomethane (Tris), glycine, sodium dodecyl sulfate (SDS), molecular weight standards and the usual electrophoresis reagents were from Bio-Rad (Richmond, CA). Recently outdated blood was kindly donated by the Children's Hospital, Boston, MA.

Preparation of resealed one-step ghosts

Red cells were washed three times in phosphate-buffered saline (PBS: 150 mM NaCl, 5 mM Na_2HPO_4 (pH 7.4)). Sealed, one-step ghosts (pink ghosts) were made by lysing the washed red cells 1 : 20 (v/v) at 4°C in one of the following osmotic lysis mediums (5 mM Na_2HPO_4 (pH 8.0); or 5 mM Hepes (pH 8.0); or 5 mM Tes (pH 8.0); or 5 mM Na_2HPO_4, 1 mM $MgCl_2$ (pH 8.0)) and centrifuging at 20000 × *g* for 10 min at 4°C. As Wood and Passow [9] pointed out, lower pH and temperature promote resealing to K^+; pink ghosts are approx. 98% resealable at lysis pH 6.0, 0°C, as compared to approx. 60% resealable at lysis pH 8.0, 0°C. The red pellet was resuspended at approx. 1 : 5 (v/v) in an 0.1 M NaCl solution containing 5 mM of the buffer used during osmotic lysis (pH 7.4). One-step ghosts were resealed by incubation for 1 h at 37°C and were diluted to their final concentration for the stopped-flow experiments without further washing. This resealing procedure is similar to that described by Wood and Passow [9] for 'pink resealable ghosts' except for the difference in the pH and temperature for the original lysis step which causes a significant reduction in the yield of resealed one-step ghosts. The ratio of the volume of resealed pink ghosts to that of the red cell has been given as 0.81 (Bjerrum [10]), 0.88 (Levin et al. [7]) or 1.32 (Brahm [11]).

Bodemann and Passow [12] describe three different types of pink ghosts: (I) ghosts that reseal immediately after hemolysis; (II) ghosts that reseal after addition of salt; and (III) leaky ghosts, that never reseal. Under our conditions (4°C hemolysis, incubation in salt at 37°C) there are mostly type II and III ghosts and relatively few type I ghosts. The presence of a large population of type III ghosts does not affect the interpretation of our results, since type III ghosts are osmotically silent.

Preparation and resealing of white ghosts

Unsealed, hemoglobin free ghosts (white ghosts) were made essentially by the method of Steck and Kant [13], by lysing the washed red cells 1 : 20 (v/v) in 5 mM Na_2HPO_4 (pH 8.0 at 4°C) centrifuging as described above, and washing the pellets three or four times in osmotic lysis medium, until white. These pellets were resuspended in PBS and resealed by incubation for 1 h at 37°C. Bjerrum [10] obtained resealed white ghosts by hemolysing

his cells at pH 3.7 in the presence of 4 mM $MgSO_4$ and then resealing them by incubating with 180 mM KCl (pH 7.1–7.3) for 45 min at 38°C. About 50% of Bjerrum's ghosts were sealed, as measured by impermeability to mannitol, and the ghosts had a mean volume of $50 \cdot 10^{-12}$ cm^3, half of the $100 \cdot 10^{-12}$ cm^3 volume of the native red cell (Jay [14]). As described above, the yield of resealed ghosts depends upon the pH and temperature at lysis. The volume of resealed pink ghosts decreases with increased KCl concentration [9], so we would expect the volume of our ghosts, made in 150 mM NaCl, to be slightly greater than, and the yield of resealed ghosts to be sharply lower than, Bjerrum's ghosts. The light scattering properties of the ghosts in our preparation are as expected for type II ghosts: the light scattering changes in response to an osmotic gradient, and the slope of the change as a function of time is linearly proportional to the osmotic gradient. Bjerrum found the diffusional water permeability (P_d) of his resealed ghosts to be $1.9 \cdot 10^{-3}$ $cm \cdot s^{-1}$ at 25°C, about half the average value of $4.2 \cdot 10^{-3}$ $cm \cdot s^{-1}$ at 20°C, for red cell water diffusion, as compiled by Dix and Solomon [15].

Permeability measurements

The time course of red cell or ghost volume changes was measured by 90° scattered light (600 nm) using a Dionex-130 stopped-flow apparatus (Sunnyvale, CA) which has a dead time of 2 ms. The analog data were digitized and averaged by a Hewlett-Packard Model 217 computer which was also used for data analysis. Cells or ghosts at approx. 2% cytocrit were mixed with an equal volume of buffer made hyperosmolar by the addition of either NaCl or urea. For each mixing solution, ten consecutive runs were averaged to improve the signal to noise ratio. All experiments were carried out at room temperature (21–24°C). The osmotic water permeability, P_f, was not calculated directly; instead, comparative ratios of permeability coefficients were computed from the time course of scattered light intensity. The zero-time slope for ghosts, obtained by fitting the data by least squares to a second degree polynomial, was compared with the zero-time slope for red cells from an experiment done under the same conditions, with the same osmotic gradients; no correction has been applied for differences in the cell volume/area ratio since we do not know the relative areas and the calculations are approximate. The urea permeability, P_{urea}, for white ghosts was estimated from the initial slope of the swelling segment of the light scattering curve. This approximation is especially valid for cases where $P_f \gg P_{urea}$, which is true for white ghosts, as will be discussed.

Separation of membrane proteins

The membrane proteins were separated by electrophoresis using the discontinuous SDS-polyacrylamide gel system as described by Laemmli [16]. Mercaptoethanol was used as the reducing agent. The ghost membranes were diluted 1:5 (v/v) in 80 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.001% (w/v) Bromophenol blue. After heating for 8 min at 90°C, the proteins were separated on a 7.5% polyacrylamide gel. The gel was fixed in acetic acid/methanol/water, stained with Coomassie blue, and destained overnight. After drying under vacuum at 80°C for 3 h, the gel was scanned in a scanning densitometer (Model GS 300, Hoefer Co., San Francisco, CA), which was coupled to our Hewlett-Packard computer. A program had been written to average 5–10 scans to improve the signal-to-noise ratio.

To sever the ankyrin from the spectrin, mild proteolytic digestion was done according to Jinbu et al. [17], who treated well washed white ghosts (2.5 mg/ml) with trypsin (25 μg/ml) for 30 s at 0°C. Jinbu et al. separated the membrane proteins on 3.3% gels; the photographs of these gels show significant loss of the ankyrin peak with no visible band 3 loss. The electron micrographs of Jinbu et al. show that the cytoskeleton becomes spatially detached from the membrane under these conditions. In our experiments, one-step ghosts made from Hepes were digested, after 1:5 dilution (into 0.1 M NaCl, 5 mM Hepes (pH 7.4)) but before the resealing step, by trypsin (25 μg/ml) for 30 s at 0°C, and the reaction was stopped by soybean trypsin inhibitor (0.5 mg/ml) or PMSF (30 μg/ml). We incubated these tryptin treated ghosts in 0.1 M NaCl, 5 mM Hepes (pH 7.4 at 37°C), 1 h, to reseal them. The ghosts were then either pelleted and prepared for electrophoresis as

described above, or diluted for permeability measurements, as described for white and one-step ghosts.

Results and Discussion

Urea and water transport lesions in white ghosts

When red cells are mixed in a stopped flow light scattering apparatus with a solution in which the osmotic pressure has been increased by 0.425 M NaCl, the cells shrink rapidly (upward deflection) for about 100 ms as shown in Fig. 1A. Fig. 1B shows a similar shrinking over the same time course in white ghosts that have been prepared by osmotic lysis and washed repeatedly, as described under Methods. Incubation with 1 mM pCMBS for 1 h at 21–24° C inhibits red cell osmotic water flux by approx. 90% (see Fig. 1A), but has no effect on osmotic flux in white ghosts (Fig. 1B) However, if white ghosts are made from red cells that had been pretreated with 1 mM pCMBS at 21–24° C for 1 h, the resulting ghost P_f was already inhibited by approx. 85%.

Although pCMBS does not inhibit osmotic water permeability in our white ghosts, Bjerrum [10] has reported that pCMB inhibited diffusional water permeability by 50% in white ghosts prepared according to his protocol. Benga et al. [18] found that pCMBS inhibited diffusional water permeability in pink ghosts, though the kinetics of inhibition were markedly slower than for red cells. Colombe and Macey [8] found that osmotic water permeability in pink ghosts prepared by the Hoffman [19] technique was inhibited by pCMBS, though the amount of inhibition was less, and the time course was slower, than in red cells. These experiments lead us to conclude that the transformation from red cell to ghost causes a change in the pCMBS site for water transport inhibition and that the nature and severity of the change depends upon the exact procedure used in making the ghost.

The sulfhydryl reagent, NEM, does not appreciably inhibit water or urea permeability of red cells [4], but we found that pretreatment of red cells with NEM (12 mM, 1 h at 37° C) makes the white ghosts susceptible to pCMBS inhibition (1 mM, 1 h at 21–24° C) of osmotic water transport by approx. 60%. Similarly, Benga et al. [18] found that NEM increases the rate and extent of pCMBS inhibition of diffusional water permeability in pink ghosts. They ascribed the NEM effect to the greater pCMBS binding by NEM-pretreated membranes which they had observed by ^{203}Hg-pCMBS uptake. We conclude that NEM reaction with band 3 sulfhydryl groups (Rao [20]) alters the configuration of band 3 in ghosts.

The permeability of red cells to urea is very high and a typical time course for red cell volume changes, following application of an 1 M urea gradient, is shown in Fig. 2A. There is a fast initial water efflux and the cells shrink rapidly for the first 150–200 ms until they reach a minimum volume at which the efflux of water is balanced by the influx of urea. Subsequently the volume changes more slowly and the return to initial cell

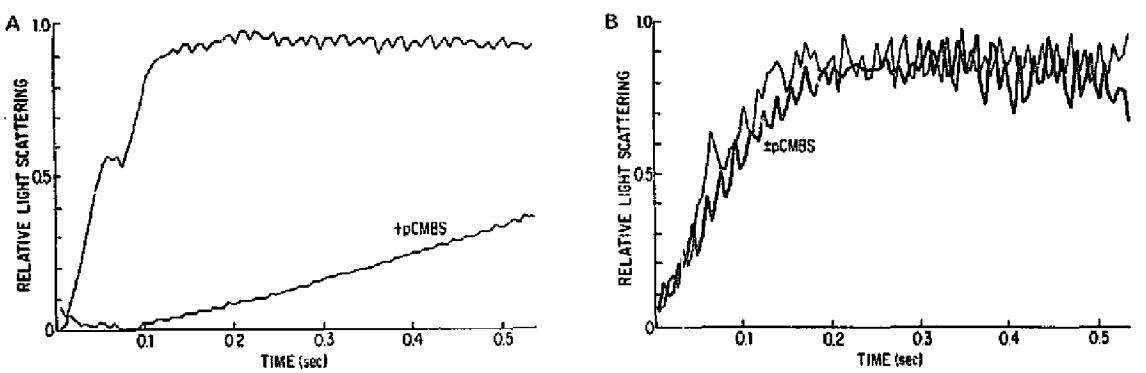

Fig. 1. (A) Red cell water permeability and its inhibition by 1 mM pCMBS (1 h, 21–24° C). Cells in isotonic medium were exposed to an 0.425 M NaCl gradient in a stopped-flow apparatus, causing the cells to shrink, which is detected by an upward deflection in the 90° scattered light. (B) water permeability of white ghosts in one of three experiments. The time course of the volume change is the same as for the red cells, but the water fluxes cannot be inhibited by 1 mM pCMBS (1 h, 21–24° C).

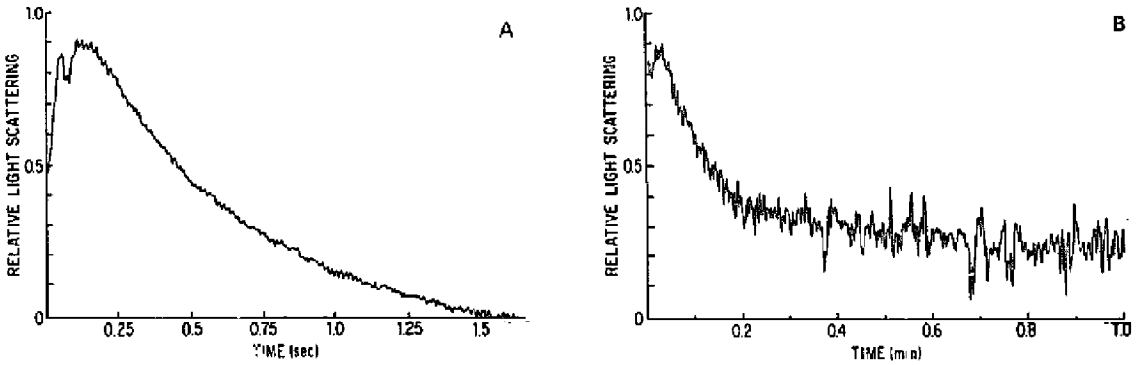

Fig. 2. (A) Urea permeability of red cells. Cells in isotonic medium were rapidly mixed with an equal volume of medium containing 2 M urea, causing the cells, first, to shrink to a minimum volume (upward deflection) and then to swell as described in the text. P_{urea} was approx. $2 \cdot 10^{-4}$ cm·s^{-1}. (B) White ghosts are also permeable to urea, but on a very much slower time scale. The urea permeability, calculated as described in Methods, in this experiment (one of three) is approx. 10^{-6} cm·s^{-1}, two orders of magnitude smaller than in red cells.

volume is driven by the influx of urea down its concentration gradient. For white ghosts, Fig. 2B shows that there is no rapid urea flux; instead the ghosts swell slowly over a 1 min period as urea enters the cell down its concentration gradient and water follows. The swelling is similar to that which follows the minimum volume in red cells, but the time constant is orders of magnitude longer. The time scale in Fig. 2B is too long to resolve the initial rapid phase of water efflux. The urea permeability coefficient, P_{urea}, in the red cell (Sha'afi et al. [21]) is $4 \cdot 10^{-4}$ cm·s^{-1}, and red cell urea flux is saturable with $K_M \approx 0.22$–0.33 M (Mayrand and Levitt [22], Brahm [23]). In contrast, $P_{urea,ghost} \approx (0.2–1.0) \cdot 10^{-6}$ cm·s^{-1} and the flux does not saturate up to 1 M urea. Phloretin inhibits red cell urea permeability with $K_i = 18$ μM (Toon and Solomon [24]) but has no effect on $P_{urea,ghost}$ up to 50 μM. These observations show that urea permeates the ghost membrane through a diffusion pathway, which we take to be the membrane lipid. $P_{urea,ghost}$ is comparable to the value of $0.4 \cdot 10^{-6}$ cm·s^{-1} given by Finkelstein [25] for urea permeation of phosphatidylcholine/cholesterol bilayers and that of $4 \cdot 10^{-6}$ cm·s^{-1} for P_{urea} in phosphatidylcholine bilayers, given by Poznansky et al. [26].

We think that protein damage may be responsible for the impairment of urea permeability and the alterations in pCMBS inhibition of water permeability in white ghosts. In the native red cell, the saturability of the high flux component of urea permeability and its inhibition by pCMBS indicates that urea transport is protein mediated [4,23]. The pCMBS inhibition of water transport is also protein associated, as shown by Ojcius and Solomon [27] who correlated pCMBS inhibition of water flux with pCMBS binding to band 3.

Some cytoskeletal links are lost when ghosts are prepared from red cells. Fowler and Bennett [28] report that 50–80% of tropomyosin dissociates from red cell ghosts prepared in the usual manner without Mg^{2+}. Beth et al. reported that red cell lysis causes a significant change in band 3 rotational mobility, due either to disruption of cytoskeletal [29] or membrane protein [30] links. Salhany et al. [31] found that preparation of white ghosts altered the configuration of at least one membrane protein, since ghost preparation exposed new binding sites on band 3 for the anion transport inhibitor, pyridoxal 5'-phosphate, that are not available in the native red cell.

We made a number of attempts to restore normal transport to white ghosts. For these experiments, unsealed white ghosts were prepared with Na_2HPO_4 as described under Methods and the various restorative measures were applied to the unsealed ghosts to allow access to the intracellular compartment. In order to make flux determinations, the ghosts were subsequently sealed by incubation with isosmolar NaCl for 1 h at 37°C. Since normal water permeability, though

not its pCMBS inhibition, was shown by our white ghosts, we checked the integrity of the seal by observing the time course of light-scattering changes, as shown in Figs. 1 and 2. We first added the supernatant from the lysing step, but found no effect on the urea permeability, which remained two orders of magnitude slower than in the red cell, though, in the presence of ATP, pCMBS inhibition of water permeability was restored. Then we tried addition of 1 mM ATP, with or without 1 mM ADP, 1 mM Mg^{2+}, 1 mM 2,3-diphosphoglycerate, and various alterations in intracellular NaCl, but none of these were able to restore the native urea permeability. Changes of buffer in the incubation medium also had no effect. Substitution of 5 mM Hepes or Tes for 5 mM Na_2HPO_4 in the resealing medium had no effect, although their use is important in the preparation of one-step ghosts, as will be discussed below. Rao [20] had found that two of the intracellular SH groups in band 3 were oxidized to cystine when white ghosts are prepared; we incubated white ghosts in 5% mercaptoethanol (v/v) for 1 h at 37°C to reduce these bonds, but found no effect on either urea permeability or inhibition of water permeability by pCMBS.

Preparation and properties of one-step ghosts

Since none of these treatments had been able to restore the water and urea permeability of white ghosts to normal, we explored the possibility that the lesion resulted from the harsh treatment of repeated washing. We devised a gentler procedure to lyse them in a single step, centrifuge them once and then reseal as described under Methods. These ghosts retained a significant amount of Hb and are called pink, or one-step ghosts; the densitometric scan of the polyacrylamide gel is shown in Fig. 3.

The scheme of cytoskeletal assembly described by Bennett [32] forms the basis of our understanding of the effects on one-step ghosts. The α and β chains of spectrin are twisted together to form a heterodimer and these dimers are joined tail-to-tail to form the spectrin tetramer which is the fundamental cytoskeletal filament. Near the tail of one heterodimer, there is a binding site for ankyrin, one site for each spectrin tetramer. Ankyrin is a 215 kDa protein which links spectrin to the specific

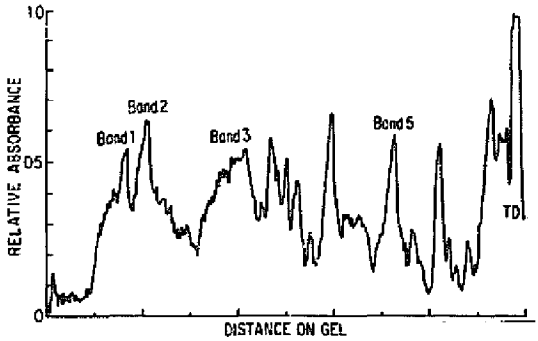

Fig. 3. Densitometric scan of a Coomassie blue-stained polyacrylamide gel of one-step ('pink') ghosts. These one-step ghosts were made by osmotically lysing red cells 1:20 (v/v) and resealing the membrane pellet from the first centrifugation step. The extra bands not normally seen in white ghosts are due to the ≈ 5% cytoplasmic proteins trapped in the resealing step.

binding site on the cytosolic pole of band 3. Since there are approx. 10^5 ankyrin molecules, compared to approx. $5.5 \cdot 10^5$ band 3 dimers, only about one band 3 dimer in five is bound to the cytoskeleton, or a correspondingly larger fraction if the basic band 3 unit is a tetramer. At the head end, the spectrin tetramers are bound to actin, which forms the hub for an ≈ five-spoked wheel of tetramers. Red cell actin is a short chain, consisting of 12–17 monomers, and tropomyosin is thought to lie in the grooves of the actin chain, where Bennett suggests that it may stabilize the actin filaments or regulate the spectrin : actin interaction. Tropomyosin is a dimer of about 56 000 kDa and is present in approx. 60 000 copies/cell, sufficient to coat about 80% of the actin. The actin filaments are bound to the membrane through band 4.1, a protein of approx. 160 000 kDa, which is thought to bind to glycophorin A, and thence possibly to another band 3 tetramer in the membrane. Many of the cytoskeletal proteins are phosphorylated, but the role of phosphorylation is not understood.

There are three treatments that affect cytoskeletal integrity that we have found also to affect urea and pCMBS-inhibitable water permeability in pink ghosts. Patel and Fairbanks [33] studied the ability of red cell ghosts to change their structure from crenated spheres to cup or disk forms, the echinocyte : discocyte transformation. They

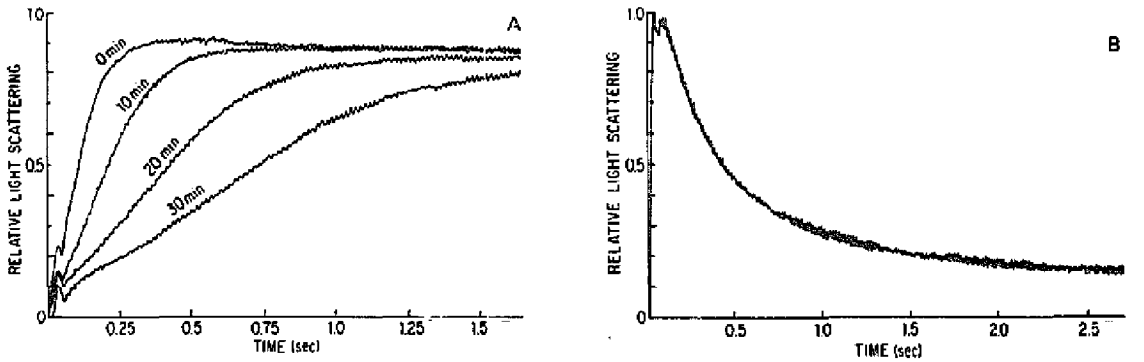

Fig. 4. (A) Inhibition by 1 mM pCMBS of the water permeability of one-step ghosts, made by hemolysis in 5 mM Hepes (pH 8.0) in one experiment, typical of five. Numbers above the curves represent the reaction times with pCMBS. The osmotic gradient was 0.425 M NaCl. (B) Urea permeability of one-step ghosts in one experiment, typical of five. The osmotic gradient was 1 M urea. Notice that the time scale is similar to that for red cells (Fig. 2A).

found that the ability of the cell to make this transformation is dependent upon the presence of MgATP and is promoted by certain hemolysis buffers, and inhibited by others. In particular, ghosts hemolysed in 5 mM Na_2HPO_4 or 10 mM Tris lose the capacity to change shape, while those prepared in 10 mM Hepes or 10 mM Tes retained it. We have found in three experiments that one-step ghosts prepared by hemolysis with 5 mM Na_2HPO_4 lose the ability for normal urea flux, having a P_{urea} that is less than in red cells by one order of magnitude or more, while those prepared in 5 mM Hepes (five experiments) or 5 mM Tes (two experiments) retain native urea permeability with results shown in Fig. 4B. These one-step ghosts also exhibit normal pCMBS inhibition of water flux, as shown in Fig. 4A and of urea flux (data not shown).

Fowler and Bennett [28] have observed that tropomyosin, which is removed in ghosts prepared without Mg^{2+}, inhibits the binding of spectrin to actin by 50–80%, and Bennett [32] has suggested that the presence of Mg^{2+} may be important in studies involving cell shape and stability of the red cell cytoskeleton. We have found in two experiments that one-step pink Na_2HPO_4 ghosts, prepared in the presence of 1 mM Mg^{2+}, retain normal urea permeability, which is lost when Mg^{2+} is omitted.

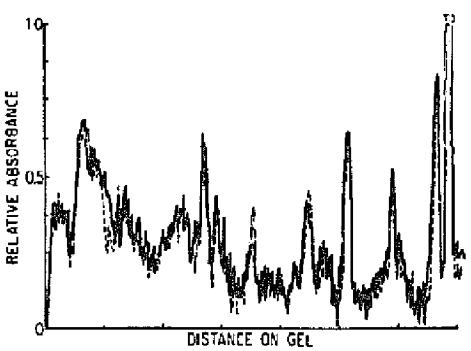

Fig. 5. Densitometric scan of a Coomassie blue-stained polyacrylamide gel of one-step ghosts, before (full line) and after (dotted line) treatment with trypsin and trypsin inhibitor, as described under Methods. Ankyrin removal by trypsin treatment causes a significant loss in the band 2.1 (215 kDa) region on the shoulder of band 2.

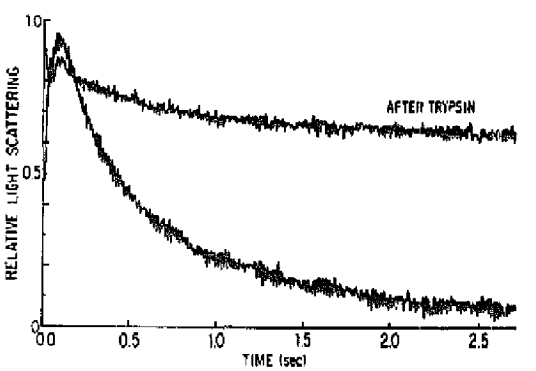

Fig. 6. Urea permeability of one-step ghosts made in the presence of Hepes. Mild trypsin digestion (25 μg trypsin/ml for 30 s at 0°C), which cleaves ankyrin, causes the urea permeability to decrease significantly.

Jinbu et al. [17] have devised a mild trypsin treatment which breaks the ankyrin link to spectrin (and/or band 3) with no effect on band 3. We have used this treatment on unsealed one-step ghosts, prior to resealing. Fig. 5 shows a densitometric scan of a 7.5% gel prepared from these ghosts with no further washing. Comparison of the control and trypsin-treated scans shows that the mild trypsin treatment has produced a significant loss in the band 2.1 region with essentially no effect on band 3, in agreement with the findings of Jinbu et al. [17]. The trypsin treatment strongly inhibits the urea permeability, as shown in Fig. 6 in one experiment, typical of two, but there is no effect of trypsin on water permeability. This experiment strongly suggests that the in situ presence of ankyrin is required for preservation of native urea permeability.

Our experiments show that the native transport properties of water and urea are sensitive to their cellular environment and treatments which can easily be tolerated by other transport systems, such as Cl^- exchange [34] and glucose transport [35], have drastic effects on the transport of water and urea. The effects produced by each of the three treatments we have used to perturb the cytoskeleton are consistent with the view that the integrity of the cytoskeleton is necessary for the preservation of the native urea and water transport systems, but they do not prove it.

Relation of water and urea transport to the properties of the native red cell

The present experiments provide three of the following four observations that need to be accomodated by any model which explains how urea and water cross the red cell membrane.

(1) pCMBS inhibition of water transport can be destroyed with little or no effect on water transport. A similar effect was found by Dix et al. [36] who reported that radiation inactivates the pCMBS inhibition of water transport, but has no effect on the transport itself. These observations impose the requirement that the pCMBS water transport inhibition site be located either on a separate domain of the water channel protein, or on an adjacent protein.

(2) There is a linkage between the pCMBS water transport inhibition site and urea transport since we have found that cytoskeletal perturbations which affect one component, usually affect the other. Assuming this linkage to be causal, a signal must be transmitted between the pCMBS water transport inhibition site and the urea channel protein, as if they were on the same or adjacent proteins, or as if a common cytoskeletal element transmitted information to both sites.

(3) Low concentrations of pCMBS can completely inhibit urea flux with no effect on water flux [4,39]. Furthermore, the present experiments show that mild trypsinization has a large effect on urea flux in one-step ghosts with no effect on water flux. This means that there must be one region in the urea channel protein in which the urea flux can be modulated with no effect on water.

(4) The reflection coefficient for urea interaction with the red cell membrane is significantly less than unity [37,38]. This means that there is a channel across the cell membrane through which both urea and water are transported so that there must be one region in which the urea channel protein and the water channel protein coincide.

The findings of Ojcius and Solomon [27] strongly suggest that the sites for pCMBS inhibition of both water and urea transport are on band 3, with stoichiometries of approx. 0.25 urea flux inhibition site/band 3 and approx. 0.75 water transport inhibition site/band 3. They suggested that a band 3 tetramer provides a useful construct for discussion, consistent with a model in which the first pCMBS molecule to reach the tetramer would bind to the urea flux inhibition site because its affinity is greater by two orders of magnitude and the kinetics of binding to this site are very much faster. This initial binding was presumed to lead to a conformational change in the other three monomers which transforms the remaining three binding sites to water flux inhibition sites. There is precedent for cooperative behavior of the band 3 monomers since both Macara et al. [40] and Verkman et al. [41] have shown that binding of a stilbene inhibitor to one monomer causes a conformational change in the other monomer in the non-covalent band 3 dimer in the membrane.

This construct also provides a useful way to understand why pCMBS inhibition of the water permeability is destroyed together with normal

urea permeability when white ghosts are prepared from red cells. The initial lesion is presumed to be in the monomer responsible for urea permeability. We speculate that this lesion prevents the subsequent conformational change which enables the sites in the other three monomers to bind pCMBS, and hence both transport-related functions usually disappear together.

In order to accomodate the observation that urea flux can be inhibited with no effect on water flux, it is necessary that urea interact with a membrane element before, or after, passage through the channel. Brahm [23] and Mayrand and Levitt [22] have shown that there is a urea binding site on at least one, and probably both, faces of the red cell membrane. The conformational change that follows pCMBS binding to the urea transport inhibition site is presumably responsible for inhibiting the urea flux. We have previously suggested [2] that passage through the membrane requires urea to exchange its waters of hydration with hydrogen bonds in the channel. Inhibition of this exchange would inhibit urea flux, but any other mechanism which is specific to urea transport would be just as satisfactory.

Thus, it seems that the tetrameric construct, which was proposed to account for the stoichiometry of pCMBS binding to band 3, also satisfies the four criteria above, and may provide a framework for constructing a model that is amenable to experimental test. The role of the cytoskeleton would be to maintain the tetrameric configuration.

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