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Some Transport Properties of Resealed Washed Human Erythrocyte Membranes

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A comparison was made between the phosphate- and glucose-transport systems of intact erythrocytes and resealed washed membranes. Glucose transport exhibits identical properties in both cases, but the phosphate-transport system does not appear to have survived the membrane isolation procedure unaltered. Evidence is presented to support the suggestion that some form of structural perturbation has occurred to the protein mediator of phosphate exchange.

Studies into the organization of membrane components, the disposition of membrane proteins and the transport properties of membrane-derived vesicles all depend for their relevance to the intact membrane on the assumption that little or no fundamental alterations occur to the membrane during the various isolation procedures. It seems a reasonable precaution therefore, before attempting labelling and reconstitution studies on integral transport proteins, to confirm that the isolated membrane preparations truly reflect the transport properties of the intact system.

These investigations were prompted by our interest in the structure and activity of the anion-transport system of human erythrocyte membranes, which has been identified with the band-3 polypeptides (Cabantchik & Rothstein, 1972; Ho & Guidotti, 1975) as seen on polyacrylamide-gel electrophoreses (Steck, 1972). The purification of erythrocyte 'ghosts' (Dodge *et al.*, 1963) and the formation of resealed membrane preparations (Bodemann & Passow, 1972; Steck & Kant, 1974) are well established and fairly gentle procedures. The present paper describes some of the passive transport properties of these resealed preparations. The results suggest that changes do occur during the isolation and washing regimen for these membranes, which may subsequently be reflected in altered properties of at least one of the integral protein components.

Materials

Blood type A Rh^+ was obtained from the Regional Blood Transfusion Centre, Leeds, U.K. Phlorrhizin, aldolase (grade I) from rabbit muscle, NAD^+ (grade III), D-fructose 1,6-bisphosphate were from Sigma Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. D-[^{14}C]Glucose, L-[^{3}H]glucose and [^{32}P]P_i were supplied by The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. 4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (disodium salt) and all other reagents were from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Abbreviation used: SDS, sodium dodecyl sulphate.

Methods

Phosphate transport in intact erythrocytes

Erythrocytes were washed with 0.3M-triethanolamine/citric acid, pH7.5, at 4°C, and centrifuged at 1000*g* for 10min. The leucocytes were removed and the erythrocytes washed twice more. The cells were diluted with 0.3M-triethanolamine/citric acid to a 30% hematocrit value. Inhibitors were added as the solid ($HgCl_2$) or from a concentrated stock solution and the volume of added buffer was adjusted to keep the cell concentration the same in all cases. A preincubation at 26°C for 45min was carried out in all instances. The temperature was then adjusted as necessary before starting the experiment by adding concentrated KH_2PO_4 in 0.3M-triethanolamine/citric acid, pH7.5, and [^{32}P]P_i (10μCi/ml) to a final phosphate concentration of 10mM. Samples were taken at timed intervals and centrifuged at 1000*g*; 100μl of the supernatant was then pipetted into 400μl of 5% trichloroacetic acid. At the end of the experiment all the trichloroacetic acid solutions were centrifuged and the supernatants sampled for radioactivity counting. A dioxan scintillation fluid containing naphthalene (100g/l) and 2,5-diphenyloxazole (5g/l) was used and the samples were counted for radioactivity in a Beckman LS 230 liquid-scintillation counter. Each experiment was repeated a minimum of three times. The points shown in the Figures represent single experiments, the maximum variation in the duplicates being within 5%.

Glucose transport in whole cells

Erythrocytes were prepared as for phosphate transport, and diluted with buffer to a 50% hematocrit value. Then 0.2ml of the cell suspension was placed in a centrifuge tube and 1 ml of 0.3M-triethanolamine/citric acid, pH7.5, containing 0.5mM-D-glucose, 1.0mM-D-galactose and 0.5μCi of D-[^{3}H]-glucose was added with thorough mixing. At the specified time, 6ml of ice-cold 0.3M-triethanolamine/citric acid/3mM-$HgCl_2$ was added and the tube kept on ice until the end of the experiment. All the samples were then centrifuged for 5min and the supernatants removed. The pellet was lysed with 0.1ml of water and the protein precipitated with 5% trichloroacetic acid. Samples were counted for radioactivity as for phosphate transport.

Preparation of resealed 'ghosts'

'Ghosts' were made after the method of Dodge *et al.* (1963). The cells were lysed in 10vol. of cold 5mM-KH_2PO_4, pH7.5, and washed three times in the same buffer. The membrane preparation was sealed by incubation at 37°C for 30min in 0.2M-triethanolamine/citric acid (pH7.5)/10mM-KH_2PO_4/1mM-$MgCl_2$, pH7.5. The resealed 'ghosts' were centrifuged at 40000*g* for 10min. The pellet was resuspended in 0.2M-triethanolamine/citric acid/1mM-KH_2PO_4, pH 7.5, and repelleted.

The percentage resealing was determined by assaying the activity of glyceraldehyde 3-phosphate dehydrogenase in the presence and absence of 0.1% Triton X-100 (McDaniel *et al.*, 1974; Steck & Kant, 1974). The assay solution consisted of 2.55ml of 30mM-sodium pyrophosphate, pH8.4, containing 4mM-cysteine, 0.1ml of aldolase (1mg/ml), 0.1ml of 3mM-NAD^+, 0.1ml of 0.4M-sodium arsenate and 50μl of the resealed 'ghosts' preparation. Triton X-100 (0.3ml, 1%) was added in place of 0.3ml of buffer where lysis was required. The solutions were equilibrated at room temperature for 5min. Then 0.1ml of 0.067M-fructose 1,6-bisphosphate was added and the A_{340} increase was continuously monitored by using an SP. 500 Unicam spectrophotometer with chart recorder.

Phosphate transport in resealed 'ghosts'

The resealed 'ghosts' (protein concentration 16mg/ml) were diluted 1:1 with 0.2M-triethanolamine/citric acid/1mM-KH_2PO_4, pH7.5; 3ml of this was further diluted with 1ml of buffer containing the required amount of inhibitor, and the mixture incubated for 30min at 10°C. The experiment was started by adding [^{32}P]P_i; 0.7ml samples were accurately withdrawn into 6ml of ice-cold 0.2M-triethanolamine/citric acid, pH5.0, at specified times. These solutions were centrifuged at 50000*g*, the supernatants removed and the pellets redissolved in 10% SDS. Samples were assayed for their radioactivity content as previously described.

Glucose transport in resealed membranes

This was performed as described for glucose transport in whole cells except that the buffer used was 0.2M-triethanolamine/citric acid containing 0.5mM-D-glucose, D-[^{3}H]glucose (0.5μCi/ml) and 1.0mM-D-galactose, pH7.5. The temperature was 10°C and 0.2ml of resealed 'ghosts' (protein concn. 8mg/ml) was added to each tube. The resealed 'ghosts' were centrifuged at 50000*g* for 5min, the supernatants removed and the pellets redissolved in 10% SDS before counting for radioactivity as before.

Results

The method used for producing washed erythrocyte 'ghosts' was very similar to that commonly used in many such preparations. The resultant membranes were light pink in colour and the virtual absence of residual haemoglobin was further confirmed by SDS/polyacrylamide-gel electrophoresis. The critical factors of the resealing procedure were the presence of bivalent cations (1mM-$MgCl_2$) and the temperature (37°C). The degree of resealing determined from the accessibility of glyceraldehyde 3-phosphate dehydrogenase was always greater than 90%. Tests for leakiness by using the availability of this enzyme were also performed in which one substrate, NAD^+, was included within the resealed 'ghosts' and the other, glyceraldehyde 3-phosphate, added subsequent to resealing. No dehydrogenase activity was detected in the absence of Triton X-100, indicating complete membrane impermeability to glyceraldehyde 3-phosphate.

To ascertain whether other physiological properties of the intact cell were preserved in the sealed preparations, a series of transport studies was performed on the washed resealed membranes. Fig. 1 illustrates the transport of [^{32}P]P_i ions into intact erythrocytes in the presence and absence of 10μM-4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulphonate. Complete inhibition was also observed at 5μM. In Figs. 2 and 3 similar transport properties were monitored, by using on this occasion the resealed washed membrane preparation. The most obvious difference between the two systems is the failure of this inhibitor to inhibit [^{32}P]P_i uptake by the vesicle system even at much higher concentrations (150μM; Fig. 3). That transport was indeed being observed in this case was confirmed by the failure of unsealed 'ghosts' preparations to exhibit any degree of radiolabel incorporation. The closely related inhibitor 4-sulphophenyl isothiocyanate also failed to inhibit the uptake of anions into these membrane vesicles.

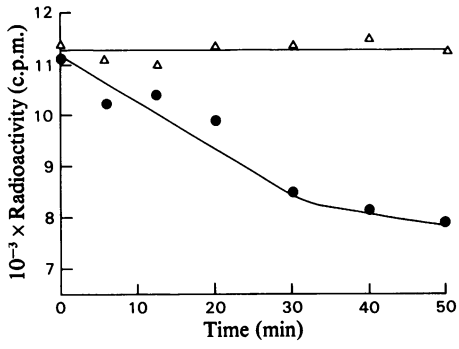

Fig. 1. *Phosphate transport in whole cells: effect of 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate*
The temperature was 24°C; other experimental details are as in the Methods section. ●, Control; △, + 10μM-4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate (which was made up fresh as a 2mM stock solution in 0.3mM-triethanolamine/citric acid, pH7.5). The ordinate represents radioactivity (c.p.m.) in the supernatant fraction after centrifugation.

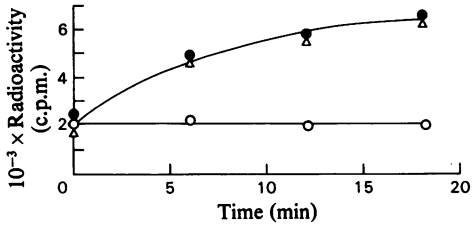

Fig. 2. *Phosphate transport in resealed washed 'ghosts': effect of 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate*
The temperature was 10°C; the protein concentration and experimentation were as described in the Methods section. ●, Resealed washed 'ghosts'; △, + 15μM-4-acetamido-4′-isothiocyanato-2,2′-stilbene disulphonate (from 2mM stock as in Fig. 1); ○, unsealed 'ghosts' (protein concentration in reaction mixture 6mg/ml). The ordinate shows the radioactivity (c.p.m.) in the 'ghost' pellet after centrifugation.

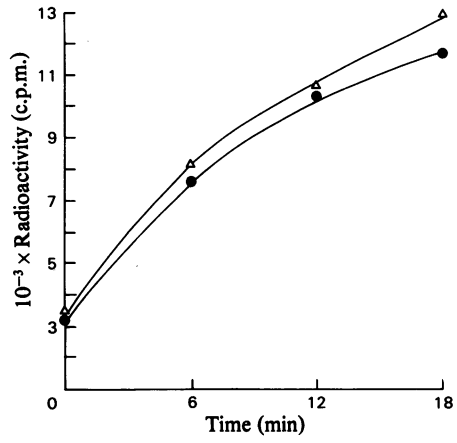

Fig. 3. *Phosphate transport in resealed washed 'ghosts': effect of 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulphonate*
The experiment was as in Fig. 2 except that the concentration of 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate was 150μM.

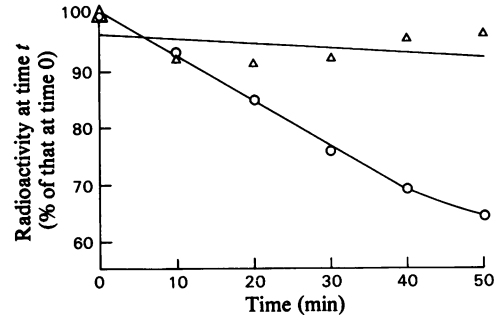

Fig. 4. *Phosphate transport in whole cells: effect of phlorrhizin*
Details were as for Fig. 1. ○, Control; △, + 2mM-phlorrhizin. The ordinate is (c.p.m. at time *t*/c.p.m. at time 0) × 100.

This lack of inhibition of phosphate transport was also demonstrated with phlorrhizin, another inhibitor of phosphate transport in whole cells (Figs. 4 and 5), whose mechanism of action is thought to be different from that of 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate and 4-sulphophenyl isothiocyanate. A third difference also seemed to be the slightly increased rate of phosphate uptake in the resealed preparation.

To determine whether this apparent disturbance in transport function in the membrane preparation represented a generalized membrane phenomenon or a specific perturbation of the anion transport system, certain characteristics of glucose uptake were also investigated. The following parameters were exhibited by both intact cells and resealed membrane preparations: (1) glucose transport, inhibitable by 3mM-Hg^{2+} (Figs. 6 and 7); (2) inhibition of transport by phlorrhizin (Figs. 8 and 9); (3) stereospecific transport of D- rather than L-glucose (Fig. 10). These results suggest therefore that the perturbation that gave rise to uninhibitable phosphate transport was not manifested by any alteration in the properties of the glucose-transport system. The inability of L-glucose to pass into the vesicles further attested to the absence of non-specific channels through the membrane.

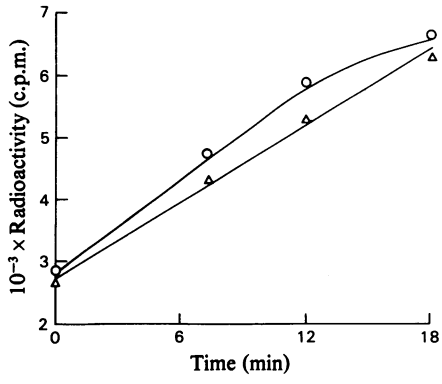

Fig. 5. *Phosphate transport in resealed washed 'ghosts': effect of phlorrhizin*
Details were as for Fig. 2. ○, Control; △, + 2mM-phlorrhizin. The ordinate represents the radioactivity in the pellet after centrifugation.

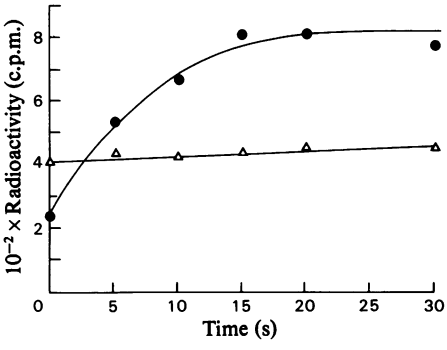

Fig. 6. D-*Glucose transport in whole cells: effect of* Hg^{2+} *ions*
The temperature was 10°C; experimental details are given in the Methods section. ●, Control; △, + 2mM-$HgCl_2$. The $HgCl_2$ was dissolved in the buffer used for diluting the erythrocytes to a 50% haematocrit value, and in the incubation solution. The ordinate represents radioactivity in the whole-cell pellet after centrifugation and removal of the supernatant.

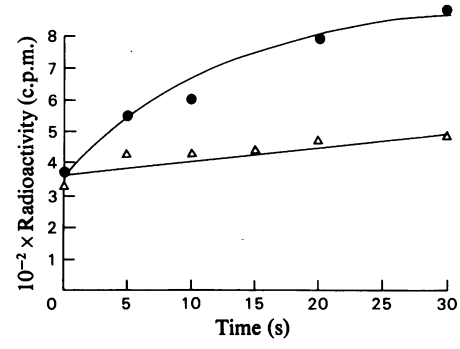

Fig. 7. D-*Glucose transport in resealed washed 'ghosts': effect of* Hg^{2+} *ions*
The temperature was 10°C; experimental details are given in the Methods section. ●, Control resealed washed 'ghosts'; △, + 2mM-$HgCl_2$. $HgCl_2$ was added as in Fig. 6. The ordinate represents the radioactivity in the resealed washed 'ghost' pellet after centrifugation.

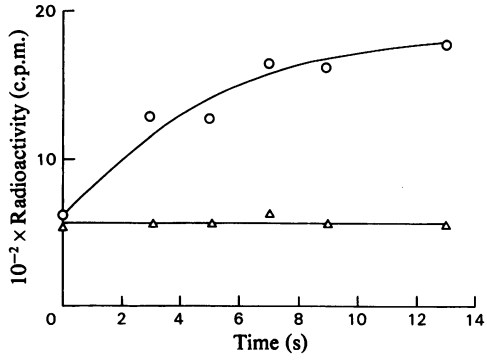

Fig. 8. D-*Glucose transport in whole cells: effect of phlorrhizin*
The experimental details are as in Fig. 6. ○, Control; △, + 2mM-phlorrhizin. Phlorrhizin was present in the cell suspension and the incubation medium.

The effect of Hg^{2+} ions on phosphate transport threw further light on the uptake process, for, although no inhibition of influx was observed in whole cells at a concentration of 3mM-$HgCl_2$ (Fig. 11), transport in the resealed membrane preparation was very effectively abolished at this concentration of Hg^{2+} ions (Fig. 12). The known interaction of these ions with accessible SH groups on protein molecules and the presence of such an available SH group at the cytoplasmic surface of the anion-transport protein (Steck *et al.*, 1976) led us to measure transport in resealed washed 'ghosts' that had been treated with iodoacetamide before sealing. Although iodoacetamide reacts with this free SH group, it did not inhibit phosphate transport. Furthermore, these pretreated resealed 'ghosts' were still susceptible to inhibition of phosphate transport by Hg^{2+} ions (Fig. 12).

It is also possible that perturbation of the membrane or the protein structure may lead to the loss of the anion-selectivity site of the transport mediator and that failure of 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate to inhibit resulted from the inability of the inhibitor to bind to the transport protein. Cells were therefore pretreated with inhibitor, 'ghosts' made from these cells and the

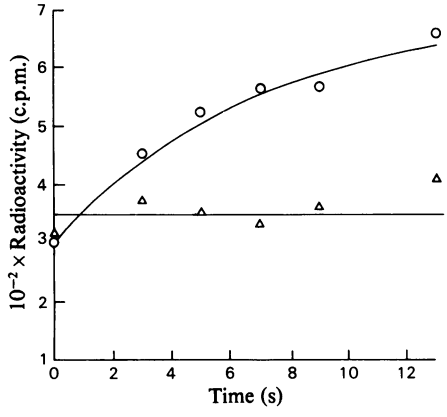

Fig. 9. D-*Glucose transport in resealed washed 'ghosts': effect of phlorrhizin*
The experimental details are as in Fig. 7. ○, Control; △, + 2mM-phlorrhizin (present as in Fig. 8).

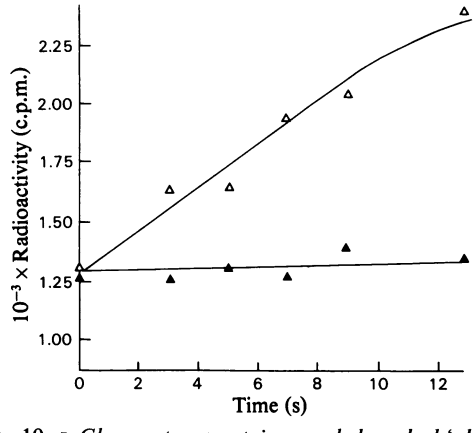

Fig. 10. L-*Glucose transport in resealed washed 'ghosts'*
The experimental details are as in Fig. 6. △, D-Glucose; ▲, L-glucose. In the incubation solution to monitor L-glucose transport 0.5mM-L-glucose and L-[^{14}C]glucose (0.25μCi/ml) replaced 0.5mM-D-glucose and D-[^{3}H]glucose (0.5μCi/ml). The ordinate represents the radioactivity in the pellet.

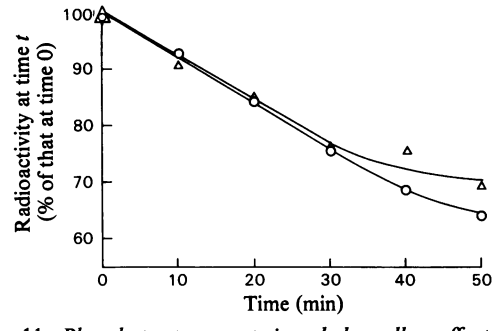

Fig. 11. *Phosphate transport in whole cells: effect of Hg^{2+} ions*
The experimental details are as in Fig. 1. ○, Control; △, + 3mM-$HgCl_2$. The mercury is added as the chloride and it is therefore necessary to make the control of 6mM-KCl to allow for the effect on phosphate transport of the chloride ions present. The ordinate represents (c.p.m. at time t/c.p.m. at time 0) × 100.

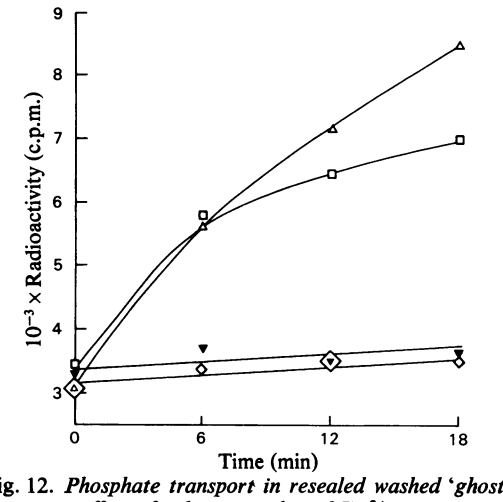

Fig. 12. *Phosphate transport in resealed washed 'ghosts': effect of iodoacetamide and Hg^{2+} ions*
The experimental details are as in Fig. 2. △, Untreated resealed washed 'ghosts'; ▼, untreated + 3mM-$HgCl_2$; □, resealed washed 'ghosts' pretreated with iodoacetamide; ●, pretreated + 3mM-$HgCl_2$. The pretreated 'ghosts' were reacted with 10mM-iodoacetamide for 1h at room temperature in 50mM-Tris/HCl, pH8.0, centrifuged, washed with 50mM-Tris/HCl, pH8.0, and resealed as in the Methods section. KCl (6mM) was added to the control tubes as in Fig. 10. The ordinate represents radioactivity in the pellet.

membranes washed and subsequently resealed. Some of the whole cells were retained and washed first in albumin-containing buffer (5mg of protein/ml) and then in buffer alone, to remove any reversibly bound inhibitor. In this case, 100% inhibition was observed in the intact cells (Fig. 13*a*), but not with the resealed preparation (Fig. 13*b*). When the experiment was repeated by preincubating with a greatly increased concentration of 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate (400μM, 2h at 25°C and 16h at 4°C), 100% inhibition was seen with intact cells, but less than 50% with the resealed 'ghosts.' The perturbation of the protein therefore cannot be solely at the level of the loss of binding of the inhibitor.

Intact cells and resealed 'ghosts' were also treated with 3mM-[^{35}S]sulphophenyl isothiocyanate

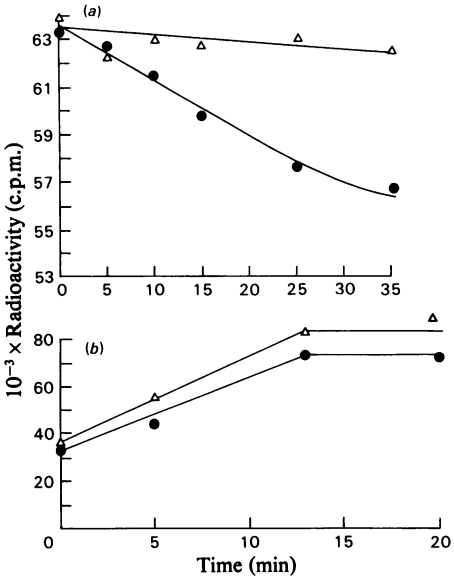

Fig. 13. *Phosphate transport: effect of pretreatment with 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulphonate*
Erythrocytes at 50% haematocrit were reacted with 20 μM-4-acetamido-4′-isothiocyanato-2,2′-stilbene disulphonate for 1 h at 26 °C. They were then centrifuged, and washed. A small amount of blood was retained and tested for phosphate transport (*a*), as in Fig. 1. ●, Control (untreated); △, pretreated with inhibitor. The remaining cells were lysed, washed and resealed as in the methods section. Phosphate transport was examined as in Fig. 2(*b*). ●, Control (untreated resealed washed 'ghosts'); △, resealed 'ghosts' pretreated with inhibitor. The ordinate represents radioactivity in the 'ghost' pellet after centrifugation.

(0.14 μCi/μmol) and the anion-transport protein was isolated in each case (W. Mawby & J. B. C. Findlay, unpublished work). The resultant purified protein preparations possessed similar specific radioactivities (2×10^{-4} μCi/mg and 3.8×10^{-4} μCi respectively), indicative of the presence of similar binding sites for the labelled reagent.

Discussion

The results presented above clearly indicate that the transport system for phosphate ions in the resealed membrane preparations does not possess the same characteristics exhibited by the intact erythrocyte. It is no longer inhibitable by 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate and sulphophenyl isothiocyanate even at concentrations much higher than those effective in the whole cell and may show a somewhat accelerated rate. Phlorrhizin, an inhibitor whose mode of action may be associated more with general membrane effects, also fails to block phosphate (but not glucose) uptake. Two possible explanations may be advanced to account for these observations.

Firstly, discontinuities in the organization of the lipid bilayer may present permeation pathways for the phosphate ion. This alternative appears the less likely explanation, for a variety of reasons. The uptake of both glyceraldehyde 3-phosphate and L-glucose, which one might also expect to occur via these non-specific channels, is prohibited. Furthermore, the movement of phosphate can in fact be completely inhibited by Hg^{2+} ions, whose mode of action is thought likely to be associated more with protein than with lipid interaction.

It seems more probable, therefore, that during the isolation procedure some form of perturbation occurs in the protein system responsible for anion transport. The effect is apparently seen with the overwhelming majority of the phosphate uptake mediators, since no significant inhibitable component was clearly observed, although the transport rate was low enough to allow such an observation to be made. This perturbation can be envisaged to occur in a number of ways, the more obvious being conformational rearrangements affecting a monomeric transport mediator and/or the interrelationship between the subunits of an oligomeric system, for molecular weight (Clarke, 1975) and cross-linking (Steck, 1972; Wang & Richards, 1974), studies indicate at least a dimeric organization for the anion-transport protein. The resultant effect is to negate the influence of the inhibitors 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate and sulphophenyl isothiocyanate at concentrations effective in the intact cell, but not their ability to bind to the polypeptide. In view of this effect, it can only be supposed that the mechanism of transport is no longer the same as in the intact cell or that these inhibitors do not bind to the true active site of the protein. In the case of phlorrhizin it can similarly be envisaged that conformational changes render the protein unresponsive to the membrane-mediated effects of this inhibitor, whereas the glucose-transport protein retains its sensitivity.

The different influences of Hg^{2+} ions on the intact cell and the resealed membrane preparation also support rearrangements in protein structure. These ions readily permeate the intact cell, and interact with an SH group located at the cytoplasmic surface of the band-III polypeptide (J. B. C. Findlay & M. A. Walker, unpublished work), but little inhibition of phosphate uptake occurs. With resealed membranes, however, inhibition is seen, indicating the exposure of a sensitive protein grouping previously protected from the influence of Hg^{2+} ions and not reactive towards iodoacetamide. Some support for the feasibility of structural alterations is also seen in the report by Jenkins & Tanner (1977), who demonstrated conformational changes in the band-III polypeptide

that were influenced by the ionic conditions. In the results reported here, the ionic conditions were varied without significant alteration in the results, suggesting that this was not the sole critical factor. It may be argued, for example, that lipid-mediated influences on protein structure may be a major determining element. Whatever the causative factor, however, it does not appear to be a general membrane phenomenon, for the glucose-transport system seems to have survived the process unaltered. In summary, therefore, the effects specifically on phosphate transport of substances acting by three different mechanisms are all markedly altered when erythrocyte membranes are prepared.

The observations detailed here do not agree at first sight with those of several groups (Schnell *et al.*, 1977; Zaki *et al.*, 1975) who demonstrated similar properties of sulphate transport in both intact erythrocytes and resealed 'ghosts'. The experimental conditions in the two sets of experiments, however, differed in one significant aspect. Whereas in our system the membranes were extensively washed free of haemoglobin before resealing, in Passow's work (Zaki *et al.*, 1975) the 'ghosts' apparently were resealed immediately after haemolysis of the erythrocytes. This may be a critical factor, for in our hands immediate resealing also gave rise to inhibitable transport in the resealed 'ghosts'. It is possible therefore that a factor or factors in the haemolysate may be responsible for the stabilization of the membrane in some way. That the washing procedure may introduce structural alterations in the membrane is also indicated by the work of Staros *et al.* (1974), who reported altered labelling patterns in the total 'ghost' protein fraction as a function of the number of washing steps.

Rice & Steck (1976, 1977) have reported the transport of pyruvate by sealed 'ghost' and vesicle preparations. In this instance, transport is inhibitable by 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulphonate, but at concentrations up to 100 times that effective for anions in whole cells. If the situation is analogous to that seen with sulphophenyl isothiocyanate, the reaction of 4-acetamido-4′-isothiocyanato 2,2′-stilbenedisulphonate may not be specific for a single reactive group at these concentrations, again perhaps indicative of some perturbation. It may also be that pyruvate transport is mediated by more than one system (Halestrap & Denton, 1974; Halestrap, 1976).

Finally, some concern may exist as to the feasibility of reconstitution studies with purified transport proteins. In the case of the glucose-transport system such experiments would appear possible, and indeed several successful reconstitutions have been reported (Kahlenberg, 1976; Kasahara & Hinkle, 1976; Zoccoli & Lienhard, 1977; Edwards, 1977). The anion-transport protein may, however, present greater problems. Reports of successful reconstitutions have revealed varying degrees of differences from the native activity (Rothstein *et al.*, 1975; Ross & McConnell, 1977; Wolosin *et al.*, 1977).

Generally, therefore, it follows from the observations described above that care must be exercised in the interpretation of both the labelling patterns and the transport characteristics of isolated membranes and vesicles.

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