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Anion Transport and Membrane Morphology

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Summary. Freeze-fracture electronmicroscopy has been used to examine the membrane ultrastructure of human red blood cells in the presence of inhibitors of chloride exchange. The extent of inhibition was correlated with a decrease of intramembrane particle density on the B-fracture face. Dimethylsulfoxide (DMSO) and glycerol, which markedly and reversibly reduced the intramembrane particle density, were shown to drastically and reversibly inhibit chloride self-exchange. DMSO was shown to be a noncompetitive inhibitor of chloride flux.

In this paper we investigate the relationship between membrane ultrastructure revealed by freeze-fracture electronmicroscopy and the membrane transport of small inorganic anions, particularly chloride. Previously in this laboratory it was shown that there was little or no relationship between the loss of intramembrane particles caused by dimethylsulfoxide (DMSO) or glycerol and changes in passive or active transport of alkali cations in sheep erythrocytes (Kirk & Tosteson, 1973). Small but reproducible reductions in the intramembrane particle density, however, were found in membranes treated with ouabain or trinitrocresol which, respectively, inhibit and enhance Na^+ and K^+ transport. From another point of view it is of interest that the number of intramembrane particles per red cell, 5×10^5 (Weinstein, 1969), approximately equals the number of major glycoprotein molecules intrinsic to the membrane and approximately equals the number of 95,000 dalton protein molecules which specifically bind an anion transport inhibitor (Cabantchik & Rothstein, 1972). The correspondence of these numbers has led to suggestions that the membrane-associated particles are complexes of these two proteins (Guidotti, 1972), both of which have been reported to span the erythro-

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cyte membrane (Bretscher, 1971; Segrest, Kahane, Jackson & Marchesi, 1973). In addition, recent findings have shown that rapid exchange of chloride isotopes is mediated by specific sites in the erythrocyte membrane (Gunn, Dalmark, Tosteson & Wieth, 1973). Chloride transport can be inhibited by 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS) which covalently binds to the 95,000 dalton protein (Cabantchik & Rothstein, 1974). These findings taken collectively indicate that intramembrane particles, the 95,000 dalton protein, and chloride transport are related.

Specifically, this paper deals with the relationship between the intramembrane particle density as modified by inhibitors of anion transport and measurements of chloride self-exchange in the presence of these modifiers. We have found a correlation between the loss of some of the intramembrane particles and the reduction of chloride transport and also have discussed the meaning of this in terms of different populations of intramembrane particles and the mechanisms of action of the inhibitors.

Methods

Preparation of Cells

Whole blood from one of us (RBG) was collected by venipuncture into a glass cylinder treated with heparin (Upjohn, Co., Kalamazoo, Michigan) and centrifuged. The characteristics of chloride transport in these cells have been reported (Gunn *et al.*, 1973). Plasma and buffy coat were removed. The erythrocytes were washed in 165 mM NaCl and titrated with CO_2 or $NaHCO_3$ at 0° C to the pH of the buffered medium. Cell samples were then repeatedly suspended in cold medium, centrifuged, separated from the supernatant and resuspended until the cells were in steady state with respect to water and anion content. One volume of cells was transfered to DMSO-containing media by washing three times in 5 volumes of these media over 90 min at 37° C and finally cooled to 0° C. Similarly, cells in glycerol-containing media were washed four times over a 120-min period.

Control medium was prepared from reagent grade chemicals and had the following millimolar concentrations at 23° C: 160 NaCl, 5 KCl, 27 glycylglycine, 5 D-glucose. This control solution was used for both the particle counts and the flux measurements to avoid any interference of phosphate on these measurements (*see* Kirk & Tosteson, 1973). Deuticke (1967) has shown that chloride inhibits phosphate transport and the converse relation may be expected (Gunn, 1972). Solutions with DMSO (Fisher Scientific Co., Fair Lawn, N. J.), glycerol (J.T. Baker Chemical Co., Phillipsburg, N.J.), trinitro-*m*-cresol (Eastman Kodak Co., Rochester, N.Y.), and phloretin (K. and K. Laboratories, Inc., Plainview, N.Y.) were made without altering the concentrations of salts, buffer or glucose per liter of final solution.

Freeze-Fracture Electronmicroscopy

The procedures used previously (Kirk & Tosteson, 1973) were followed for preparation of samples, freeze-fracturing and measuring membrane particle densities. These procedures

were found to give reproducible values of particle densities within experiments and between experiments. Droplets of cell suspensions were placed on small brass holders and immediately frozen in Freon 12 (E.I. Dupont de Nemours & Company, Wilmington, Delaware), which resulted in random orientation of the cells and the final fracture faces. Replicas were prepared in a Berkeley device (C.W. French, Inc., Weston, Massachusetts) and examined in an AEI-EM6B electronmicroscope. Particle densities were determined by projecting Electron Image Plates (Eastman Kodak, Co., Rochester, New York) directly on graph paper. In these studies measurements of particle densities were limited to areas which contained particles with shadows and diameters of equal length. No selection was made on the basis of the degree of aggregation. There was no evidence for heterogeneity of particle density in any of the cells studied except those treated with butanol. Examination of treated erythrocytes by light microscopy did not reveal crenation, spiculation or highly abnormal shapes.

Chloride Flux Measurements

After cells reached steady state with the DMSO or glycerol-containing media at 0 °C, chloride-36 as NaCl (ICN, Irwin, California) was added (0.6 μC/ml of suspension) and allowed to equilibrate for more than six half times of the subsequent efflux. Cells were packed in small nylon tubes to form a column of cells with approximately 2.6% (w/w) extracellular fluid. These cells were injected into a well-stirred, thermostated chamber at 0 °C containing the wash medium. Samples of cell-free supernatant were taken using the method of Dalmark and Wieth (1972) and counted in a liquid scintillation counter (Beckman LS30, Intertechnique LS30CP). The efflux was calculated from the product of the chloride efflux rate coefficient and the cellular chloride content. The latter was determined by titration of perchloric acid extracts of duplicate columns of packed cells using a chloride titrator (Radiometer, London Co., Westlake, Ohio) (*See* Gunn *et al.*, 1973, for further details of efflux measurement).

Cation Flux Measurements

Water and anions were allowed to reach steady-state conditions. Then sodium and potassium influxes were measured by adding $Na^{22}Cl$ (ICN, Irvine, California) or $K^{42}Cl$ (New England Nuclear, Boston, Mass.) and sampling, using either the dibutyl phthalate technique (Gunn & Tosteson, 1971) or repeated washing of cell samples with isotonic $MgCl_2$ with added glycerol or DMSO. Samples were taken at 5, 65 and 125 min. The activity of the Na-K pump was estimated by the difference between the K influx in the absence and presence of ouabain, 8×10^{-5} M.

Results

The Effect of DMSO and Glycerol on the Intramembrane Particle Density of Fracture Face B

In this study DMSO and glycerol greatly reduced the particle density in human red blood cells. This is similar to the previously reported effect of DMSO and glycerol on sheep erythrocytes (Kirk & Tosteson, 1973). The particle density on fracture face B, which is adjacent to

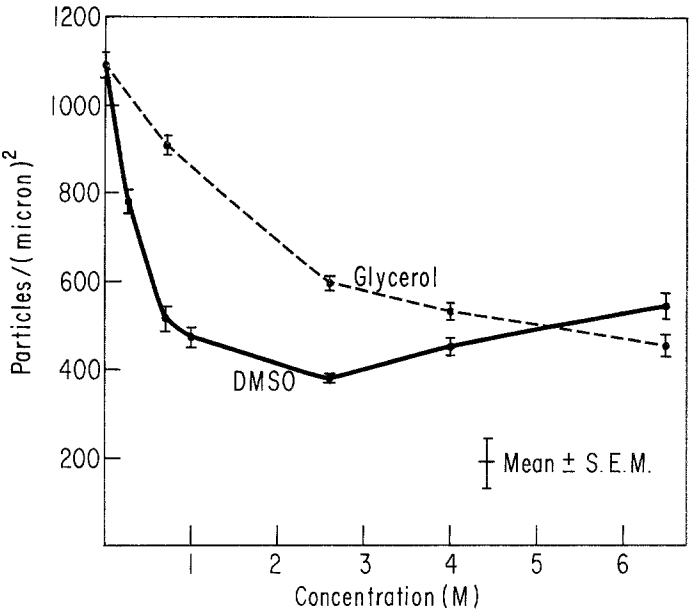

Fig. 1. Each data point represents particle densities determined from at least two experiments and 11 fracture faces. The data point limits are the calculated standard errors of the means

the extracellular space, decreased with increasing concentration of glycerol as shown in Figs. 1 and 2 and Table 1. DMSO was more potent than glycerol in reducing membrane particles up to 4 M. The 2.6 M DMSO caused a fall in the particle density from 1100/μ^2 in control medium without DMSO to 390 particles per μ^2. Glycerol (2.6 M), on the other hand, caused the particle density to fall to only 590 particles per μ^2. In contrast to the increasing effect of glycerol on particle density, DMSO removed fewer particles/μ^2 above 2.6 M, than at lower concentrations.

The effect of DMSO and glycerol on the particle density of fracture face A (the intramembrane plane adjacent to the cytoplasm) was more difficult to determine because of the larger number of smaller particles found on this surface (*see* Table 1) and because of their tendency to aggregate. In the control medium, fracture face A had 2580 ± 40 (SE) particles/μ^2, while in 2.6 M DMSO the particles/μ^2 slightly increased to 2700 ± 70 and was similarly increased (2750 ± 110) in 6.5 M glycerol. The loss of particles on fracture face B was much greater than the gain in particles on fracture face A.

The losses of particles from fracture face B were in large part reversible as shown by the following experiment. A portion of cells which had

been prepared for freeze-fracture and chloride flux determination were washed in gradually decreasing concentrations of DMSO and finally in the control medium before freezing and fluxing in the control medium. The particle densities returned to 84% of normal after 2.6 M DMSO treatment and to 97% after 1.0 M DMSO treatment.

The Effect of Anion Transport Inhibitors, Trinitro-m-cresol, Butanol and Phloretin on the Intramembrane Particle Density

In 10^{-3} M concentration of 2,4,6-trinitro-*m*-cresol (TNC^-), chloride self-exchange flux was reversibly reduced by more than 300-fold (Gunn & Tosteson, 1971; Gunn & Horton, *unpublished observations*). In this concentration of TNC^- (Table 1, Fig. 2), we found the intramembrane particle density of fracture face B of human red cells was 1110 ± 40 particles per μ^2, unchanged from the control, while the intramembrane particle density of fracture face A was slightly increased from 2580 ± 40 to 2810 ± 40.

Phloretin is the most potent known inhibitor of chloride flux (Wieth, Dalmark, Gunn & Toteston, 1973), but is also known to inhibit other carrier-mediated transport systems such as those for hexoses (Wilbrandt, 1950), urea and methyl urea (Wieth, Funder, Gunn & Brahm, 1974). As shown in Table 1 and Fig. 2, phloretin (2.5×10^{-4} M) slightly increased the freeze-fracture intramembrane particle density on surface B (1240 ± 30) and surface A (2710 ± 60).

Butanol, which readily penetrates red cells, is an inhibitor of mediated transport processes in erythrocytes including active alkali cation transport (Tosteson, 1955) sulfate transport (Parpart, 1940) and chloride transport (Wieth *et al.*, 1974). Butanol (1 M) produced distorted intramembrane particles and uneven particle distribution (Fig. 2). Thus, the quantitation of particle densities was difficult; however, it did not alter the particle density by more than 50%.

Human erythrocytes do not lose significant chloride when washed in a low pH and low chloride sucrose medium where $H_3O^+ \times Cl^-$ in the solution equals that within the red cell. An intramembrane carrier molecule transferring equal numbers of chloride between the high chloride interior and the very low chloride concentration outside might be found more frequently at the outer surface waiting to be loaded from the low chloride medium. With this notion in mind, we determined the intramembrane particle densities in acid-sucrose solution (Table 1, Fig. 2). The particle density on fracture face A was increased 270 parti-

Fig. 2. (*A*) Fracture face B of human red cells fractured in (*a*) control media, (*b*) 4 M glycerol, (*c*) 4 M DMSO, (*d*) TNC, (*e*) phloretin, (*f*) acid sucrose, and (g) butanol. (× 90,000)

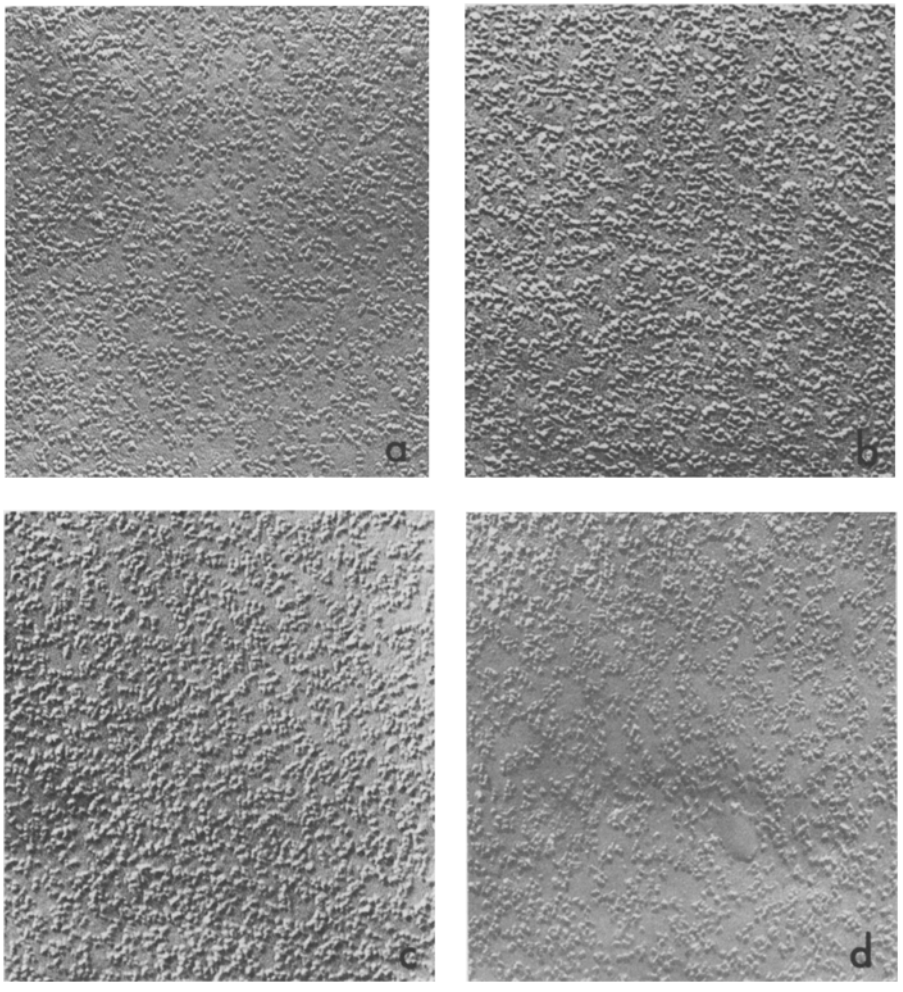

Fig. 2. (*B*) Fracture face A of human red cells fractured in (*a*) control media, (*b*) 6.5 M glycerol, (*c*) 2.6 M DMSO, and (*d*) butanol. (× 90,000)

cles/μ^2 (2850 ± 60) and on fracture face B was reduced 230 particles/μ^2 (870 ± 30). These reciprocal changes are in the opposite direction from those predicted.

Inhibition of Chloride Self-exchange Flux by DMSO

The striking loss of particles resulting from addition of DMSO to the medium (Fig. 1) was accompanied by an equally striking loss in chloride transport capacity (Fig. 3). The chloride flux was little affected by 0.3 M or 0.7 M DMSO but was progressively reduced in higher

Table 1. Membrane particle densities of drug-treated human red cells

Sample	Fracture face A (particles per μ^2)	Fracture face B (particles per μ^2)
Control	2580±40 (*SE*)[a]	1100±30 (*SE*)
TNC 10^{-3}M	2810±40	1110±40
Phloretin 2.5×10^{-4}M	2710±60	1240±30
Acid sucrose	2850±60	870±30
6.5M Glycerol	2750±110	450±30
2.6M DMSO	2700±70	390±10
2.6M DMSO reversal[b]		920±20
1.0M DMSO reversal[b]		1070±30

[a] Each entry is a particle density with standard error of the mean.
[b] Reversibility of 2.6M and 1.0M DMSO-treated red cells was measured by washing samples in gradually decreasing concentrations of DMSO and finally in control medium before freezing.

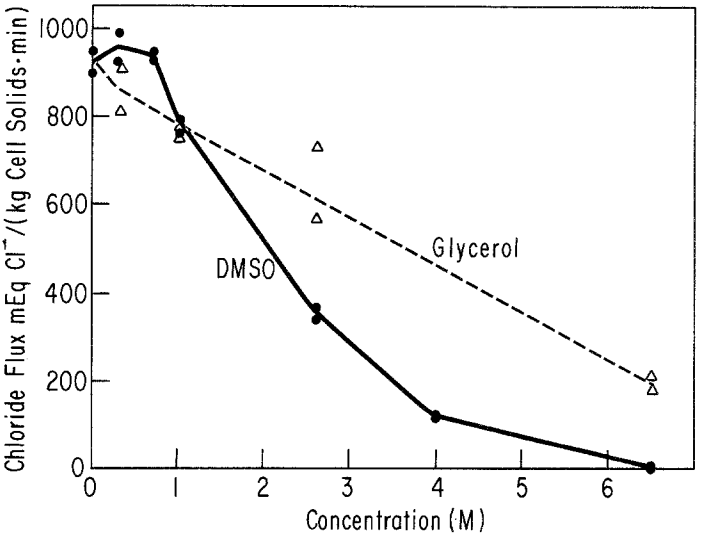

Fig. 3. Chloride self-exchange flux in human red cells measured in different concentrations of DMSO and glycerol at 0°C, Cl_{cell}=140–165mM

concentrations of DMSO. In 6.5M DMSO the flux was only 0.5% of the value measured in the absence of DMSO. Similar results were obtained in a solution containing phosphate, although the fluxes were 5–10% lower at each concentration of DMSO and some inhibition was observed at the lower concentrations of DMSO. During these chloride effluxes the red cell volume and chloride concentration were constant. Cell water contents in the experiments shown in Fig. 3 ranged from

1.6 to 1.3 kg cell water/kg cell solids and 1.6 was the water content of the control cells. The DMSO inhibition of chloride flux was reversible to the same extent as the morphological loss of intramembrane particles on fracture face B. Chloride flux returned to 96% of control values after being washed in DMSO-free solutions. While no direct effort was made to determine the kinetics of DMSO reaction with the transport mechanism, we observed that the chloride efflux from cells loaded with Cl^{36} in control medium without DMSO was inhibited when the cells were injected into DMSO-containing media at 0 °C, and showed first-order kinetics after the first 5 sec. The rapid water shifts accompanying the DMSO equilibration precluded these fluxes from being true steady-state chloride self-exchange measurements.

The dependence of chloride flux on chloride concentration in the presence of 2.6 M DMSO was determined at 0 °C and pH 7.8 in order to ascertain whether DMSO decreased the number of transport sites or decreased the affinity of the sites for chloride. The results in Fig. 4, when compared with previously published measurements, show that the dominant effect of DMSO was a reduction of the maximal flux at high chloride concentration without significant shift in the concentration of chloride required to obtain half-maximal fluxes. $K_{1/2}$ in Fig. 4 is between

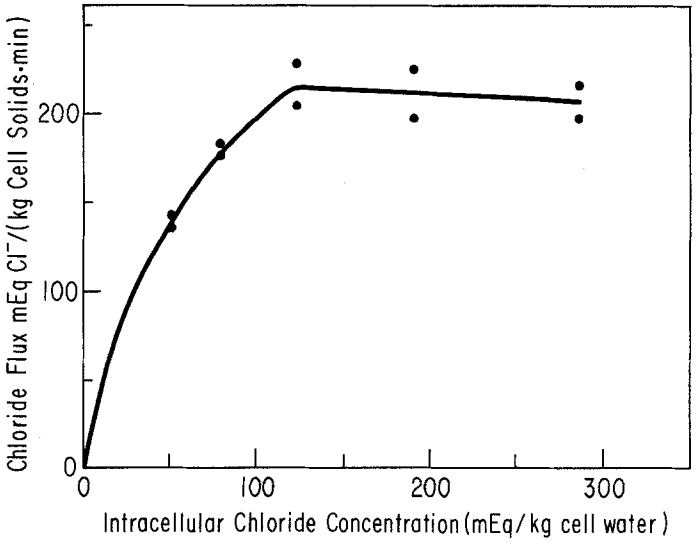

Fig. 4. Chloride self-exchange flux in 2.6 DMSO and different chloride concentrations. The efflux from cells pre-equilibrated with different concentrations of NH_4Cl added to isotonic Na-acetate shows saturation at 210 mEq Cl^-/(kg cell solid × min) and half-maximal flux at 35 mEq Cl^-/kg cell water

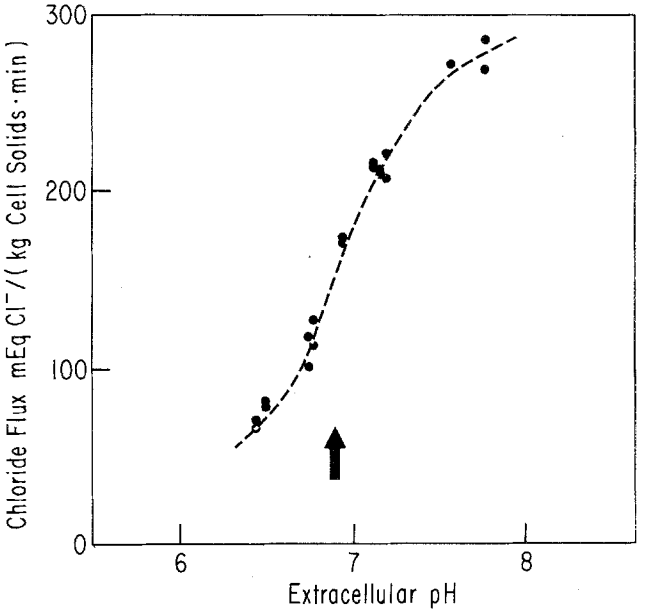

Fig. 5. pH dependence of chloride self-exchange flux in the presence of 2.6 M DMSO. The inflection point and half-maximal flux occur at pH 6.9, unchanged from the control previously reported (Gunn, 1973)

30 and 40 mM which differs little from the $K_{1/2}$ (26–30 mM) we obtained earlier using the same method to alter both intra- and extracellular chloride concentrations (Gunn *et al.*, 1973). This together with the 99.5% inhibition shown in Fig. 3 indicated that the apparent affinity $(K_{1/2})^{-1}$ was unaltered by DMSO and that either 99.5% of the transport sites were blocked or all sites functioned at less than 1% of their normal rate in 6.5 M DMSO. The maximum flux at high chloride concentrations in Fig. 4 was lower than that shown in Fig. 3 at 2.6 M DMSO. This probably is a result of the additional noncompetitive inhibition by acetate anions on chloride fluxes shown in Fig. 4.

The pH Dependence of Chloride Flux in 2.6 M DMSO

Since DMSO can raise the pK_a of organic compounds (Szmant, 1971) and thus potentiate the titration of groups at any fixed pH, we investigated the possibility that the DMSO inhibition was the result of a shift in the apparent pK_a of the titratable chloride carrier (Gunn, 1973). Red cells titrated with CO_2 to different pH values at 0 °C were washed in control medium containing DMSO at the same pH and temperature

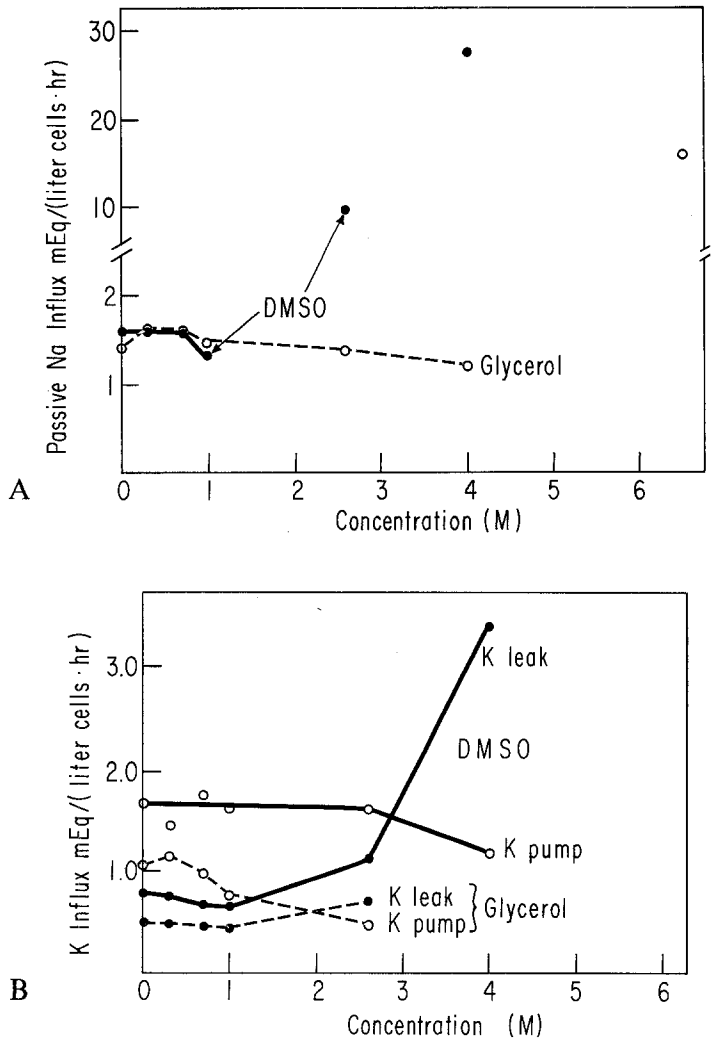

Fig. 6. (*A*) Passive Na influx at 37 °C in DMSO and glycerol. Only at high concentrations was the leak increased. Some hemolysis occured at these higher concentrations. (*B*) K pump and leak determined from fluxes with and without ouabain. Only at high concentrations were the fluxes altered in DMSO. 37 °C

until the steady state for water and chloride were obtained. These cells after loading with chloride-36 were effluxed into the same DMSO-containing medium. The results are shown in Fig. 5.

The titration of the chloride transport system to low pH reduced the flux of chloride in the presence of 2.6 M DMSO in the same manner as in the absence of DMSO. That is, all of the flux values were lower in the presence of DMSO, but the apparent pK_a of the transport system

remained at 6.9 at 0 °C, the same value as previously reported (Gunn, 1973). These two apparently noncompetetive inhibitors of chloride self-exchange flux, the hydrogen ion and DMSO, appear to have effects through mechanisms which do not interact.

Inhibition of Chloride Flux by Glycerol

Chloride flux was proportionally inhibited by glycerol up to a 6.5 M solution in which the flux was 22% of control valves. Except below 1 M, the inhibition at each concentration was less than that observed in DMSO (Fig. 3). Glycerol was, therefore, less effective than DMSO both in decreasing the chloride self-exchange flux and intramembrane particle density at concentrations below 5 M.

Effect of DMSO and Glycerol on Na and K Fluxes at 37 °C

The passive Na and K influxes, and the ouabain-sensitive K influx were unchanged in DMSO or glycerol solutions of lower than 2.0 M (Fig. 6). Above this concentration the passive permeabilities were increased several-fold. These results confirm those reported earlier for sheep red cells (Kirk & Tosteson, 1973). In contrast to chloride transport, alkali cation fluxes were unaltered at concentrations which reduced the B face intramembrane particle density and were increased at high concentrations. We conclude that DMSO and glycerol interact selectively with the several different transport systems of the erythrocyte membrane.

Discussion

Relationship Between Inhibition of Anion Flux and Membrane Ultrastructural Changes

From these observations, DMSO and glycerol are not specific for a particular class of particles which are related to chloride transport. In low concentrations of DMSO a large number of particles in one class were no longer visible by freeze-fracture microscopy, although the flux was unaltered or slightly increased (Fig. 7). But, in an intermediate concentration range a second class of particles was apparent. Over a small change of particle densities the apparent loss of particles in the micrographs was associated with a sharp decrease of chloride flux. In high concentrations of DMSO the total particle density increases with

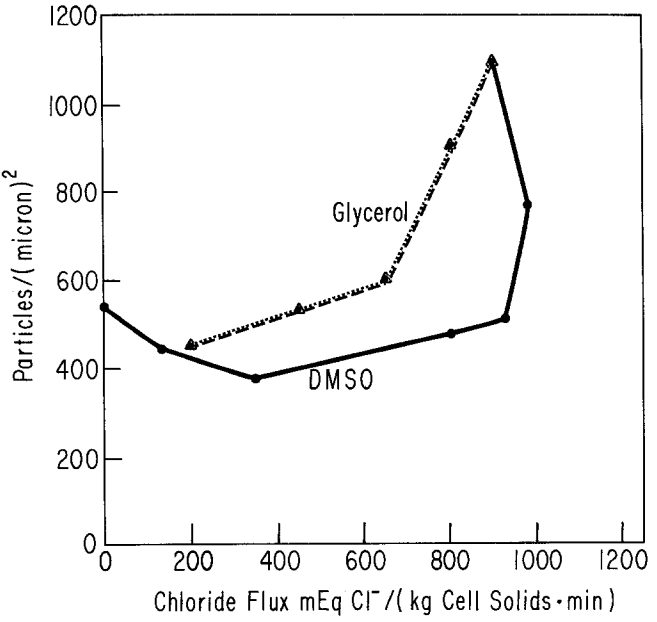

Fig. 7. Intramembrane particle density of fracture face B as a function of chloride flux in glycerol and DMSO. This graph is constructed from the data in Figs. 1 and 3. In this Figure as one moves along the curves beginning at the far right, the concentrations of both DMSO and glycerol increase. Moving from right to left on the DMSO curve there is a large decrease in particle density accompanied by no change in chloride flux. Then, at 510 particles/μ^2 a large change in flux begins and is associated with only a small decrease in particle density and then a small increase in particle density. The rise in the numbers of particles at high DMSO concentrations may be a separate phenomenon. Glycerol, however, monotonically reduces particle density with decreasing chloride flux. 400 particles per μ^2 are always found, even in the highest concentrations of DMSO or glycerol

a concomitant inhibition of chloride flux. This increase in particle density may be a separate and independent effect of DMSO and may be due to the reappearance of particles lost at low DMSO concentrations, lost at intermediate concentrations, or due to the visualization of new intramembrane particles. In low concentrations of glycerol there is a rapid loss of particle density with the decrease in chloride flux, while at higher concentrations the rate of loss of particle density is less with a given reduction in chloride flux. A third separate class of particles are those which were not susceptible to either DMSO or glycerol. These constituted 400 particles/μ^2 which had no morphological changes related to chloride transport, since this number of particles remained in the fracture face when chloride self-exchange was reduced 100-fold. These differential effects of the penetrating agents DMSO and glycerol may be accounted

for if the intramembrane particles are of several different classes with different functions. Only one class may be responsible for anion transport.

The DMSO effects on particle density and chloride transport are reversible. Therefore, DMSO does not remove the molecules responsible for the visualization of the intramembrane particles from the erythrocyte membrane and does not remove the chloride transport mechanism. Nor does DMSO cause a reduction of particles on fracture face B by transferring their apparent position to fracture face A because the slight gain of 120 particles on fracture face A is not equal to the great loss of 715 particles from fracture face B in 2.6 M DMSO. Like DMSO, the addition of TNC^- or phloretin causes over 99% inhibition of chloride flux; but, unlike DMSO and glycerol, these agents cause no loss of intramembrane particles. While morphological changes may be associated with loss of chloride transport, the lack of morphological changes in the presence of phloretin or TNC^- clearly shows that anion inhibition does not necessarily result in these morphological changes.

The several morphological classes of particles, determined by sensitivity to DMSO and other agents, may correspond to classes of transport systems for different molecules such as hexoses, chloride, alkali cations and glycerol. Glycerol does not inhibit hexose or erythritol transport in erythrocytes (Bowyer & Widdas, 1955), but, as reported here, it does inhibit chloride transport. Butanol, as referred to earlier, inhibits cation and hexose transport. DMSO has been recently found to inhibit hexose transport (J.O. Wieth, *personal communication*) and the number of hexose transport sites is less than 200,000 per cell ($<1300/\mu^2$) (LeFevre, 1961, as quoted by Stein, 1967). It may be, therefore, that many of the particles are related to hexose transport.

There is evidence of molecular heterogeneity of the 95,000 dalton band (band III, component *a*) of protein seen on SDS gel electrophoresis. The total number of copies of protein molecules in this band is 10^6 per red cell (Steck, 1974), but within the red cell membrane most of these may behave as dimers giving 5×10^5 dimers per erythrocyte. The (Na + K)-ATPase which actively transports the alkali metal cations is one of the proteins in this band. From studies on the binding of tritiated ouabain (Hoffman, 1969) it is concluded that there are only 250 of these sites per erythrocyte or $2/\mu^2$, making it a very minor even though physiologically a crucial fraction of this protein band. Recently, band III has been shown to have three components by the differential reactivity of plant lectins (Adair & Kornfeld, 1974; Findlay, 1974). The 95,000 dalton band has a small amount of carbohydrate covalently linked

to the protein. Unlike glycophorin, the major glycoprotein which has over 40% carbohydrate by weight, approximately 5% of the weight of the 95,000 dalton proteins is sugar moieties. Presumably it is differences in these sugars which confer different reactivity with plant lectins although specificity in the amino acid sequences may be involved. The inhibition of chloride transport by DMSO and glycerol at concentrations which have little effect on cation transport indicates that these agents can act differently on the function of proteins in band III. For the same unknown reasons these agents may act differently on the morphology of band III proteins if this is what is visualized by freeze-fracture microscopy. We, therefore, believe that band III proteins are heterogeneous in composition and function and that this is reflected in the different classes of particles defined by their sensitivity to DMSO and glycerol in our freeze-fracture electronmicroscopy studies and also reflected in the differences in anion transport accompanying these morphological changes.

From Fig. 7 it appears that not more than 600 particles/μ^2 can be related to chloride transport. With this value the number of transport sites per cell can be estimated and the turnover number for the chloride carrier calculated. Assuming an area of 150 μ^2 per cell, there are 9.0×10^4 sites per cell. Since 3×10^{13} cells have one kilogram of dry cell solids, there are 2.7×10^{18} sites per kg cell solids. The maximum chloride flux at 0 °C is 1000 mEq Cl^-/ (kg cell solid $\times$ min) and thus the turnover number for each site is 3.3×10^4 ions/sec at 0 °C. By extrapolating to room temperature, using the careful determination of activation energy, E_a, = 32.3 kcal/mole, measured by Dalmark and Wieth (1972) between 0 and 10 °C, and extended by J. Brahm to 15 °C and E_a = 22 kcal/mole above 15 °C (Brahm, 1975) the flux would be increased 62-fold; and the turnover number for each of the 600 sites/μ^2 would be 2.3×10^5 ions/sec at 23 °C. This value at 37 °C would be 9.6×10^5 ions/site $\times$ sec, nearly the same value calculated by Sachs, Knauf and Dunham (1975). However, they used the number of DIDS binding sites at 100% inhibition of SO_4 flux, 300,000 per red cell, and used the observed higher temperature coefficient between 0 and 10 ° for the entire temperature range. The use of a larger number of sites and a higher temperature coefficient above 15 °C just compensate to give 5 to 8×10^5 ions/site $\times$ sec. These values are very large but not beyond reason. Carbonic anhydrase catalyzes the hydration of carbon dioxide at a rate of 10^6 molecules per sec at 25 °C (Kahlifah, 1971).

The mechanism of inhibition of chloride transport by DMSO is of

interest and not without implication for the loss of intramembrane particles which accompany the inhibition. As shown in Fig. 4, DMSO reduces the maximum chloride flux but does not shift the concentration of chloride which gives a half-maximum flux. This noncompetitive inhibition by DMSO thus acts as if sites for transport are blocked or removed, while the remaining sites behave as they would in the absence of DMSO. Furthermore, there appears to be no interaction between the titration of the chloride carrier by hydrogen ions, a noncompetitive inhibitor down to pH 5.5 at 0 °C (Gunn, Wieth & Tosteson, 1975), and the noncompetitive inhibition caused by DMSO. The industrial use of DMSO as a solvent in organic synthesis is to increase the basicity of certain organic groups. However, this effect is not seen in our experiments since the apparent pK_a of the titratable group on the carrier is unchanged from 6.9 in the presence of 2.6 M DMSO. In addition, the affinity of the carrier for chloride as judged by $(K_{1/2})^{-1}$ is not altered by DMSO; and, thus, the environment of chloride binding site is not significantly altered by the high concentration of DMSO.

The observation that the gain of particle density on fracture face A was equal to the loss on fracture face B in acid-sucrose solutions may indicate that charge groups on the particles play a role in their visualization on the A or B face. Since the chloride concentration ratio between the cell interior and the medium was reversed in these acid-sucrose solutions, the membrane became depolarized with the cell interior strongly positive. The shift of intramembrane particles from the fracture face adjacent to the extracellular space (B) to that adjacent to the cytoplasm (A) may, therefore, indicate a response to the membrane potential. This in turn would indicate that the movement of negative groups is responsible for the shift in the visualization of the particles between the fracture faces but would not explain the normal asymmetrical distribution of particles between the two fracture faces (A > B).

The asymmetry of the transport mechanism within the membrane and the asymmetry of the 95,000 dalton protein within the membrane are established. Kaplan and Passow (1974) have shown that chloride transport which can be inhibited by phlorizin in the external medium has no effect on the internal surface although the inhibition of xylose transport is identical whether phlorizin is on the external or internal surface. Thus, either the site or reactivity with phlorizin is absent on the inside or the access of phlorizin to the site is prohibited on the inside but not on the outside. In both circumstances the carrier is not situated symmetrically within the erythrocyte membrane. The membrane phospholipids themselves are asymmetrically distributed between the two monolayers. Red cell membrane lipids are arranged with sphingomyelin

and phosphatidyl choline on the outside half of the lipid bilayer, while phosphatidyl ethanolamine and phosphatidyl serine are primarily on the inside (Bretscher, 1972). It is suggested that this asymmetry of lipid composition is responsible for the differences in the number of particles visualized on the two fracture faces. The constancy of phosphatidyl serine and phosphatidyl ethanolamine on the inner half of the bilayer in erythrocytes from different species (Nelson, 1967) may be responsible for the constancy of the number of particles on the B fracture face plucked during freeze-fracture from that mileu (Kirk & Tosteson, 1973). The variation in particles on fracture face A in several species may be due to the changes in the relative amounts of phosphatidyl choline and spingomyelin within the outer half of the bilayer from which the particles on fracture face A are plucked (Kirk & Tosteson, 1973). With regard to the morphological changes reported in this paper, this thesis suggests that the presence of DMSO or glycerol in the inner half of the bilayer reduces the ability of the freeze-fracture technique to pluck particles from this region and reduces the particle density visualized on the B fracture face. It would not necessarily be in this region, however, that DMSO reacts with the chloride carrier.

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