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Cation Transport and Membrane Morphology

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Summary. Freeze-fracture electron-microscopy has been used to study membrane ultrastructure in (1) red cells from five species of mammals which have naturally occurring differences in cation transport and (2) red cells which have been treated with various drugs known to affect transport. A reproducible method for estimation of the intramembrane particle density is described. Considerable differences in the densities of intramembrane particles on the A-fracture faces were noted in five species of red cells studied. Such differences were not noted on the B-fracture faces. The differences correlated with species differences in active potassium transport and membrane phospholipid composition. Ouabain and trinitrocresolate-treated red cell membranes were found to have small but reproducible reductions in intra-membrane particle densities on the B-fracture faces, but such differences were not seen in valinomycin and amphotericin B-treated red cells. It was found that dimethylsulfoxide (DMSO) and glycerol drastically reduced intra-membrane particle densities. However, over the range of glycerol and DMSO concentrations in which membrane morphology was altered, no effects on either passive or active potassium transport were observed. It appears that the particles which are altered by DMSO and glycerol are not involved in cation transport.

This paper reports experiments designed to explore the relation between ion transport and membrane ultrastructure as revealed by freeze-fracture electron-microscopy. We have directed particular attention to the intramembrane particles revealed by this method (Branton, 1966). We have used three approaches to this problem. First, we have examined the ultrastructure of the membranes of red cells from two genotypes of sheep and four other species of mammals known to have different red cell cation transport systems (Shaw, 1955; Rettori, Rettori, Maloney & Villamil, 1969; Beaugé & Ortiz, 1971; Hoffman & Tosteson, 1971; Israel, MacDonald, Bernstein & Rosemann, 1972). Second, we have studied the ultrastructure of the membranes of sheep red cells exposed to compounds such as valinomycin (Tosteson, Cook, Andreoli & Tieffenberg, 1967), ouabain (Dunham &

Hoffman, 1971), trinitrocresolate (TNC) (Gunn & Tosteson, 1971), and amphotericin B (Dennis, Stead & Andreoli, 1970), which are known to alter dramatically the ion permeability of these cells. Finally, we report both observations of membrane ultrastructure and measurements of cation transport in sheep red cells exposed to high concentrations of glycerol and dimethylsulfoxide (DMSO). We have found previously that intramembrane particle densities of sheep red cells can be altered by treatment with DMSO and glycerol (R. G. Kirk, *in preparation*). These cryoprotective agents have been used routinely in freeze-fracture electron-microscopy to prevent freeze-damage (Bank & Mazur, 1972). The red cell membranes were found to differ in both their intra-membrane particle densities and in the sensitivity of these particles to DMSO treatment. Also, sheep red cells frozen and fractured in the presence of ouabain were found to have a small but statistically significant reduction in the number of intra-membrane particles. The magnitude of this reduction is about 14,000 particles per cell, roughly 300 times more than the number of ouabain binding sites in these cells (Dunham & Hoffman, 1971).

Materials and Methods

Preparation of Cells

All experiments were performed with freshly drawn blood containing 10 units of heparin (Upjohn Co., Kalamazoo, Michigan) per ml of blood. Plasma and white cells were removed by aspiration after the blood had been centrifuged. The cells (5 ml) were then washed twice in 40 ml of salt medium (Solution A) which contained the following: 130 mM NaCl, 5 mM KCl, 9 mM NaH_2PO_4, 17 mM $Na_2HPO_4 \cdot H_2O$, and 11 mM dextrose (pH 7.4, osmolarity 0.310 to 0.320). Other solutions were obtained by modification of this medium. The sedimented washed cells were used for all experiments.

In the freeze-fracture microscopy experiments, cells were incubated at room temperature in the various solutions for varying lengths of time prior to freezing. The hematocrit was 6% in these solutions. Trinitrocresol (Eastman Kodak Co., Rochester, N.Y.) was converted to Na-trinitrocresolate (TNC) by titration to pH 7.4 with NaOH. The TNC samples were treated 15 min in Solution A medium containing 10 mM Na–TNC. TNC control samples were prepared by incubating cells in Solution A without TNC. A stock solution of 3×10^{-4} M valinomycin (Calbiochem, La Jolla, California) was prepared in methyl alcohol. Cells were treated for 30 min in Solution A containing 10^{-6} M valinomycin and 0.4% (v/v) methanol while the valinomycin control samples were incubated 30 min in a solution of the same composition except that it contained no valinomycin. Ouabain samples (Sigma, St. Louis, Mo.) were treated 15 min in Solution A containing 10^{-4} M ouabain and ouabain control samples were incubated in Solution A. The stock solution of amphotericin B contained 1 mg of Fungizone (Squibb, New York) per ml of Solution A. Fungizone is a mixture of amphotericin and sodium desoxycholate (NaDC). Amphotericin-treated cells were incubated for 30 min in Solution A containing 0.02 mg/ml Fungizone while amphotericin control samples were treated with Solution A containing 0.007 mg/ml of sodium desoxycholate (NaDC).

Procedure for Freeze-Fracture Electron-Microscopy

Small droplets of samples of cell suspensions were placed in brass holders and frozen immediately in liquid Freon 22 (E. I. Dupont de Nemours and Company). The specimens were freeze-fractured and shadowed with platinum-carbon in a Berkeley device (C. W. French, Inc., Weston, Massachusetts), according to methods described by Bullivant and Ames (1966). Replicas were cleaned with Clorox for 1 hr and with distilled water three times. Replicas were examined in an AEI-EM6B electron-microscope. The micrographs were printed as positives and arranged so that the direction of shadowing was from the bottom toward the top of the page.

Membrane particle densities were determined by projecting 6.5×9 cm Electron Image Plates (Eastman Kodak Co.) directly on graph paper with a photographic enlarger. As the particles were counted, each particle was marked with an ink pen. Areas were only counted if they contained particles with shadows equal to the diameters of the particles.

Measurement of Potassium Influx

The procedures for measurement of potassium influx were similar to those described by Hoffman and Tosteson (1971). Cells were washed in Solution A and 0.2 ml aliquots of packed cells were incubated in 10-ml Erlenmeyer flasks containing 2 ml of Solution A medium modified with various concentrations of DMSO or glycerol for 90 min (DMSO) or 180 min (glycerol). Isotope was added (10 μC of $^{42}K^{+}$) to each of the incubation flasks and 1-ml samples were taken after 1 hr and transferred to centrifuge tubes filled with 10 ml of ice-cold isotonic $MgCl_2$ solution containing the concentrations of DMSO or glycerol used in the flux media. Cells were immediately centrifuged at $12{,}000 \times g$ for 2 min and then washed 3 times using the $MgCl_2$ solutions.

Results

Structure of Cell Membranes with Known Naturally Occurring Transport Differences

All freeze-fractured red cells were characterized by two distinct membrane fracture faces as shown in Fig. 1. Fracture face (A) is adjacent to the cytoplasm and has a higher density of particles than fracture face (B), which is adjacent to the extracellular space. Particle densities for individual fracture faces are reported from six experiments in Table 1 to demonstrate the variation within experiments and between experiments. Note that the standard error was always less than 3% of the mean. This precision was made possible by limiting counting to areas which contained particles with shadows and diameters of equal length. We observed no significant differences between particle densities on the B surfaces and very small, questionably significant differences on the A surfaces of membranes of HK and LK sheep red cells (Table 1) despite substantial differences in cation composition and transport in these cells (Table 2). On the other hand, large differences were found among the five species studied in the

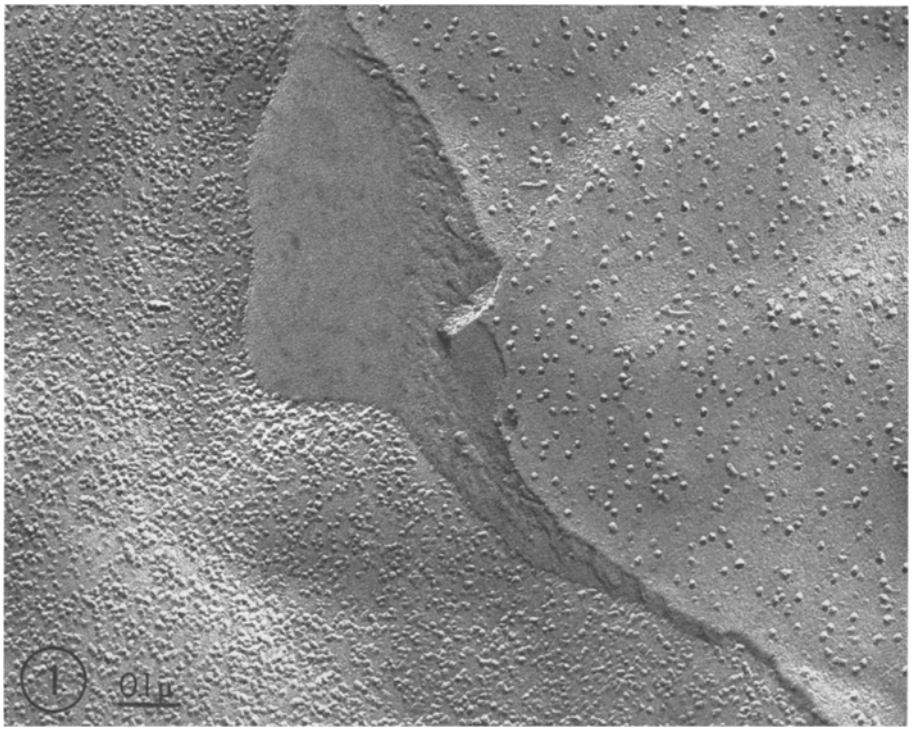

Fig. 1. Fracture faces of sheep red cells fractured in 2.6 M glycerol. × 90,000

particle densities of fracture face A but not of fracture face B (*see* Table 3). Sheep and bovine red cells have low potassium influxes and high particle densities for the A fracture faces. However, human, rat and rabbit have higher influxes and lower particle densities (*see* Fig. 4).

Structure of Membranes with Drug-Induced Transport Differences

Cation transport was altered in sheep red cells by treatment with ouabain, valinomycin, amphotericin or TNC, and the cells were examined for changes in membrane morphology (*see* Table 4). Control samples for red cells treated with valinomycin and amphotericin were treated with methanol and Na-desoxycholate, respectively, since these agents were used to solubilize the antibiotics. Small reductions in particle densities were observed in membranes treated with ouabain and TNC, and no differences were observed with the antibiotic-treated membranes (*see* Table 4). Although the changes were small, the ouabain and TNC effects were reproducible and statistically significant. No effects of these agents on the particle densities on the A fracture faces were observed.

Table 1. Red cell membrane particle densities of individual HK and LK sheep (particles per μ^2)

Fracture face A					
HK 2563	HK 177	HK 109	LK 188	LK 103	LK 108
3930[a]	3926	4318	5100	3920	4023
4048	4335	3744	4950	3520	4028
3728	3666	3871	4500	4470	3666
4183	4028	4267	4300	3728	4689
4073	4023	3879	4950	4010	4073
3934			4700	3795	4643
4065				3972	4689
3846				4693	4470
4313				3981	4453
					3896
4013 ± 59[b]	3996 ± 107	4016 ± 116	4750 ± 125	4100 ± 121	4263 ± 117
HK average 4008 ± 62			LK average 4371 ± 195		
Fracture face B					
HK 2563	HK 177	HK 109	LK 188	LK 103	LK 108
1226	1299	1106	1200	1302	1133
1256	1236	1185	1400	1262	1236
1383	1164	1218	1460	1191	1290
1271	1240	1204	1220	1187	1149
1416	1214	1214	1220	1360	1230
		1383	1280	1271	
		1171	1500	1234	
		1171	1240		
		1281			
1310 ± 37	1231 ± 22	1215 ± 26	1315 ± 42	1258 ± 23	1208 ± 29
HK average 1252 ± 29			LK average 1260 ± 31		

[a] Each entry is a particle density determined from a single fracture face.
[b] Limits are calculated standard errors.

Structure and Cation Transport of Cells Treated with DMSO and Glycerol

In the DMSO and glycerol-treated membranes, it was difficult to quantitate the particle densities on fracture face A due to the tendency of the particles to aggregate (*see* Fig. 1). In contrast, for fracture face B, measurements of particle densities were highly reproducible. The relationship of particle densities on the B faces and the concentration of these

Table 2. Cation composition and transport properties of red cells from various species

Cells	$(K)_i$[g]	$(Na)_i$	$(K)_0$	$(Na)_0$	K Influx[e]	
					Total	With cardiac glycoside
	(mmoles/liter cells)		(mmoles/liter)		(10^{-15} moles cm^{-2} sec^{-1})	
HK sheep[a]	85	15	5	135	7.2	0.6
LK sheep[a]	12	88	5	135	4.7	3.3
Bovine[b]	27	65	5	150	5.3	2.2
Human[c]	89	11	5	150	25	6.9
Rabbit[c]	96	10	5	150	31	4.5
Rat[d]	106	4	5	150	49[f]	—

[a] Hoffman and Tosteson, 1971.
[b] Israel *et al.*, 1972.
[c] Rettori *et al.*, 1969.
[d] Beaugé and Ortiz, 1971.
[e] The uptake rates were converted into fluxes by relating them either to known values of surface area or to values calculated by Emmons' formula (*cf.* Ponder, 1948, p. 25).
[f] Rubidium influx.
[g] $(K)_i$ and $(Na)_i$ are the intercellular ion concentrations and $(K)_0$ and $(Na)_0$ are the extracellular concentrations.

Table 3. Red cell membrane particle densities from five species

Species	Fracture face A (particles per μ^2)	Fracture face B (particles per μ^2)
Sheep	4170 ± 56 (6,44)[a]	1256 ± 14 (6,39)
Bovine	3634 ± 67 (3,17)	1172 ± 34 (5,37)
Human	2658 ± 60 (3,18)	1218 ± 39 (3,20)
Rat	2489 ± 119 (2,14)	1193 ± 34 (3,20)
Rabbit	2189 ± 132 (4,21)	1197 ± 42 (4,24)

[a] Each entry is a particle density determined from n experiments and s fracture faces. The n and s values are reported in the parentheses (n, s) following each standard error limit.

agents is shown in Figs. 2 and 3. The effect of glycerol and DMSO treatments of cells are reversible by repeated washing of the samples with Solution A (R. G. Kirk, *in preparation*).

We studied the cation transport in sheep red cells treated with glycerol and DMSO by measuring the potassium influx in the presence and absence of ouabain. Ouabain was used to inhibit the energy-dependent "active" fraction of the total cation transport (Schatzman, 1953). In the transport

Table 4. Membrane particle densities of drug-treated sheep red cells

Experiment	Sheep #	Control (Sol. A) (particles per μ^2)	+Ouabain (10^{-4} M) (particles per μ^2)
1	HK 177	1230±22 (8)	1180±40 (4)
2	HK 177	1235±20 (5)	1080±121(5)
3	HK 177	1310±37 (5)	985±62 (5)
4	LK 104	1238±21(11)	1084±21(12)
5	HK 109	1215±29 (9)	1185±23 (5)
6	LK 112	1233±37 (8)	1113±22 (5)
Avg.		1238±11(46)	1099±22(36)
Experiment	Sheep #	Control (MeOH) (particles per μ^2)	+Valinomycin (10^{-6} M) (particles per μ^2)
1	LK 110	1136±28 (6)	1112±29 (7)
2	LK 115	1151±12 (5)	1101±44 (5)
3	HK 100	1117± 5 (5)	997±27(12)
4	HK 106	1085±34 (6)	1064±20 (7)
Avg.		1126±11(22)	1055±17(31)
Experiment	Sheep #	Control (Sol. A) (particles per μ^2)	+TNC (10^{-3} M) (particles per μ^2)
1	HK 177	1230±22 (8)	1106±66 (6)
2	HK 2562	1310±37 (5)	1208±41 (6)
3	HK 106	1129±27 (7)	1106±36 (5)
4	LK 110	1229±19 (6)	1129±22 (6)
Avg.		1218±16(26)	1141±22(23)
Experiment	Sheep #	Control (NaDC) (particles per μ^2)	+Ampho (10^{-5} M) (particles per μ^2)
1	LK 104	1093±56 (6)	1136±58 (5)
2	HK 106	1166±28 (5)	1175±28 (6)
3	LK 115	1097±16 (5)	1083±20 (6)
4	LK 110	1103±53 (7)	1098±36 (5)
Avg.		1113±22(23)	1122±15(22)

studies using cells treated with glycerol, it was necessary to incubate for periods of 3 hr for complete glycerol penetration. Penetration was determined by measuring the hematocrit at various times during the incubation (*see* Table 5). Despite the fact that DMSO penetrated very rapidly (in a few seconds), we exposed cells to DMSO for 90 min prior to adding ^{42}K, since this was the length of incubation used in the freeze-fracture studies for maximum morphological effect. No hemolysis was observed in the DMSO or glycerol-treated samples.

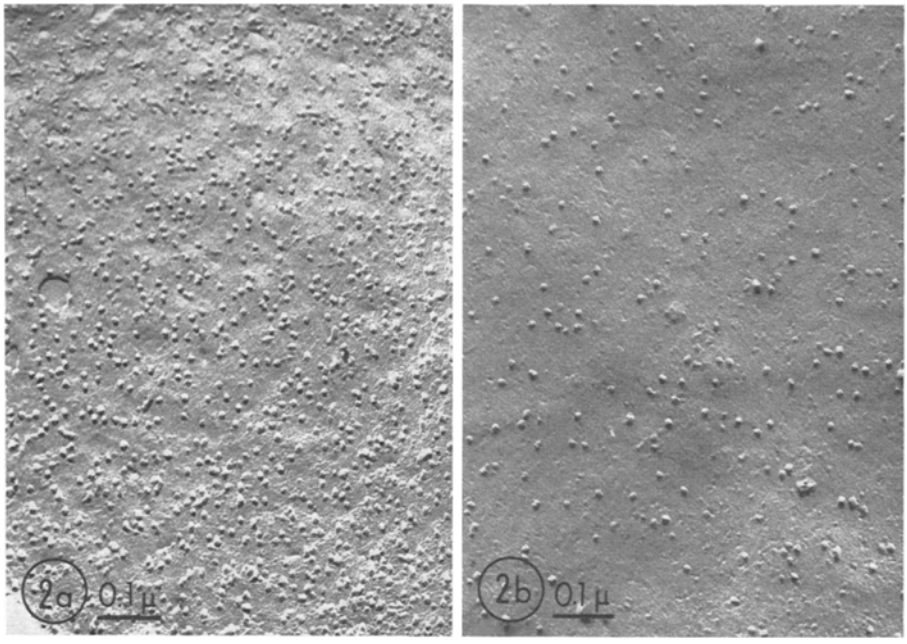

Fig. 2. (*a*) Fracture face B of a sheep red cell fractured in isotonic salt solution (Solution A). (*b*) Fracture face B of a sheep red cell fractured in 2.6 M DMSO solution. × 90,000

Table 5. Penetration of glycerol into sheep red cells

Glycerol conc. (M)	% of original volume at various times of incubation in glycerol		
	15 min	60 min	145 min
0.7	82	94	99
1.0	80	94	98
2.6	73	89	96

Our potassium influx results are given in Table 6. It is somewhat surprising to find that over the range of glycerol and DMSO concentrations in which membrane morphology is altered, no effects on either passive or active transport were observed.

Discussion

The B fracture faces of red cell membranes of the five species studied are morphologically similar despite the fact that these membranes differ

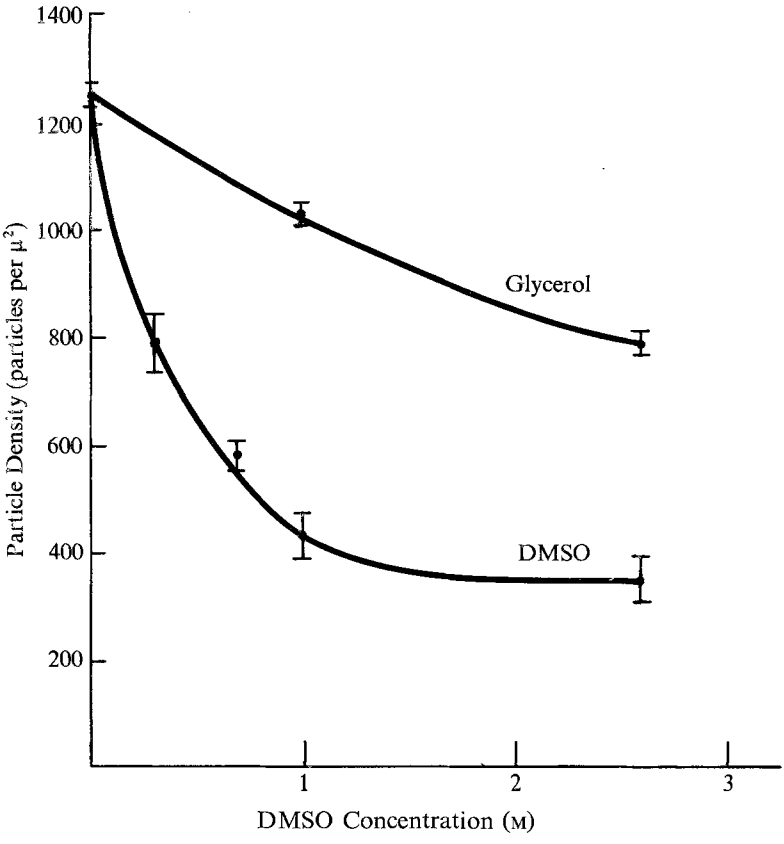

Fig. 3. Effect of DMSO and glycerol on membrane particle density of fracture face B of sheep red cells

Table 6. Effect of DMSO and glycerol on K^+ influxes in HK sheep red blood cells

Treatment	$(K)_i$	$(Na)_i$	K Influx	
	(mmole/liter cells)		Total mmole/(liter cells · hr)[a]	With ouabain
Control	70	23	0.75	0.06
0.7 M Glycerol	74	29	0.77	0.05
1.0 M Glycerol	74	22	0.73	0.06
2.6 M Glycerol	70	24	0.69	0.07
0.7 M DMSO	72	23	0.65	0.08
1.0 M DMSO	72	23	0.65	0.08
1.0 M DMSO	75	23	0.60	0.13
2.6 M DMSO	75	25	0.57	0.14

[a] Equivalent volume of packed cells of the original whole blood.

in both their transport characteristics (*see* Table 2), and their composition (Nelson, 1967). The HK and LK sheep membranes differ in their transport characteristics (Hoffman & Tosteson, 1971), but show no differences in their morphology and overall membrane lipid composition (J. de Gier, *personal communication*; Nelson, 1967). Substantial differences are found in the A fracture faces. The sheep and bovine membranes have high particle densities, and human, rat and rabbit have low densities. Sheep and bovine membranes have been found to have lower active potassium transport rates (ouabain-sensitive) than human, rat and rabbit membranes, while the "leak" (ouabain insensitive) rates are relatively low for all five species (*see* Table 2). In Fig. 4, membrane particle density is plotted on the ordinate and active K influx on the abscissa for red cells of five species. Whether the apparent inverse relation indicates a functional correlation between these membrane properties is not clear. It is interesting to note that sheep and bovine membranes are known to contain high proportions of sphingomyelin and low proportions of lecithin, while human, rat and rabbit membranes show the reverse pattern (Nelson, 1967). Thus, it seems possible that the differences found in the fracture face A particle densities, as well as the differences in the potassium permeability, are reflections of the phospholipid composition of the mammalian red cells (*see* Fig. 5).

There are small morphological differences in the sheep membranes treated with drugs. Although the differences were about 10% in ouabain-treated cells as compared with control samples, they were reproducible and statistically significant. Taking into account the total surface area of a sheep red cell (about 100 μ^2), one calculates that ouabain removes about 14,000 particles per cell. The number of specific ouabain binding sites determined by Dunham and Hoffman (1971) is about 45 per HK cell and 7 per LK cell. Thus, if the reduction in intra-membrane particle densities is due to binding of the drug at the sites which inhibit cation transport, a single molecule of ouabain affects over 1,400 particles and an area of $>10\ \mu^2$ in LK cells. However, the reduction in intra-membrane particle density may be due to binding of ouabain molecules at nonspecific loci. The latter possibility is particularly likely in view of the high concentration of ouabain used in our experiments. Further work will be required to establish the relation between ouabain binding and intra-membrane particle density. Both TNC and valinomycin produced slight reductions of questionable significance in particle density on the B-fracture surfaces. Valinomycin at 10^{-6} M increases K^+ permeability of sheep red cell membranes by more than two orders of magnitude (Tosteson *et al.*, 1967), while 10^{-2} M TNC reduces Cl^- permeability by about four orders of

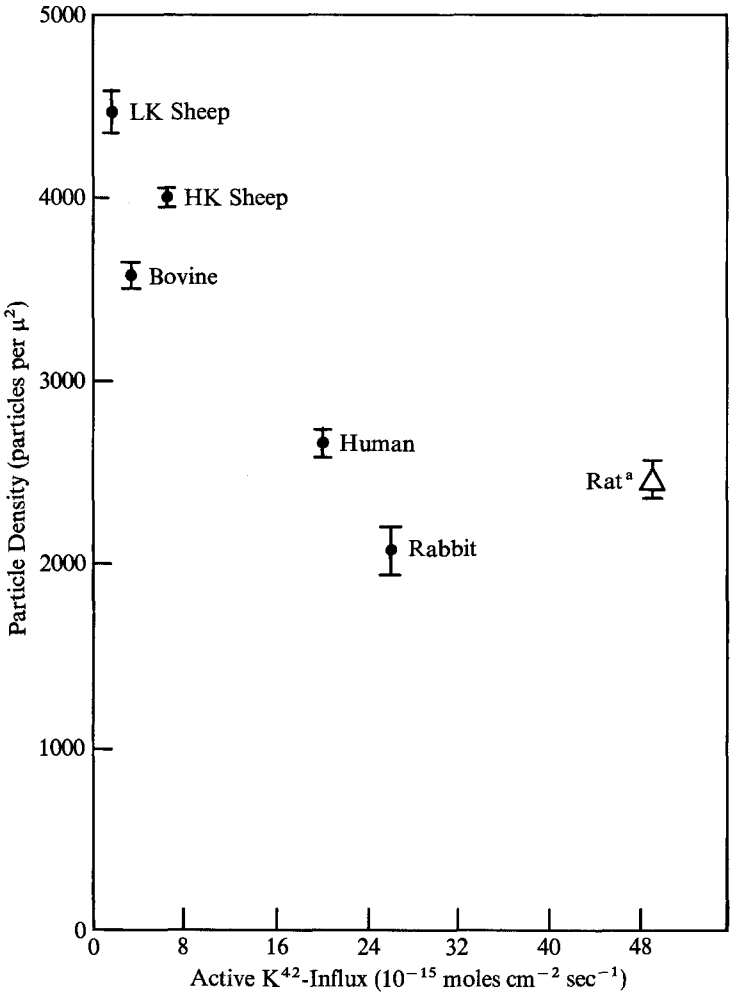

Fig. 4. Relationship between particle density of fracture face A and membrane "active" transport. The transport values were calculated from Table 2. *Closed circles:* ouabain-sensitive K-influx. *Open triangle:* total rubidium influx. Superscript [a]: Ouabain-sensitive potassium influx was not available for rat

magnitude. Thus, if intra-membrane particles are involved in K^+ and Cl^- transport, either the changes produced by these agents are not revealed by freeze-fracture electron-microscopy or only a very small fraction of the total number of particles participate in ion transport. Amphotericin is thought to form membrane pores of about 5 Å in diameter (Dennis, Stead & Andreoli, 1970). The failure to observe these membrane pores is most likely due to the limited resolution of the freeze-fracture technique.

Membrane morphology was found to be altered drastically by treatment of the sheep red cells with either DMSO or glycerol. DMSO treatment

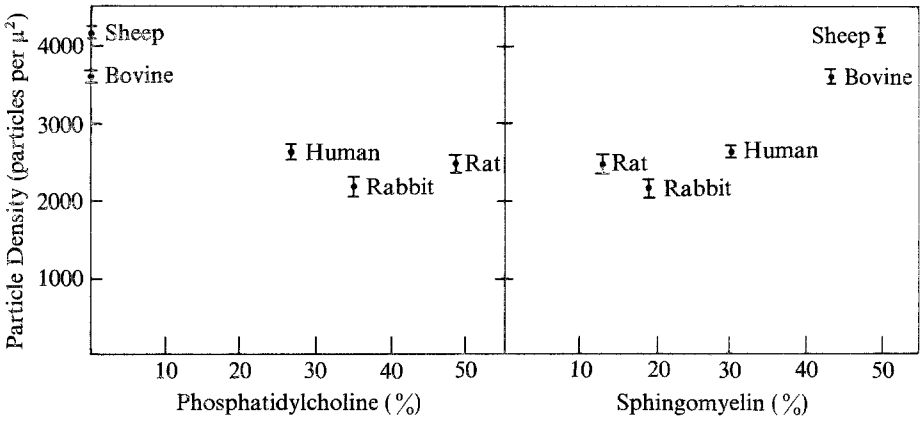

Fig. 5. Relationship between particle density of fracture face A and membrane phospholipids in mammalian red cells. Phospholipid values taken from Nelson (1967), are expressed as percentages of the sum of phospholipids

results in a 70% reduction of fracture face B particle density as compared to cells frozen in isotonic saline solution. This effect is completely reversible, which we interpret as evidence that the phenomenon is not a loss of molecules responsible for the visualization of the intra-membrane particles. Cells incubated in glycerol have reductions of about 35% in their membrane fracture face B particle densities; and similar to the DMSO effect, glycerol treatments were found to be completely reversible. The reduction of particle density cannot be explained on the basis of increased membrane surface area since increases in surface area were not observed in DMSO and glycerol-treated cells (R. G. Kirk, *in preparation*).

Over the range of glycerol and DMSO concentrations in which membrane morphology is altered, no effects on both passive and active transport were found. It appears that the particles which are altered by DMSO and glycerol are not involved in cation transport. Heterogeneity of particle populations is indicated by the varying sensitivities and reversibilities to these agents.

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