ONLY SOLID RED BLOOD CELL GHOSTS TRANSPORT K' AND Na^+ AGAINST CONCENTRATION GRADIENTS: HOLLOW INTACT GHOSTS WITH K' – Na' ACTIVATED ATPASE DO NOT

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• ***Human red blood cell ghosts, prepared by simple hypotonic lysis are filled with cytoplasmic proteins. These solid ghosts can transport K^+ and Na^+ against concentration gradients in the presence of ATP. In hollow red cell ghosts prepared by repeated hypotonic lysis followed by high salt wash, this ability to transport K^+ and Na^+ in the presence of ATP is lost even though these hollow ghost membranes remain intact and are equipped with Na^+ – K' activated ATPase.***

INTRODUCTION

In 1963 Hashimoto and Yoshikawa demonstrated an ATP-dependent Rb' uptake by human red cell ghosts against a concentration gradient.[1,2] Ten years later Freedman demonstrated a similar ATP-dependent net movement of K' into red cell ghosts, accompanied by a net loss of Na^+.[3,4] Assuming that these red cell ghosts were membrane-enclosed hollow sacs, Freedman concluded that his findings offered support for the membrane pump theory, according to which K^+ accumulation and Na^+ exclusion are due to the activities of postulated pumps in the cell membrane, and against the association-induction hypothesis, according to which K' accumulation and Na^+ exclusion reflect primarily properties of the cytoplasmic protein-water system.

A review of the literature revealed that the procedures used for preparing demonstrably hollow ghosts (e.g., Marchesi and Palade;[5] Dodge et al[6]) differ from that used by Freedman, which was a modification of the method of Bodeman and Passow.[7] Therefore, it was not surprising that Ling and Balter[8] soon found that EM plates of red cell ghosts prepared by the method of Bodeman and Passow,[7] and those prepared by the method of Marchesi and Palade[5] showed different pictures: Whereas the red cell ghosts prepared by the Marchesi and Palade method, involving an additional step of high salt wash following repeated hypotonic lysis, are indeed primarily intact hollow sacs (see Figure 1); those prepared by the method of Bodeman and Passow were solid bodies. Subsequently Hazlewood and coworkers[9] confirmed the findings of Ling and Balter and showed that the methods of Garrahan and Glynn[10] and of Freedman,[4] produced solid ghosts.

These findings showed that Freedman's demonstration of K' and Na' movements against concentration gradients, offered no specific support for the membrane theory, because the observed phenomena can also be readily explained on the basis of the association-induction hypothesis as reflecting properties of the remaining cytoplasmic protein-water system inside the solid ghosts.

The procedures for red cell ghost preparation described by Marchesi and Palade produce hollow ghosts; from the representative EM picture given by these authors and reproduced here as Figure 1, the membranes of

these hollow sacs remain intact.' The intactness of these hollow sacs was further confirmed by the inability of externally added lead phosphate to penetrate into the sacs. Ghosts prepared by their methods including repeated lysing and high salt wash, also retain normal activities of K' − Na' activated ATPase, widely believed among proponents of the membrane pump theory to be the Na pump itself."

To probe deeper into the basic mechanism of K^+ accumulation and Na' exclusion in living cells, we undertook an investigation to answer three questions:

1. Can we confirm in general the findings of Hashimoto, Yoshikawa, and Freedman? And, in particular, can we confirm Freedman's finding of the ATP-dependent accumulation of K^+ and extrusion of Na' from red cell ghosts against concentration gradients?

2. Are red cell ghosts prepared by precisely the same procedure as that used by Freedman hollow?

3. Finally, can the hollow "Marchesi - Palade" ghosts accumulate K' and extrude Na' against concentration gradients?

The present communication reports the results of investigations aimed at answering these questions.

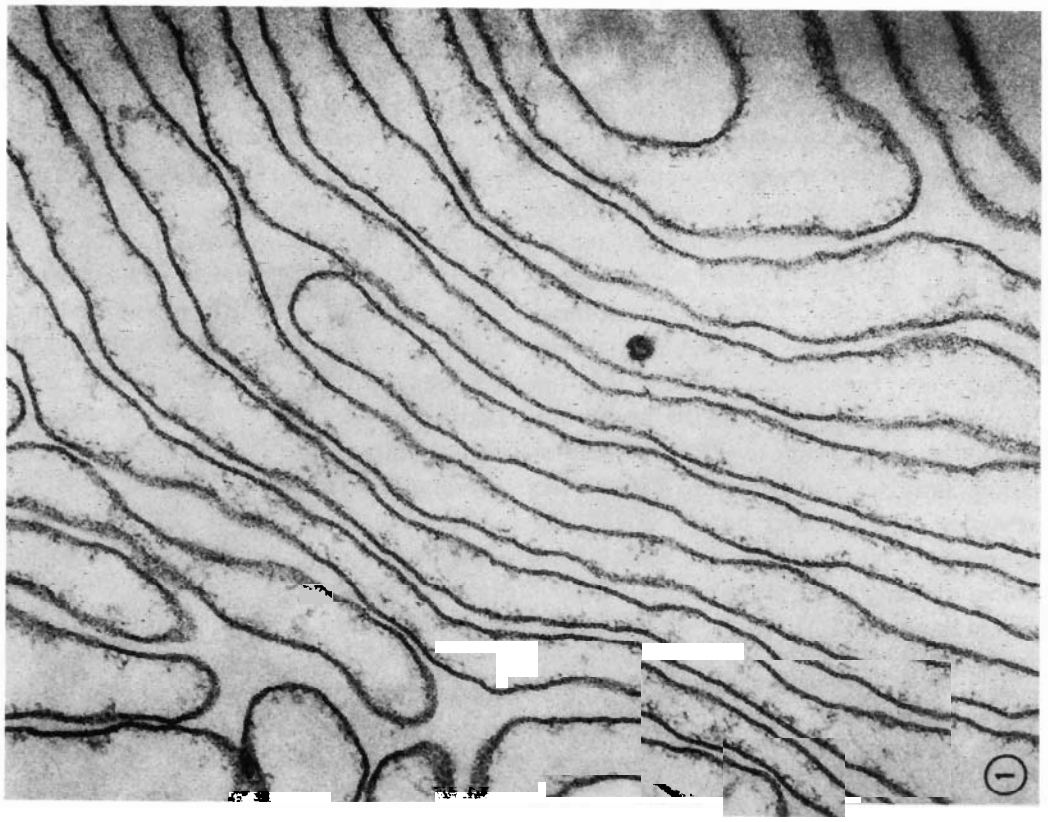

FIGURE 1. Electron micrograph of a representative area in a section through ghost membrane pelleted by high speed centrifugation (100,000 g, 30 min) and fixed in glutaraldehyde-$0s0_4$). The ghosts appear as empty sacs bounded by continuous unit membranes. Fibrillar material is seen along the inner surfaces of the ghost membranes. Magnification 90,000×. (from Marchesi and Paade,[5] by permission of the J. *Cell Biol.)*

MATERIALS AND METHODS

All ghost preparations were made from freshly drawn human blood from young adults.

The methods for the preparation of "Type II" ghosts used in Freedman's ion transport studies, to be referred to as "Freedman's ghosts," loading the ghosts with Na^+ and ATP, resealing, and fractionation with a sucrose cushion were entirely as Freedman had described.[3,4]

"Marchesi-Palade ghosts" were prepared by essentially the method described by these authors.' The modification of the Marchesi-Palade procedure we introduced was to make the treatment less severe than the one they employed. Thus instead of 5 mM Tris-HCl and 1 mM EDTA, which Marchesi-Palade used for lysing, our hypotonic lysing solution contained Na_2 ATP (5 mM), $MgCl_2$ (7 mM), L-cysteine (1 mM), Tris HCl (10 mM), Na_2 EDTA (0.1 mM), adjusted to pH 6.0 with NaOH at 3°C. This solution was in fact that used by Freedman and shown by Freedman (and ourselves as well, see below) to produce no serious impairment of the ion transport mechanism. After lysing at 1°C (10 min.) the ghosts were exposed for a total of 10 min. (including centrifugation time) at 4°C to a high salt solution containing 0.5 M NaCl, washed twice more in the ATP-containing lysing solution and then resealed by adding to each 100 ml of the ATP-containing lysing solution-ghost suspension, 20 ml of a hypertonic mixture of NaCl(0.5 M), KCl (15 mM), sucrose (2 M).

The procedure for demonstrating K^+ and Na^+ movements, also followed Freedman.[3,4] Briefly the resealed "Type II" ghosts were incubated at 37°C as a 20% suspension (v/v) in an incubation solution containing the following: NaCl (50 mM), KC1(10 mM), $MgCl_2$ (2 mM), Tris-HCl (10 mM), Na_2EDTA (0.1 mM), inosine (10 mM), adenosine (10 mM), sucrose (160 mM). The pH of the medium was 7.4 measured at 37°C. At intervals, aliquots of the ghost suspension were taken out, spun in 0.4 ml polypropylene microcentrifuge tubes before assay for K, Na^+ (by atomic absorption spectroscopy) and water contents (by drying at 100°C. in vacuo). Following Freedman,[3,4] we made no corrections for ions in the extracellular space which in intact red cell pellets usually amounts to 1.5 to 3.0% (Maizels and Remington[11]).

For visualization by transmission electron microscopy, pellets of the ghost preparations obtained by centrifugation were fixed in glutaraldehyde, dispersed to avoid layering and non-random sampling errors and then post-fixed in osmium tetraoxide, dehydrated through alcohol and propylene oxide, imbedded in Epon, and then stained in uranyl acetate and lead citrate. Essentially the same procedure was used by Marchesi and Palade[5] for their EM plate shown as Figure 1 below.

RESULTS

Are the Red Cell Ghosts Hollow? Figure 2 shows an EM plate of "Type II" red cell ghosts prepared by the Freedman procedure ("Freedman's ghosts"). Like those prepared by the original method of Bodeman and Passow, these ghosts are also solid. We chose to present a picture of not too low a magnification so that it can be compared with that of Marchesi and Palade.[5] For other pictures where larger populations of "Freedman's ghosts" were shown, see Hazlewood et al[9] whose conclusion Figure 2 confirms. Since human cells contain little or no polynucleotides, the uranium and lead staining material seen inside the ghosts most likely reveals the presence of substantial amounts of cytoplasmic proteins. In contrast, Figure 1 reproduced from Marchesi and Palade[5] shows intact but hollow "ghosts."

Can the "Freedman Ghosts" Transport K^+ and Na^+ Against Concentration Gradi-

enu? In Figure 3, the K' and Na' concentrations in the "Freedman ghosts" are expressed as a ratio (p-value) to the concentration of the respective ion in the external incubation solution and are shown against the time of incubation. ATP was introduced into the ghosts only during hemolysis and resealing in both cases. The "Freedman ghosts" showed moderate K' accumulation and Na^+ exclusion against concentration gradients as Freedman described earlier; the time course of K' concentration and that of Na' extrusion from the ghosts diverged with time. The lowest level of Na' reached was about 60% of that in the external solution. The highest level of K^+ reached in the cell was 30mM, 6 times higher than that of the K' concentration in the surrounding medium which was 5 mM. In normal intact human erythrocytes, the Na^+ concentration is about 16% that in the external solution; the K' concentration is about 155 mM, more than 27 times higher than the

FIGURE 2. Electron micrograph of human red cell ghosts prepared by the method of Freedman ("Freedman Ghosts") Magnification 18,000x (Plate No. 383).

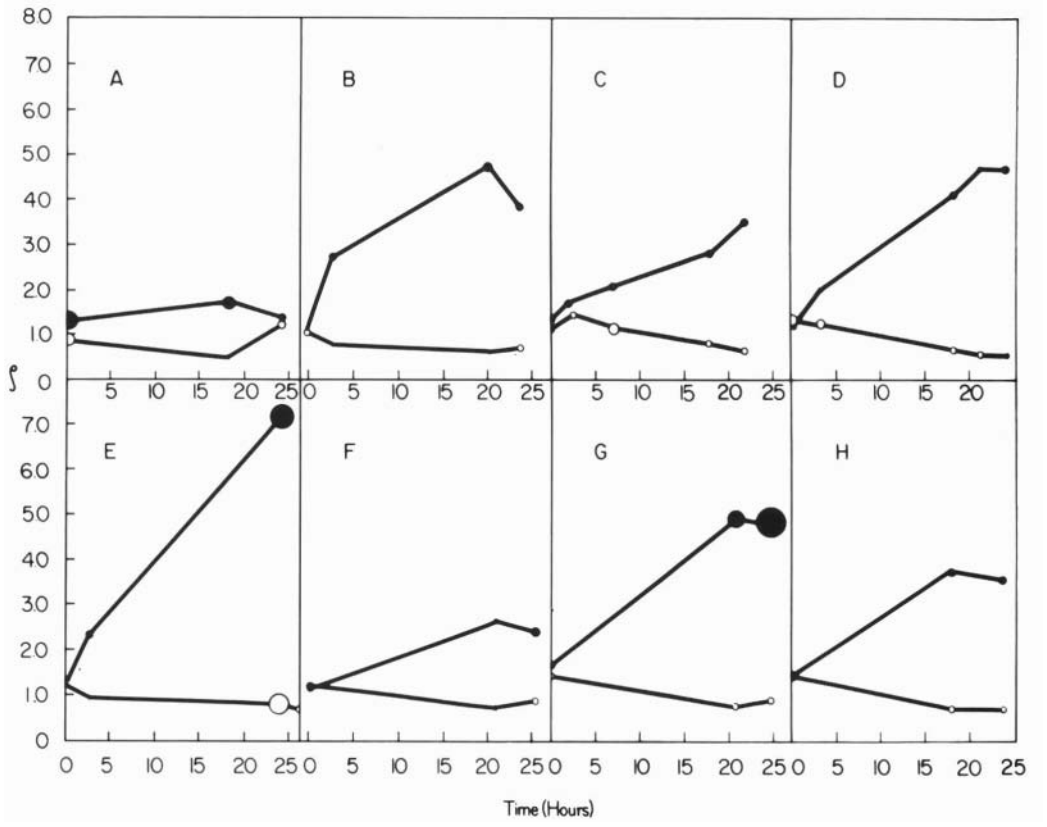

FIGURE 3. Demonstration of active transport of K^+ and Na^+ against concentration gradients in the "Freedman Ghosts." Ordinate represents the ratio of the concentration of K^+ or Na^+ ion in the ghost water over the concentration of the same ion with the incubation media. This ratio is called the p-value. Each point is the average of at least four deteminations. The diameter of the solid circles (K^+) and hollow circles (Na^+) represent twice the standard errors.

K' concentration in the plasma which is also 5 mM.[12]

Can the "Marchesi-Palade Ghosts" transport K^+ and Na^+ against concentration gradients? As shown in Figure 4 the "Marchesi-Palade ghosts" behaved differently under the same experimental conditions. The concentrations of K' and Na^+ remained essentailly unchanging: there was no active transport of K' or Na^+. When the K^+ and Na^+ concentrations did exhibit minor changes, they rose and fell with time in a parallel manner. These parallel K' and Na' changes, in contrast to the divergent changes shown in Figure 3 probably reflected minor fluctuation of the water contents of the ghosts. It may be mentioned that we also prepared ghosts according to the procedures of Dodge et al,[6] a method also known to produce truly hollow ghosts. The results were quite similar to those shown in Figure 4.

DISCUSSION

Confirmation of Freedman5 Demonstration of K^+ and Na^+ Movements Against Concentration Gradients in Ghosts that are Solid. This paper is not the first to report that simple hypotonic lysis does not produce

hollow ghosts; besides those earlier mentioned,[8,9] Eric Ponder reached the same conclusion from a totally different approach[13] three years after the publication of his authoritative monograph, "Hemolysis and Related Phenomena" in 1948.[14] Taken together, these findings invalidate Freedman's claim that his observation specifically supported the pump theory since this conclusion was based on a wrong assumption. However, the data shown in Figure 3 do confirm the factual findings of Freedman concerning transport of K' and Na' against concentration gradients in ghost preparations now known' to be not hollow but solid. In a general way these findings of course also confirm the conclusion of Hashimoto and Yoshikawa.[1]

Failure of Pure and Intact Plasma Membrane-enclosed Sacs to Pump K and Na^+. Marchesi and Palade showed that the majority of the ghosts prepared by their procedure, which we followed, are not leaky. As mentioned above, in the context of the membrane pump theory, these ghosts (which were subjected to a much milder version of the preparatory procedures of Marchesi and Palade) also possess normal Na pump, i.e., the K' – Na^+ activated ATPase, as these authors demonstrated.[5] It would seem that the necessary conditions for pumping Na^+

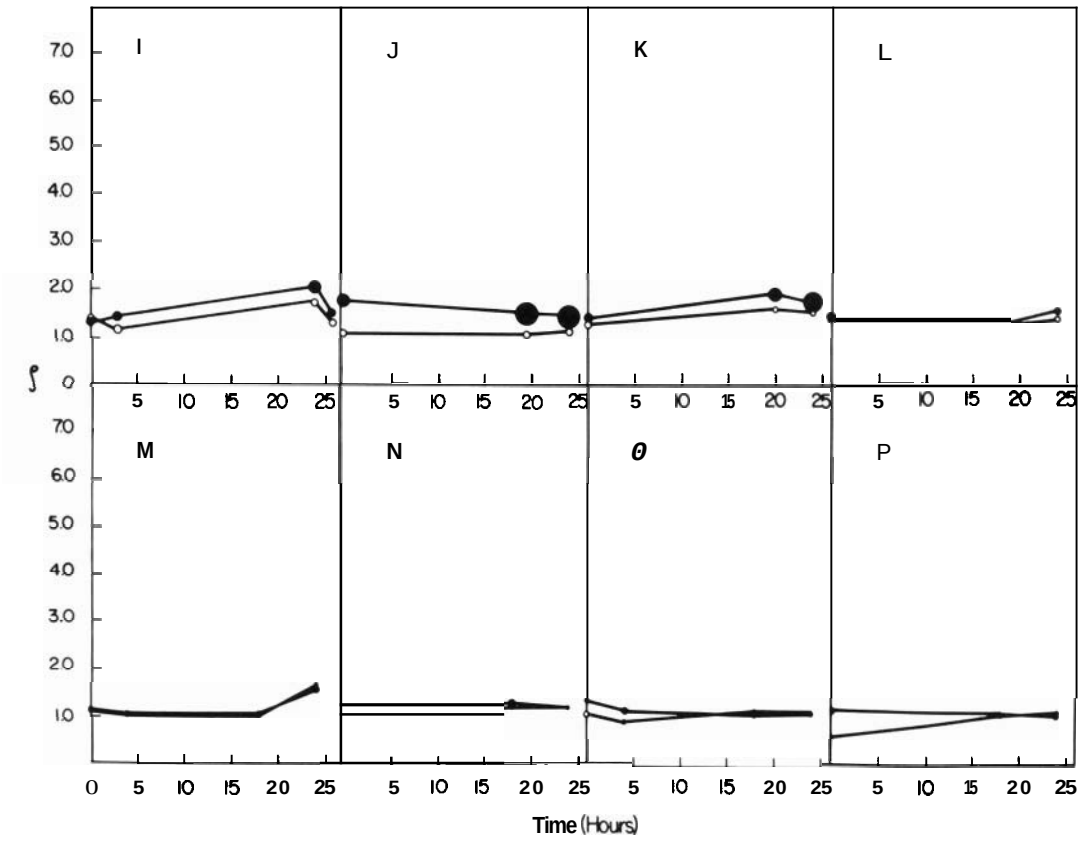

FIGURE 4. Demonstration of a lack of active transport of K^+ and Na^+ against concentration gradients in the "Marchesi-Palade ghosts." Ordinate represents the ratio of the concentration of K^+ or Na^+ ion in the ghost water over the concentration of the same ion in the incubation media. This ratio is called the p-value. Each point is the average of at least four determinations. The diameter of the solid circles (K^+) and hollow circles (Na^+) represents twice the standard error.

out and accumulating K' in the ghosts were fulfilled and that active transport of K' and Na' should occur in the presence of ATP. Yet this was not the case.

Confirmation of the reported K' accumulation and Na^+ exclusion in the ghosts now shown to be solid can be compared with the success in demonstrating both K' accumulation in and Na^+ exclusion from a muscle-cell preparation whose postulated cell membrane pumps were incapacitated, in part by surgical amputation, and in part by the deprivation of "sinks" or "sources" for the ions involved.[15,16] This effectively membrane-pump-less open ended cell preparation, called EMOC preparation, demonstrates that the ability to handle K' and Na^+ distribution resides not in the cell membrane but in fact within the cytoplasm itself.

On the other hand, the failure of an intact but hollow membrane pump preparation obtained with the "Marchesi-Palade" procedure actively to transport Na^+ or K^+ agrees with a similar failure to demonstrate active transport of K' and Na^+ in squid-axon membrane sacs from which the bulk of cytoplasm had been removed.[17,18] Here the anatomical as well as functional intactness of the axonal membrane was also clearly established, by electron microscopy[19] and by the full and normal electrical behaviors observed,[19,20] which according to the membrane pump theory indicate normal membrane and healthy pumps.

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