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ASYMMETRIC MASS DISTRIBUTION OF ($Na^+ + K^+$)-ATPase IN MEMBRANES STUDIED BY FREEZE-FRACTURE-ETCH ELECTRON MICROSCOPY

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SDS-purified porcine kidney ($Na^+ + K^+$)-ATPase was studied by thin-section and freeze-etch electron microscopy. Freeze-fracturing of resealed membrane fragments shows no difference in the distribution of intramembranous particles of approx. 9.0 nm in diameter between convex and concave fracture faces. However, two types of convex face are found: $\widehat{F}_A$, which shows a rather smooth background with many intramembranous particles, and $\widehat{F}_B$, which shows a textured background with very few or no intramembranous particles. Etching the fractured samples further reveals that $\widehat{F}_A$ faces are covered with many intramembranous particles, while the etched external faces (E_A) are either irregularly granulated or reveal many particles half the size of intramembranous particles. $\widehat{F}_B$ faces are covered with distinct pits of 9 nm or larger. The etched external surfaces (E_B) are covered with many particles of intramembranous particle size. These results suggest that there are two vesicle orientations in our resealed purified membrane preparation: right-side-out, as in vivo, and inside-out. The majority of the protein mass is distributed only on one side of the membranes. Right-side-out resealed membrane vesicles after fracturing and etching show particulated $\widehat{F}_A$ convex fracture faces and irregularly granulated or smooth etched E_A surfaces, indicating that the $\widehat{F}_A$ face is the protoplasmic fracture face and that the majority of the protein mass of the ($Na^+ + K^+$)-ATPase is located on the cytoplasmic half of the membrane.

Introduction

The asymmetric orientation of membrane-bound ($Na^+ + K^+$)-ATPase or the Na^+ pump within the membrane is implicated by: (a) both the ATPase activity and ion transport are activated by Na^+ and ATP only from the inside of the cell, while K^+ and the inhibitor, ouabain, act only from the outside [1–3], and (b) a photoaffinity analogue of ouabain and the antibody against the catalytic subunit can bind simultaneously to the enzyme. Since both these ligands bind to the catalytic α subunit and only bind on opposite sides of the membrane, the α subunit is presumed to span the membrane [4–6].

The ultrastructure of purified ($Na^+ + K^+$)-ATPase from rabbit and rat kidneys (using SDS extraction) has been studied by negative stain, thin-section and freeze-fracture electron microscopy [7–8]. Fracture faces of membrane fragments showed intramembranous particles, 9–11 nm in diameter, which distributed preferentially on one surface while the opposite face had none or few

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intramembranous particles [7]. In a later study using pig kidney ATPase, Maunsbach et al. [9] showed no difference in the distribution density of intramembranous particles on the two fracture surfaces. Similar results were reported by Van Winkle et al. [10] in vesicles of purified canine kidney ATPase. Negative staining of membrane fragments revealed similar and smaller surface particles of 3–5 nm in diameter on both surfaces. However, edge-on views showed that one side of the membrane is covered with stalked knobs projecting from the surface and 'fuzzy' ill-defined substructures on the other side [11]. These stalked knobs have been interpreted as catalytic centers and are situated on the protoplasmic side. The 'fuzzy coat' is presumed to be the glycoprotein situated on the exterior surface. In contrast, Haase and Koepsell [8], reported that surface particles protruding from both surfaces of the membrane are of equal size and frequency.

Thus, conclusive evidence as to the true cytoplasmic and outer surface on isolated $(Na^+ + K^+)$-ATPase, as well as the mass distribution of the enzyme, have not been presented so far. The present work was undertaken to examine further the mass distribution by freeze-etch techniques. The data presented are consistent with the proposal that the mass of the enzyme is located mostly on one half of the membrane, and that the majority of the protein mass is located on the cytoplasmic half of the membrane.

Materials and Methods

Membrane preparation

Fresh pig kidneys which were obtained from a local slaughterhouse were either used immediately or stored at −70 °C. $(Na^+ + K^+)$-ATPase was isolated from the outer medulla and purified by SDS-ATP treatment followed by a discontinuous sucrose density gradient centrifugation [12]. The specific activity of the enzyme preparations used in this study was 25–30 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$. The purified membrane fragments were washed twice in 10 mM imidazole/1 mM EDTA (pH 7.5) (imidazole/EDTA) or dialyzed against imidazole/EDTA buffer overnight before processing for electron microscopy. Some purified enzyme preparations were shipped from the University of South Dakota packed in ice.

All chemicals were reagent grade with the exception of a specially pure grade of SDS from Gallard-Schlesinger Chemical Manufacturing Corp. (Carle Place, New York).

Formation and assay of right-side-out sealed membrane vesicles

Purified membranes were incubated in imidazole/EDTA buffer containing 1 mg/ml octylglucoside (*n*-octyl-β-D-octylglucopyranoside, Calbiochem) at a protein concentration of 1 mg/ml for 1 h at room temperature (approx. 23 °C). The suspension was centrifuged at 40 000 rpm (149 000 $\times g_{av}$) for 45 min in a Beckman SW 50.1. rotor. The pellet was resuspended in imidazole/EDTA buffer and dialyzed against the same buffer for 18 h at 4 °C.

Membrane vesicles were assayed for the $(Na^+ + K^+)$-ATPase activity in the presence and absence of SDS (0.65 mg/ml) according to the procedure of Forbush [13].

Electron microscopy

Thin sectioning. Pellets of membrane fragments were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4 °C. After three washes with 0.1 M cacodylate buffer, the pellets were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature and rinsed with 0.1 M sodium acetate buffer (pH 5). The pellets were block-stained overnight in 1% uranyl acetate in 0.1 M sodium acetate buffer and then dehydrated, embedded in Epon and sectioned. Sections with a gray interference color were picked up on carbon-coated grids and stained further with uranyl acetate and Sato's lead [14].

Freeze-etching. Small samples of concentrated purified membrane suspension (approx. 0.1 μl) were sandwiched between two copper strips and were ultra-rapidly frozen (rates in excess of 10 000 °C/s) by plunging into liquid propane at −190 °C [15,16]. Frozen samples were mounted in a double replica device adapted for use in a Balzers 360 M freeze-fracture apparatus (Balzers Co., Nashua, NH) and fractured at −150 °C and 10^{-7} Torr. The fracture samples were immediately replicated with Pt/C at a 45° angle followed by C at a 90° angle. Etched samples were fractured at −105 °C and etched for 30 s prior to replication.

Replicas were either floated off the copper strips onto distilled water or separated from the strips by dissolving the strips with dilute chromic acid [17]. Replicas were cleaned with Clorox and picked up on 400-mesh uncoated grids.

The replicas were examined with a Philips 301 electron microscope at 80 kV and 50 μm objective aperature. In all micrographs, the deposit metal is black and shadows are white. Shadow direction is indicated by a black arrow at the bottom of the micrograph.

Results and Discussion

Thin sectioning

Thin sections of membrane pellets show that the membrane fragments are mostly vesicular in shape and single-walled. Few of them appear flat or cup-shaped (Fig. 1). At high magnifications, a trilaminar structure is evident (inset of Fig. 1). The overall membrane thickness is about 9.5 nm and the width between the two dense lines is about 4.5 nm. Substructure, however, is not observed in thin sections.

Freeze-etching

Consistent with the results of thin sectioning, it can be seen that most membrane fragments (in freeze-fracture specimens) appear to be mainly vesicular rather than semicircular (Fig. 2). In general, there is no difference in the distribution of intramembranous particles between the two fracture faces (convex, $\widehat{F}$ and concave, $\widecheck{F}$), and the size of the particles is approx. 9 nm in diameter. How-

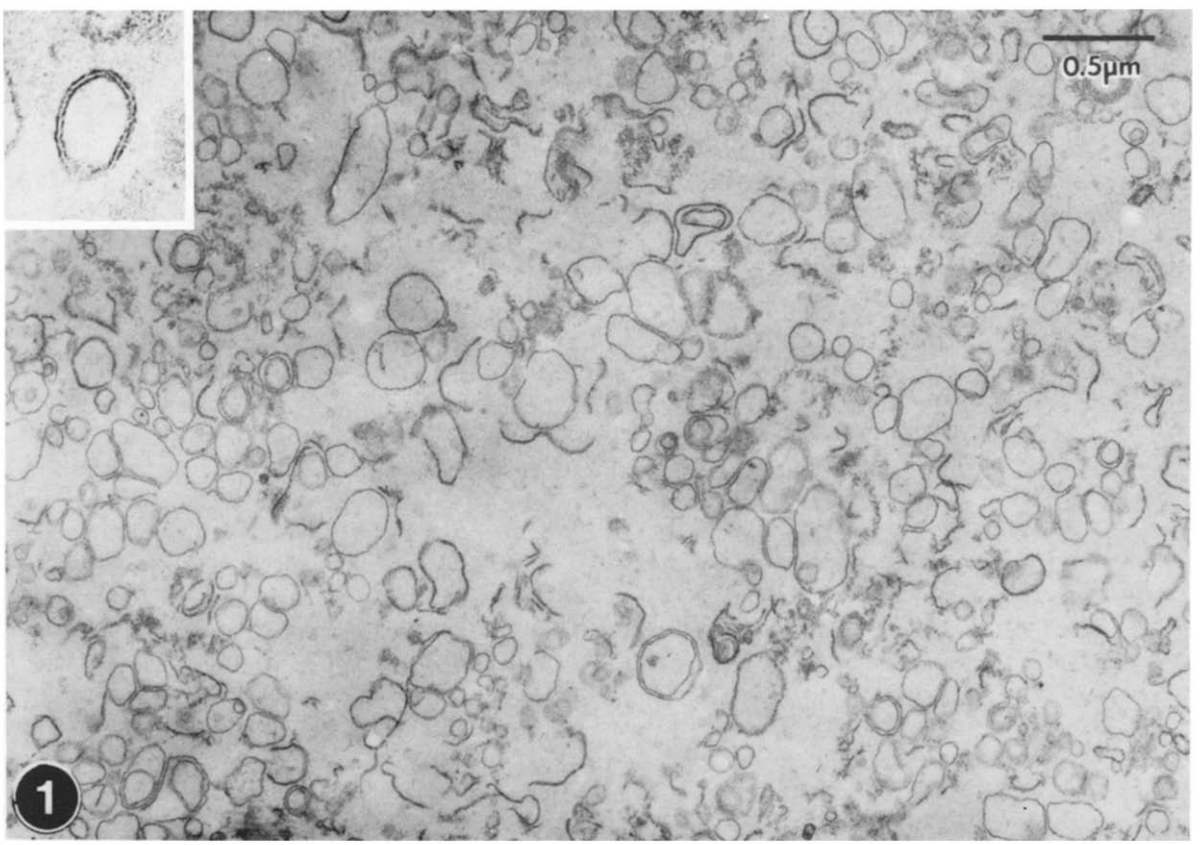

Fig. 1. Thin section of pellet of purified porcine renal microsomal membrane fragments containing ($Na^+ + K^+$)-ATPase. Membrane fragments were washed twice in 10 mM imidazole and 1 mM EDTA (pH 7.4) before processing for electron microscopy. The pellet appears to contain primarily membrane vesicles and some fragments which are flat or cup-shaped. The inset, which was printed 3.5 × the magnification of the main figure, shows the appearance of trilaminar structure of the membrane.

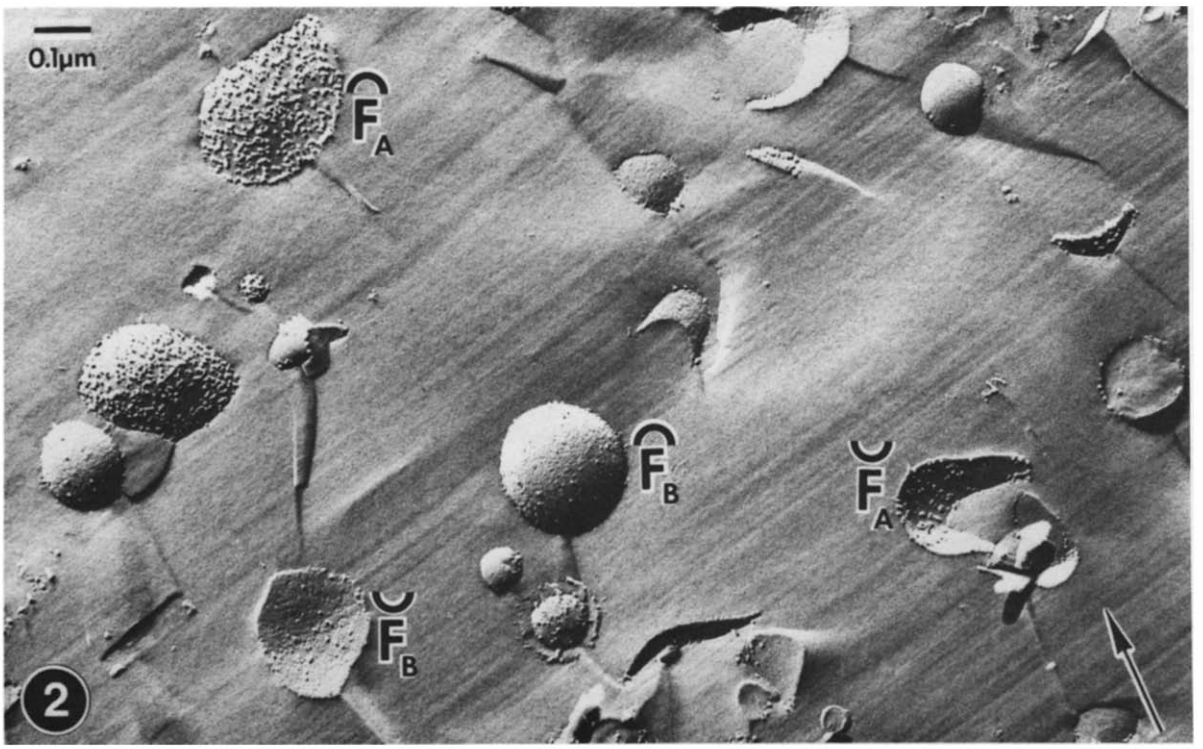

Fig. 2. Freeze-fracture micrograph of purified porcine renal ($Na^+ + K^+$)-ATPase. A small sample of concentrated membrane suspensions (approx. 0.1 μl) was sandwiched between two copper strips and ultra-rapidly frozen, fractured and followed by unidirectional shadowing at an angle of 45°. Fracture faces show both convex ($\widehat{F}$) and concave ($\breve{F}$) surfaces which either have many ($\widehat{F}_A$ and $\breve{F}_A$) or few ($\widehat{F}_B$ and $\breve{F}_B$) intramembranous particles of 8.5–9.5 nm diameter.

ever, on close examination, there are two distinct features revealed on both concave and convex faces. One population of convex faces ($\widehat{F}_A$) has a rather smooth background but is covered with many 8.5–9.5 nm in diameter particles, while the other population of convex faces ($\widehat{F}_B$) has a rough textured background covered with very few or no particles (Fig. 2). Similar features are observed on two populations of concave faces (labelled as $\breve{F}_A$ and $\breve{F}_B$).

In order to observe the substructure within the membrane and membrane surfaces simultaneously, samples of membranes which have been fractured were etched for 30 s at −105°C. Subliming away the surrounding ice, reveals further the differences between the two convex faces (Fig. 3a and b). After etching, fracture faces ($\widehat{F}_A$) are covered with many intramembranous particles, while the etched external faces (E_A) are either covered with irregular granular structures (Fig. 3a) or many small particles half the size of intramembranous particles (Fig. 4). Some of these irregular structures are seen located adjacent to the fracture surface and apparently continuous with intramembranous particles. Fracture $\widehat{F}_B$ faces, on the other hand, have distinct 9-nm or larger pits instead of textured surfaces and it appears that the protein molecules have been pulled out leaving behind holes (Fig. 3b). The texture of the unetched $\widehat{F}_B$ faces may be due to closely spaced unresolved pits. The etched external surfaces (E_B) are covered with many particles the size of intramembranous particles. It is apparent that we are dealing with two types of vesicle: right-side-out and inside-out vesicles. Indeed, by biochemical techniques, it has been shown that purified ATPase membrane fragments can reseal themselves in two different orientations [18]. Some are in the form of inside-out

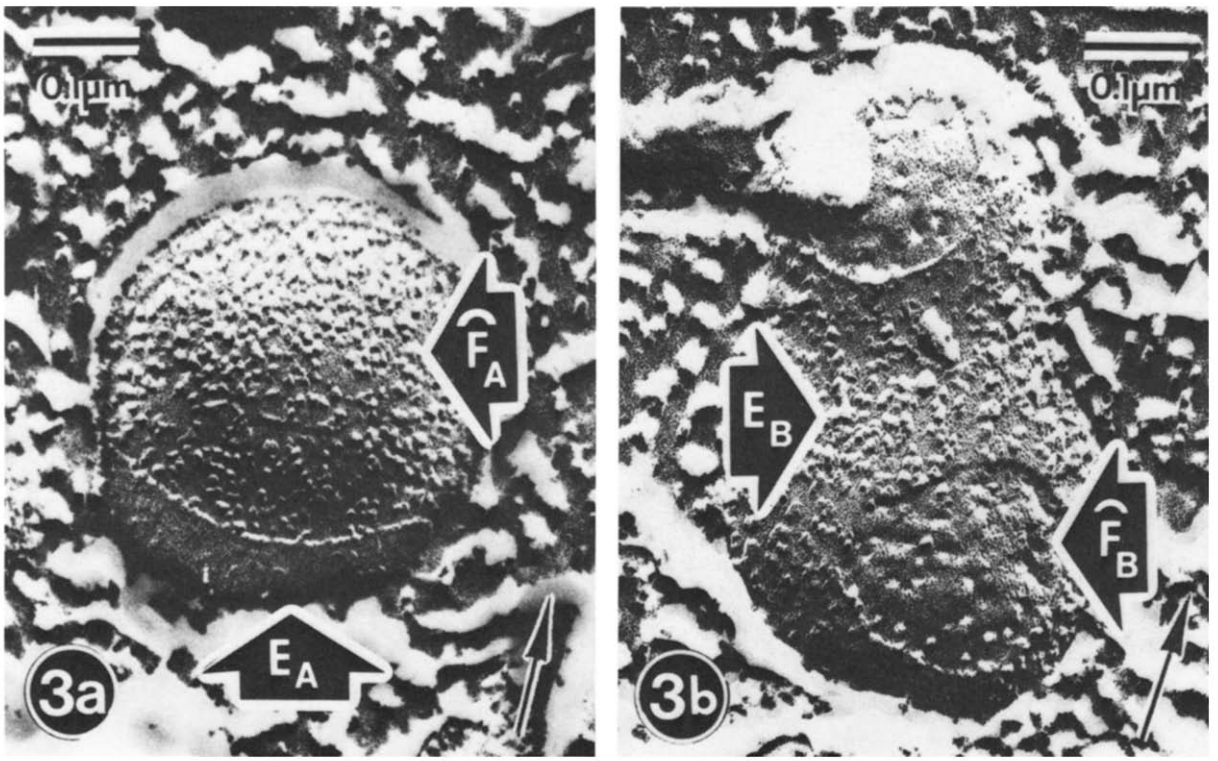

Fig. 3. Purified porcine renal ($Na^+ + K^+$)-ATPase after freeze-etching followed by unidirectionally shadowing at 45°. Fracture $\widehat{F}_A$ face shows many intramembranous particles and E_A shows granulated structures (a) while pits can be seen on the fracture $\widehat{F}_B$ face and intramembranous particles on etched E_B surface (b). The huge particulate structures in the background of the freeze-etch specimens were probably ice crystals. For details see text.

vesicles and some are in the form of right-side-out vesicles, as in the in vivo orientation. This might explain contradictory results in the distribution of intramembranous particles on both fracture faces [7,9].

In most cases, the fracture plane does not break the polypeptide chains of globular integral membrane proteins; instead, it deviates around them, leaving the whole molecule within one or the other fracture face [19,20] and pits on the corresponding half. As in the case of ($Na^+ + K^+$)-ATPase molecules, where there is a pronounced structural asymmetry in the molecule in that a large (about 5 nm) domain protrudes from the cytoplasmic surface and a small one (about 2 nm) is found on the external surface of the membrane [21], particles should be observed on one half of the fracture face, presumably on the cytoplasmic half, and pits on the corresponding external half. This is what we see in our freeze-etch results which confirms further that the majority of the protein mass is distributed on one side of the membrane.

To distinguish further the inside-out from the right-side-out vesicles as in vivo orientation, we used right-side-out resealed membrane vesicles to repeat the above freeze-etch experiments. Purified ATPase membranes after treatment with and removal of octylglucoside reseal themselves into vesicles. By biochemical analysis, 71% are tightly sealed right-side-out membrane vesicles and 13% are tightly sealed inside-out. The other 16% are leaky membrane vesicles of both orientations and small membrane fragments. Characterization of these membrane vesicles will be published elsewhere. Fig. 5 is a representative micrograph showing freeze-etch results of these vesicles. Most convex etched faces are covered with many distinct intramembranous particles, while the etched surfaces are either granulated or covered with particles half the size of the intramembranous par-

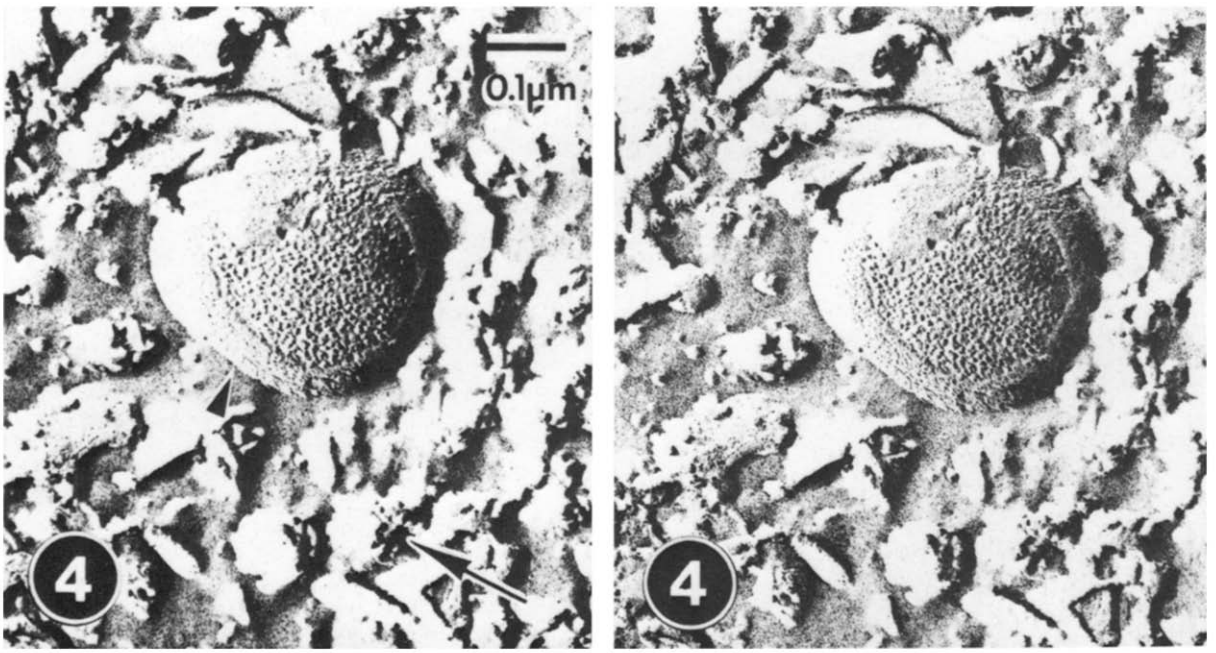

Fig. 4. Stereographic views of freeze-etched purified ($Na^+ + K^+$)-ATPase membrane vesicles. The etched external surface reveals many small particles half the size of intramembranous particles (arrow head).

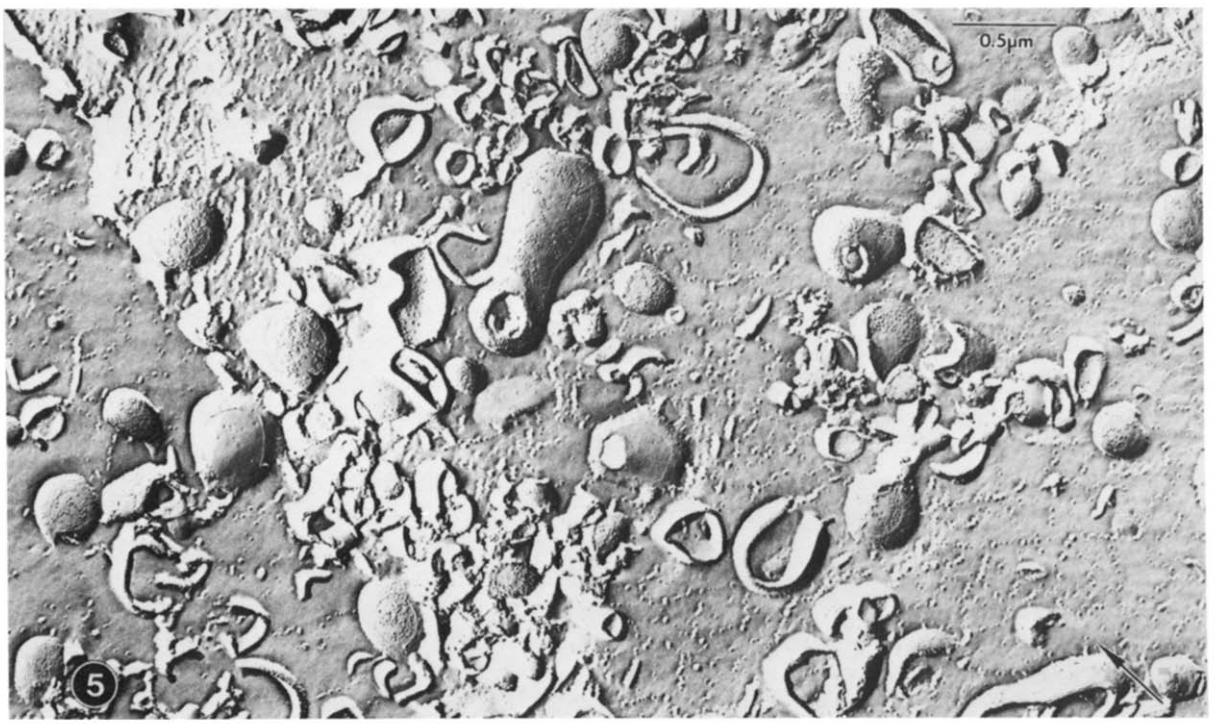

Fig. 5. Freeze-etch micrograph of purified ($Na^+ + K^+$)-ATPase membrane vesicles resealed with octylglucoside. The convex protoplasmic fracture faces are covered with intramembranous particles of 8.5–9.5 nm, while the etched external surfaces are granulated or covered with particles half the size of the intramembranous particles

TABLE I

COMPARISON OF $\widehat{F}_A$ AND $\widehat{F}_B$ FRACTURE FACES BEFORE AND AFTER OCTYLGLUCOSIDE TREATMENT

Purified membranes (1 mg/ml) were incubated in the imidazole/EDTA buffer containing 1 mg/ml octylglucoside for 60 min, pelleted, resuspended in imidazole/EDTA buffer and dialyzed against the same buffer for 18 h at 4 °C. Totally, 270 convex fracture faces were sampled.

	$\widehat{F}_A$ (%)	$\widehat{F}_B$ (%)
Before octylglucoside	46	54
After octyglucoside	82	18

ticles, characteristic of F_A fracture faces seen in Figs. 3a and 4. Table I shows a tabulation of $\widehat{F}_A$ and $\widehat{F}_B$ faces before and after treatment with octylglucoside. The convex fracture faces are primarily $\widehat{F}_A$ faces (82%) as compared to 18% $\widehat{F}_B$ faces. This is consistent with the biochemical data above that membrane vesicles are sealed mostly in one orientation (right-side-out) after octyglucoside treatment. Since these vesicles are sealed right-side-out, the convex fracture faces shown as $\widehat{F}_A$ faces are the protoplasmic fracture faces. Thus, the majority of the protein mass of $(Na^+ + K^+)$-ATPase is located on the cytoplasmic half of the membrane.

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References

1 Whittam, R. (1962) Biochem. J. 84, 110–118
2 Whittam, R. and Ager, M.E. (1964) Biochem. J. 93, 337–48
3 Perrone, J.R. and Blostein, R. (1973) Biochim. Biophys. Acta 291, 680–689
4 Kyte, J. (1974) J. Biol. Chem. 249, 3652–3660
5 Kyte, J. (1975) J. Biol. Chem. 250, 7443–7449
6 Ruoho, A. and Kyte, J. (1974) Proc. Natl. Acad. Sci. USA 71, 2352–2356
7 Deguchi, N., Jørgensen, P.L. and Maunsbach, A.B. (1977) J. Cell Biol. 75, 619–634
8 Haase, W. and Koepsell, H. (1979) Pflugers Arch. 381, 127–135
9 Maunsbach, A.B., Shriever, E. and Jørgensen, P.L. (1979) in Na, K-ATPase Structure and Function (Skou, J.C. and Norby, J.G., eds.), pp. 3–13, Academic Press, New York
10 Van Winkel, W.B., Lane, L.K. and Schwartz, A. (1976) Exp. Cell Res. 100, 291–296
11 Vogel, F., Meyer, H.W., Grosse, R. and Repke, K.R.H. (1977) Biochim. Biophys. Acta 470, 497–502
12 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
13 Forbush, B. (1983) Anal. Biochem. 128, 159–163
14 Sato, T. (1967) J. Electron Microsc. 17, 158–159
15 Costello, M.J. and Corless, J.M. (1978) J. Microsc. 112, 17–37
16 Costello,M.J. (1980) Scanning Electron Microsc. 2, 361–370
17 Costello, M.J., Fetter, R. and Hochli, M. (1982) J. Microsc. 125, 125–136
18 Jackson, R.L., Verkleij, A.J., Van Zoelen, E.J.J., Lane, L.K., Schwartz, A. and Van Deenen, L.L.M. (1980) Arch. Biochem. Biophys. 200, 269–278
19 Robertson, J.D. (1981) in Demyelinating Disease and Basic and Clinical Electrophysiology (Waxman, S.G. and Ritchie, J.M., eds.), pp. 419–477, Raven Press, new York
20 Pinto da Silva, D., Parkison, C. and Dwyer, N. (1981) Proc. Natl. Acad. Sci. USA 78, 343–347
21 Zampighi, G., Freytag, J.W. and Kyte, J. (1984) J. Cell Biol. 98, 1851–1964