Ultrastructure of Na,K-transport Vesicles Reconstituted with Purified Renal Na,K-ATPase

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ABSTRACT   To study the size and structure of the Na,K-pump molecule, the ultrastructure of phospholipid vesicles was examined after incorporation of purified Na,K-ATPase which catalyzes active coupled transport of Na^+ and K^+ in a ratio close to 3Na/2K. The vesicles were analyzed by thin sectioning and freeze-fracture electron microscopy after reconstitution with different ratios of Na,K-ATPase protein to lipid, and the ultrastructural observations were correlated to the cation transport capacity.

The purified Na,K-ATPase reconstituted with phospholipids to form a very uniform population of vesicles. Thin sections of preparations fixed with glutaraldehyde and osmium tetroxide showed vesicles limited by a single membrane which in samples stained with tannic acid appeared triple-layered with a thickness of 70 Å. Also, freeze-fracture electron microscopy demonstrated uniform vesicles with diameters in the range of 700–1,100 Å and an average value close to 900 Å. The vesicle diameter was independent of the amount of protein used for reconstitution. Intramembrane particles appeared only in the vesicle membrane after introduction of Na,K-ATPase and the frequency of intramembrane particles was proportional to the amount of Na,K-ATPase protein used in the reconstitution. The particles were evenly distributed on the inner and the outer leaflet of the vesicle membrane. The diameter of the particles was 90 Å and similar to our previous values for the diameter of intramembrane particles in the purified Na,K-ATPase. The capacity for active cation transport in the reconstituted vesicles was proportional to the frequency of intramembrane particles over a range of 0.2–16 particles per vesicle. The data therefore show that active coupled Na,K transport can be carried out by units of Na,K-ATPase which appear as single intramembrane particles with diameters close to 90 Å in the freeze-fracture micrographs.

Na,K-ATPase purified from the outer renal medulla in membrane-bound form (12) consists of membrane fragments that show surface particles after negative staining (19) and intramembrane particles after freeze-fracture (6). We have previously established a relationship between the sizes and the frequencies of these particles and the protein components of the purified Na,K-ATPase and proposed that the intramembrane particles represent an oligomer of the surface particles (6, 20).

Enzymatic and chemical studies of the purified renal Na,K-ATPase have provided evidence that its molecules are organized in units ($\alpha_2\beta_2$) of mol wt close to 280,000 consisting of two α-subunits with mol wt 104,000, and two sialoglycoproteins (β-subunit) with mol wt close to 40,000 (13, 14), but it is not known whether the Na,K pump in the cell membrane has the same structure. One way to examine both the structure and the function of the Na,K pump is to investigate the molecule after reconstitution into phospholipid vesicles, which show active coupled transport of sodium and potassium (1, 7, 9, 25). In the process of reconstitution, excess lipid is added and the frequency of intramembrane particles in the vesicle membrane could be expected to vary in proportion to the protein lipid ratio in the recombinant as in the vesicles reconstituted with band 3 protein (35). In this work we have reconstituted vesicles with different amounts of Na,K-ATPase and correlated the transport properties of the vesicles with the substructures observed in the vesicle membrane by electron microscopy.

Our results, parts of which have been reported in preliminary form (31), demonstrate that active Na,K transport is catalysed by protein units, which by freeze-fracture electron microscopy

appear as single intramembrane particles with a diameter of 90 Å.

MATERIALS AND METHODS

Preparation of Na,K-ATPase

Na,K-ATPase was purified from the outer medulla of pig kidney by incubation of a microsomal fraction with SDS and ATP followed by an isopycnic zonal centrifugation in a Ti 14 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The preparations had sp act of 28–36 μmol P_i/min·mg protein and the binding capacities for ATP or ouabain were in the range of 2.9–3.7 nmol/mg (15).

Preparation of Na,K-transport Vesicles

A modification of the previous procedure (1, 2) was employed. Transport vesicles were formed during dialysis of a mixture of phosphatidylcholine and Na,K-ATPase protein which were dissolved in buffered sodium cholate as follows: Aliquots of the purified Na,K-ATPase containing 1 mg of protein were solubilized at 20°C, in 660 μl of 1% sodium cholate in NaCl 30 mM, KCl 120 mM, $MgCl_2$ 5 mM, cysteine chloride 1 mM, EDTA 1 mM, imidazole 30 mM, pH 7.1 (buffer A). The suspension was centrifuged for 15 min at 100,000 g in a type 65 rotor (Beckman Instruments Inc., Spinco Div.). A provisional determination of the protein concentration in the supernate was obtained by measuring absorbance at 280 nm using samples of solubilized Na,K-ATPase with known protein concentrations as standards. Later, proteins were determined accurately by the Lowry method (18) after precipitation with trichloroacetic acid as before (12). The lipid suspension was prepared by drying L-α-phosphatidylcholine (type III-E, Sigma Chemical Co., St. Louis, Mo.) in hexane in a rotary evaporator (Rotavapor, Büchi, Switzerland). The lipid was washed twice with ether and suspended in 1% sodium cholate in buffer A to a concentration of 10 mg/ml.

Aliquots containing from 20 μg to ~700 μg of the solubilized Na,K-ATPase protein were mixed by shaking in a Whirlimixer (Fisons, Leichestershire, England) with 10 mg of lipid in 2 ml of buffer A with 1% cholate. Transport vesicles were formed during dialysis for 90 h at 8°–10°C against buffer A in 6-mm dialysis tubing (Union Carbide Corp., New York). Excluding preliminary experiments performed to test different preparatory procedures, transport properties and ultrastructure were studied in six different preparations with protein contents varying from 0 to 669 μg of protein/10 mg of lipid.

Determination of Cation Transport

For equilibration with isotope before transport assay, the vesicles were incubated in buffer A with either ^{22}Na (0.5 μCi/100 μl) or ^{42}K (16 μCi/100 μl) for 270 min at 24°C. Transport was started by adding 1/10 vol of ATP (Tris salt, Boehringer, Mannheim, W. Germany) to a final concentration of 3 mM, except in one experiment (Fig. 9, see below) where a low concentration of 100 μM ATP was used and 2 mM phosphenol pyruvate and pyruvate kinase 0.1 mg/ml were added to keep the ATP level constant. After 3–5 min, or at the times indicated in the graph, duplicate samples of 10–15 μl were layered on cooled (2°C) Sephadex G 50 columns (1 × 25 cm) and eluted with cooled (2°C) buffer A. The radioactivity eluting with the vesicles in the void volume was collected within 2–3 min and measured by counting in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The data were expressed as equivalents of cation transported per milligram of phospholipid or per milligram of protein.

Thin-section Electron Microscopy

For electron microscopy, the freshly prepared vesicles were concentrated either by centrifugation at 100,000 rpm for 30 min at 20°C in a Beckman Airfuge or by pressure dialysis in SM 13200 collodion bags mounted in an SM 16304 filter holder (Sartorius, Göttingen, W. Germany) using a vacuum of 10–20 mm Hg created by a water pump. Pellets of vesicles were divided into aliquots that were fixed at 4°C in different ways. Some were fixed with 1% osmium tetroxide in Veronal buffer, pH 7.2, for 0.5 h. Others were fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h and postfixed in 1% osmium tetroxide in the same buffer for 0.5 h. In addition, some aliquots were fixed in glutaraldehyde and osmium tetroxide in the same way and then rinsed thoroughly in 0.1 M cacodylate buffer and stained with 1% tannic acid in the same buffer for 0.5 h (29). The pellets were stained en bloc in 0.5% uranyl in Veronal buffer, pH 5.5, dehydrated in acetone, and embedded in Vestopal. Ultrathin sections were stained with lead citrate.

Freeze-fracture

Freeze-fracture was carried out in a Balzers freeze-fracture apparatus (BAF 300, Balzers AG, Lichtenstein). Pellets prepared as described above were resuspended and the vesicles were equilibrated with glycerol to a final concentration of ~20% vol/vol glycerol in buffer A for 2–3 h at 0°–2°C. Vesicle aliquots were frozen quickly in Freon 22 cooled by liquid nitrogen. The specimens were fractured at −100°C and immediately shadowed with platinum at an angle of 45° and replicated with carbon. After cleaning in sodium hypochlorite, the replicas were analyzed in a Jeol JEM 100 B electron microscope at an accelerating voltage of 80 kV.

Quantitative Analysis of Electron Micrographs

The vesicle preparations were analyzed with respect to their ion transport properties and by electron microscopy to determine the vesicle diameter and the frequency of intramembrane particles in the freeze-fracture replicas.

ELECTRON MICROGRAPHS: Random micrographs were taken within regions of the replicas where the true shadow angle was ~45°. To locate such regions, the lengths of the shadows in concave fracture faces were observed. When the shadow angle is 45°, the longest shadows are present in equatorially fractured vesicles and reach the center of concave fracture faces. The shadows exceed the centers of concave fracture faces when the true shadow angle is below 45°, and no shadows reach the centers of concave fracture faces when the true shadow angle is above 45°. Micrographs for quantitative analysis were taken at an initial magnification of 50,000 and enlarged three times for determination of vesicle diameter and particle density. Calibration was carried out by means of a carbon grating replica (2,160 lines per millimeter).

DIAMETER OF PHOSPHOLIPID VESICLES: The profiles of essentially all phospholipid vesicles in replicas were round (see Results). In the following analysis, we therefore assume that the vesicles are spherical and that the true diameter of a fractured vesicle is obtained when the fracture plane hits the equatorial plane of the vesicle. This fracture can follow either of the two halves of the vesicle membrane. The diameters of concave faces from equatorially fractured vesicles were identified and measured on profiles where the shadow reached the center of the fracture face (17, 33). The diameters of convex faces from equatorially fractured vesicles were identified and measured on those vesicles where the shadow reached approximately one fifth of the vesicle diameter outside the periphery, as the shadow of a convex fracture face of an equatorially fractured vesicle reaches $r(\sqrt{2} - 1)$ beyond the perimeter of a vesicle with a radius r where the shadowing angle is 45°. The diameters of both concave and convex fracture faces were measured at right angles to the direction of shadowing.

PARTICLE DENSITY: Particle densities were determined on the electron micrographs with the aid of a hexagon with a side of 2 mm (corresponding to an area of 4.6 × 10^{-4} μm^2 on the specimen when the magnification was 150,000). The hexagon had extended exclusion lines and was surrounded by a steering circle and was placed in the center of all convex or concave fracture faces which were fractured and shadowed in such a way that the edge of the platinum shadow passed outside the hexagon, thus ensuring that all intramembrane particles located within the hexagon were demonstrable. Particles that were totally enclosed and those solely intersecting the upper half were counted; those intersecting the lower half of the hexagon or its exclusion lines were rejected (8). The frequency of particles per square micrometer was determined for convex and concave fracture faces. The particle density in the reconstituted vesicle membrane was taken to be the sum of these particle densities. The number of intramembrane particles per phospholipid vesicles was calculated from the frequency of particles per square micrometer and the mean value of the diameter of the vesicles in the preparation.

DIAMETER OF INTRAMEMBRANE PARTICLES: The diameter of all intramembrane particles was measured on micrographs with a final magnification of 200,000. All particles except those touching the edge of the fracture face of the vesicle were measured. Each division of the measuring scale corresponded to 5 Å on the electron micrographs.

RESULTS

Ultrathin Sectioning

Electron microscopy of thin sections of the vesicle pellets demonstrated a very uniform population of vesicles (Fig. 1). The diameter of the vesicles was ~900 Å when they were cut through their largest diameter. Amorphous or multilamellar lipid material was only occasionally observed. The vesicles were limited by a single membrane which in preparations
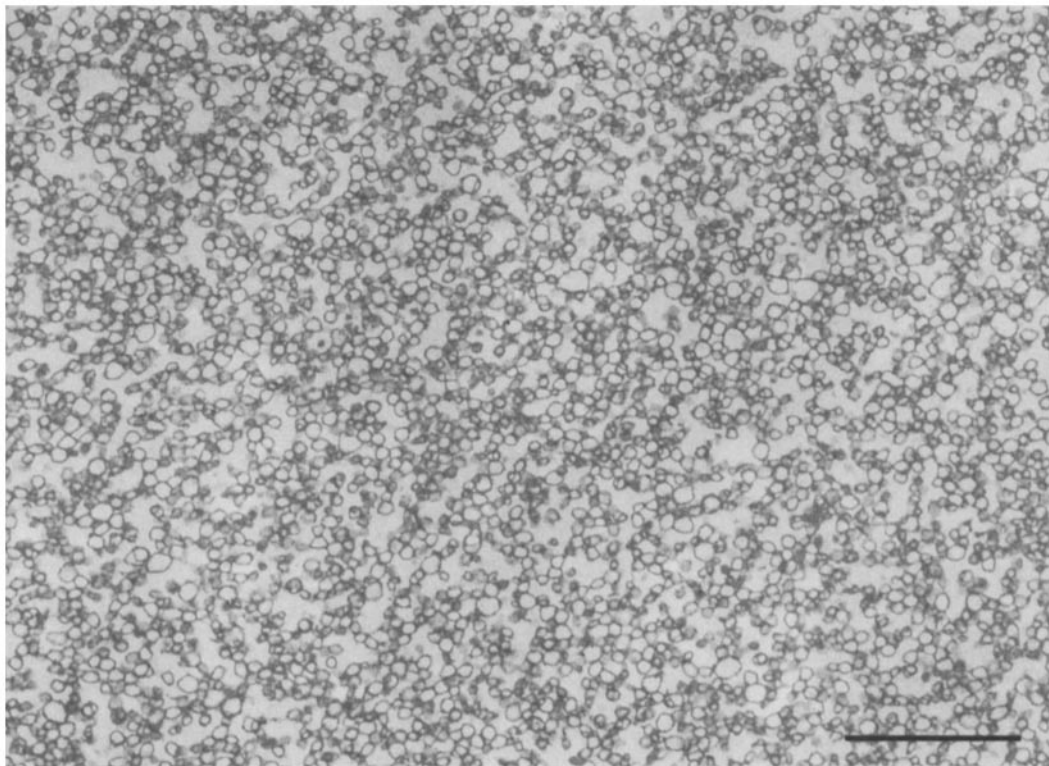
FIGURE 1 Electron micrograph of thin section from pellet of phospholipid vesicles reconstituted in the presence of Na,K-ATPase and fixed in sequence with glutaraldehyde and osmium tetroxide. The pellet consists of a uniform population of vesicles. Bar, 1 μm. × 23,000.

treated with tannic acid had an approximate thickness of 70 Å and appeared triple-layered (Fig. 2).

Freeze-fracture

Freeze-fracture of the reconstituted vesicles showed a uniform population of fractured membrane faces which were either convex or concave. As seen in Figs. 3 and 4, the shadows on both fracture faces were of different lengths, indicating that the vesicles were fractured at various levels in relation to their largest diameter ("equator"). Convex profiles of the vesicles represent the inner leaflets and concave profiles the outer leaflets of the vesicle bilayer, in agreement with a split in the hydrophobic region (3, 24). The ratio between the total number of convex and total number of concave fracture faces was in six preparations close to unity (0.98 ± 0.08 [SD]), in agreement with the concept that a convex profile is formed when the fracture plane hits the vesicle above the equator and a concave fracture face is formed when the fracture plane hits the vesicle below the equator (33).

The diameters of most vesicles fractured at their equatorial plane fell in the range of 700–1,100 Å whether or not Na,K-ATPase was added during reconstitution. In all six preparations the mean diameters of concave fracture faces were smaller than those of convex fracture faces (Table I). The average diameter of convex fracture faces in the six preparations was 919 ± 164 Å (SD) and the average diameters for concave fracture faces was 867 ± 167 Å (SD). Thus, the concave faces were on the average 52 ± 9 Å (SEM, $n = 6$) smaller in diameter than the convex faces. The paired t test showed that this difference was significant ($2P < 0.005$). Only occasionally were vesicles with
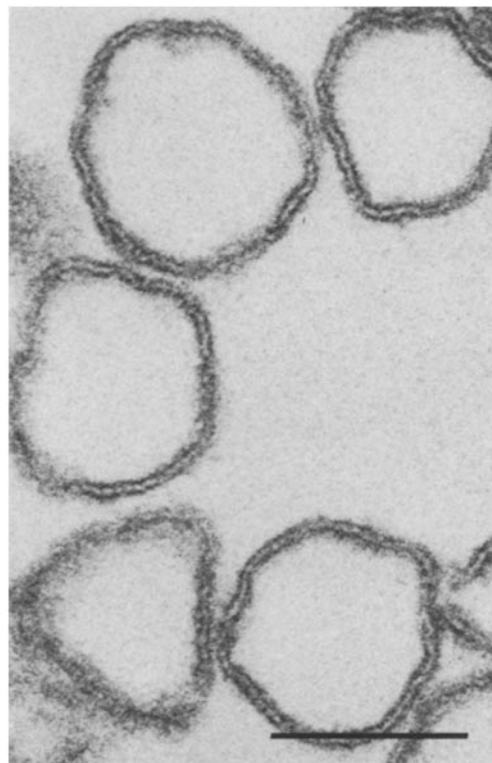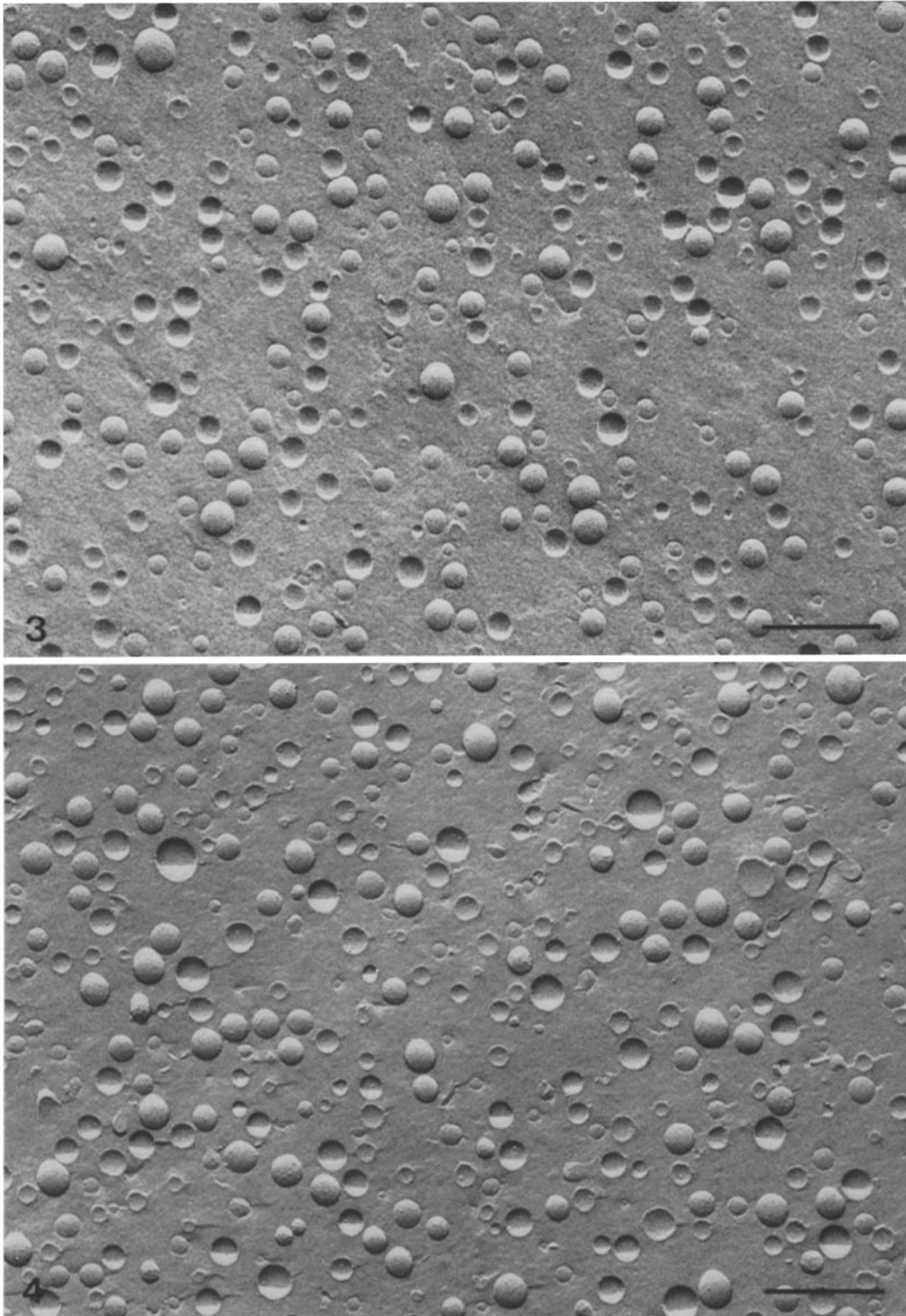

FIGURE 2 Higher magnification of the same preparation as shown in Fig. 1, but stained with tannic acid after glutaraldehyde and osmium tetroxide fixation. The membranes of the vesicles are triple-layered. Bar, 0.1 μm. × 260,000.

FIGURES 3 and 4   Electron micrographs of freeze-fracture replicas of phospholipid vesicles showing uniform populations of vesicles. The vesicles in Fig. 3 were reconstituted without enzyme, while those shown in Fig. 4 were reconstituted in the presence of Na,K-ATPase. The distribution and dimensions of the vesicles are similar. Direction of shadowing from below. Bars, 0.5 μm. × 36,000.

diameters of 2,000–3,000 Å observed. The range of diameters was the same in vesicle samples concentrated by dialysis and by centrifugation, except that an additional population of small vesicles with diameters of ~300 Å and a total volume of <1%

of the total vesicle volume were observed in preparations concentrated by pressure dialysis.

Phospholipid vesicles reconstituted without Na,K-ATPase were devoid of intramembrane particles (Figs. 3 and 5). When

TABLE I

Ultrastructural Properties of Phospholipid Vesicles Reconstituted with Different Amounts of Na,K-ATPase

Protein/lipid	Diameter (Å)		Particle density (particles/μm^2)		Particles per vesicle	
	Convex	Concave	Convex	Concave	Observed	Calculated
$\mu g/\mu g \cdot 10^4$						
0	944 ± 98 (110)	899 ± 96 (111)	0 (338)	0 (317)	0	0
20	823 ± 160 (315)	804 ± 128 (283)	3.4 (630)	5.4 (410)	0.2	0.5
275	921 ± 172 (104)	857 ± 150 (101)	116 (539)	86 (577)	5	8
359	891 ± 177 (128)	846 ± 227 (100)	144 (350)	109 (384)	6	10
548	962 ± 254 (124)	878 ± 204 (104)	241 (546)	321 (573)	15	15
669	971 ± 120 (101)	915 ± 198 (106)	237 (634)	329 (597)	16	18

Values are given as mean ± SD. Numbers in parentheses represent numbers of vesicle profiles, on which the diameter or particle density was determined.

reconstitution was carried out in the presence of Na,K-ATPase proteins, intramembrane particles were observed on both convex and concave fracture faces. The particles were equally distributed between convex and concave fracture faces because the average ratio between particle frequencies on convex and particle frequencies on concave fracture faces was 0.95 ± 0.30 (SD) in the five preparations. The particles occurred as single particles and only rarely were two or more particles located close to one another (Fig. 6). The average particle diameter was 90 ± 15 Å (SD) (Fig. 7). This size and also the shape of the particles were similar to those seen previously in membranes of purified Na,K-ATPase.

Particle frequencies obtained when applying the test area on all suitably shadowed fracture faces in a series of electron micrographs from each experiment are shown in Table I. The total number of particles per square micrometer of vesicle membrane is obtained by addition of the particle frequencies for convex and concave fracture faces. The total number of particles per vesicle was derived from the number of particles per square micrometer of vesicle membrane, using the average diameter for each preparation (Table I). The particle frequencies (particles per square micrometer of membrane) as well as the absolute number of particles per vesicle showed a striking correlation with the protein lipid ratio of the preparations (Fig. 8 and Table I).

Active Ion Transport

After addition of ATP to the incubation medium, sodium ions from the medium were accumulated inside the vesicles and potassium ions were extruded from the vesicle interior to the medium (Fig. 9). At the low protein concentration the content of $Na^+(^{22}Na)$ in the intravesicular fluid was increased and the content of $K^+(^{42}K)$ was decreased almost linearly during the first 5 min of incubation with ATP. The Na:K transport ratio calculated from the data after 0.5–5 min of incubation with ATP was 2.6–4.9 Na/2K and close to the previously observed ratio of 3Na/2K (1, 7, 9).

Active Na^+ transport was not present in phospholipid vesicles reconstituted without Na,K-ATPase, but was already demonstrable in a preparation with an average of 0.2 particle per vesicle which was obtained after reconstitution with a protein lipid ratio of 0.002 (Fig. 10). The rate of active Na^+ transport per milligram of lipid was increased in proportion to the intramembrane particle frequency in the range from 0.2 to 16 particles per vesicle. The correlation between particle frequency and active transport thus shows that the rate of active cation transport per intramembrane particle was about the same in

this series of five different preparations. The proportionate increase in the rate of active cation transport per vesicle with the increase in protein lipid ratio of the recombinant is therefore caused by the higher particle frequency of Na,K-pump molecules per unit membrane area and not to a higher turnover rate per pump site.

The rate of active Na^+ uptake per milligram of protein was 0.071–0.082 μmol Na^+/min per mg protein in four preparations, but was lower, 0.024 μmol Na^+/min per mg protein, in the preparation with an average content of 0.2 particle per vesicle. This may be caused by a less efficient incorporation of the protein into the vesicle at the very low protein lipid ratio of 0.002 (Table I). These transport rates were compared to the rate of ATP splitting by the purified Na,K-ATPase before reconstitution. In the conditions of the transport assay, at 24°C, pH 7.1 and a high K/Na ratio, the sp act of purified Na,K-ATPase was only 0.43 μmol P_i/min per mg protein as compared to 34 $\mu mol/min$ per mg protein in optimum conditions at 37°C. Assuming random distribution of the Na,K-pump molecules in the vesicle membrane and a transport stoichiometry of 3Na/ATP, the Na^+ transport across the vesicle membrane of 0.071–0.082 $\mu mol/min$ per mg protein corresponds to a rate of ATP hydrolysis which is 11–13% of the turnover rate of the purified Na,K-ATPase in vitro.

DISCUSSION

Ultrastructure of Reconstituted Vesicles

The present observations clearly demonstrate that the purified Na,K-ATPase reconstitutes with phospholipids to form a very uniform population of vesicles. It is important that both freeze-fracture and thin-section electron microscopy showed that the vesicles were limited by a single membrane and that their variability in size was limited. Thus, from an ultrastructural point of view these vesicles appear suitable for studies of ion transport processes.

The intravesicular volume is an important factor for measurements of cation transport, as the relatively small intravesicular pool of ions may be rate limiting for the coupled Na,K-transport process. It is therefore noteworthy that the diameter of reconstituted phospholipid vesicles depends upon the method used for reconstitution. Thus, phospholipid vesicles reconstituted by dialysis, as in this work, are comparatively large, whereas vesicles reconstituted with other proteins by sonication procedures are smaller with diameters of ~300 Å (22, 32). After sonication and freeze-thaw, a considerable variation in size has been observed (16). Ultrastructural studies of
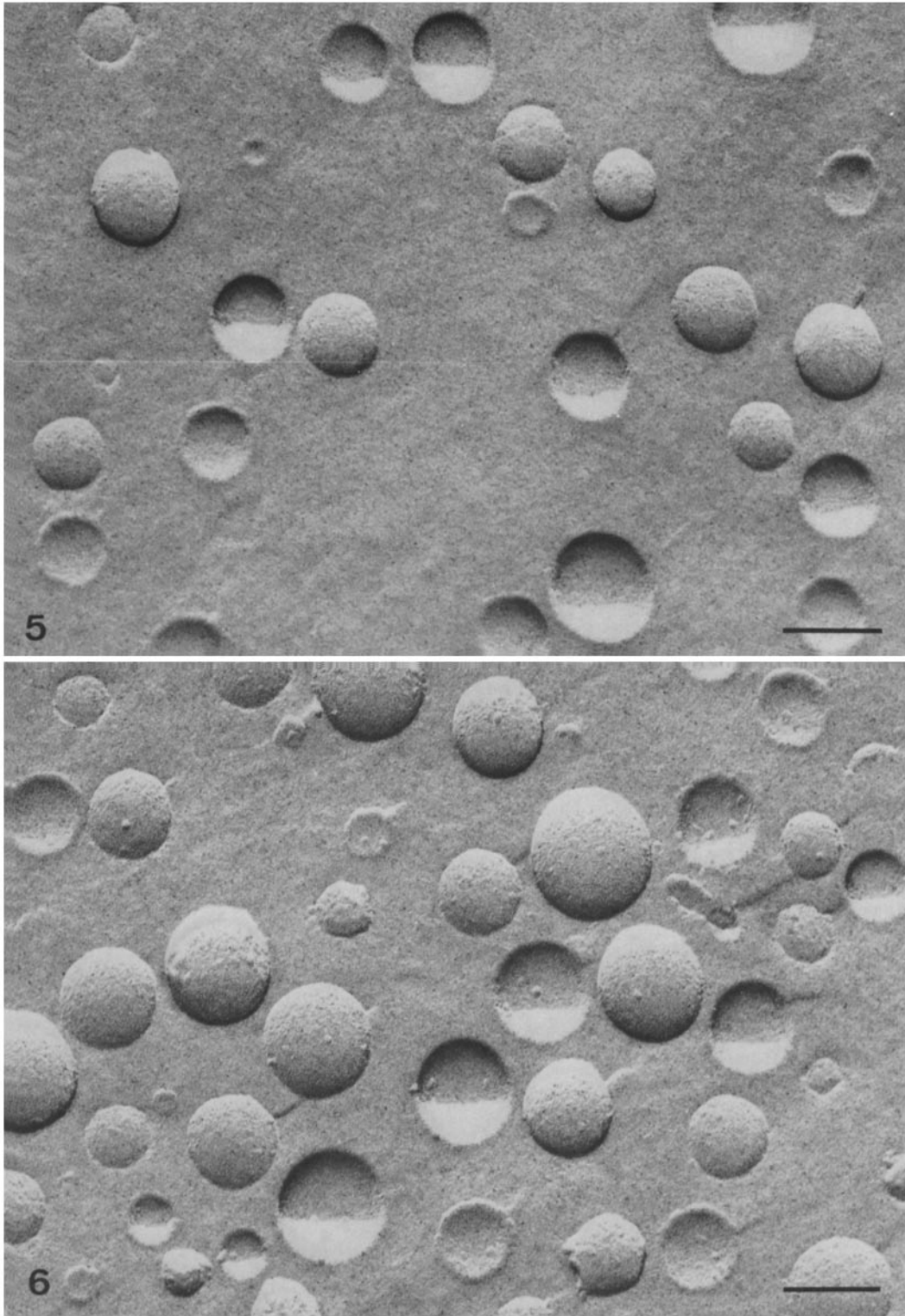
FIGURES 5 and 6   Electron micrographs of freeze-fracture replicas showing convex and concave fracture faces. The vesicles in Fig. 5 were reconstituted without enzyme and exhibit no intramembrane particles. The vesicles in Fig. 6 were reconstituted with Na,K-ATPase (protein lipid ratio $669 \cdot 10^{-4}$) and show occasional intramembrane particles on both convex and concave fracture faces. Direction of shadowing from below. Bars, 0.1 μm. × 150,000.

vesicles reconstituted with Na,K-ATPase capable of ion transport have not appeared before, but the diameter of Na,K-transport vesicles was estimated by gel chromatography to be 400–600 Å (7). Vesicles reconstituted with cytochrome oxidase which have grown from 300 Å to diameters in excess of 1,000

Å by calcium-induced fusion seem to lose the ability to fuse further (22). Comparison with these data suggests that the size of our vesicles is close to a maximum value and that the diameters of our vesicles vary within a relatively narrow range (700–1,100 Å).
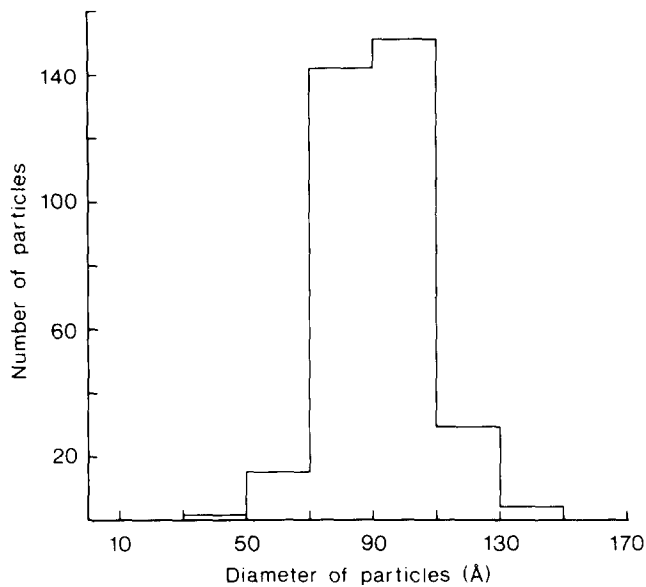
FIGURE 7 Distribution of diameters of intramembrane particles in phospholipid vesicles reconstituted with Na,K-ATPase. The total number of particles was 342.

evaporated on the convex fracture face (e.g., the inner half of the vesicle membrane) increases the diameter of the convex fracture face. In contrast, Bullivant (5) found in complementary replicas that the concave faces were larger in diameter than the convex faces and ascribed this to lipid collapse. We can offer no explanation for this apparent discrepancy, but note that both the origin and composition of the vesicles and the measuring procedures were different.

Ultrastructure and Significance of Intramembrane Particles

The particles appear in the vesicle membrane only after introduction of the purified Na,K-ATPase protein into the recombinant. This together with the proportionality between the frequency of intramembrane particles and the Na,K-ATPase protein lipid ratio in the reconstitution medium represent strong arguments for the concept that the intramembrane particles reflect the presence of Na,K-ATPase in the membranes. It has been demonstrated in several experimental systems that intramembrane particles appear on fracture faces only after incorporation of protein in the membranes (11, 21,
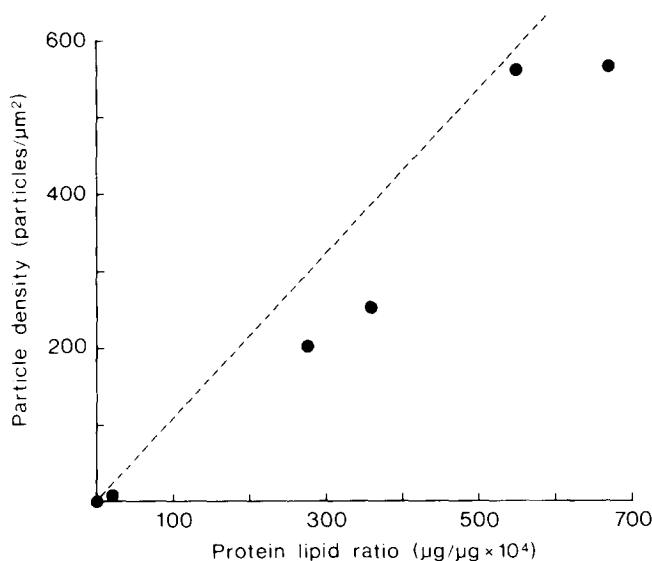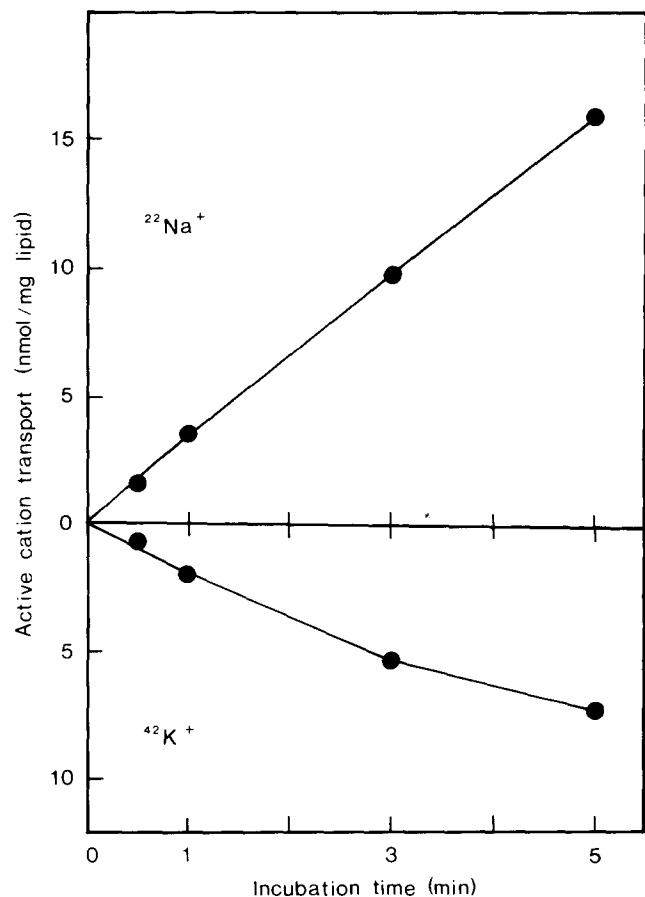

FIGURE 8 Correlation between frequency of intramembrane particles and protein lipid ratio in phospholipid vesicles reconstituted with different amounts of Na,K-ATPase. The dotted line indicates the particle density that can be calculated from the protein lipid ratio, assuming that the particle has a mol wt of 280,000, that the protein is inserted in a lipid bilayer as in the fluid mosaic membrane model (30), and that the efficiency of reconstitution is 100%.

In our preparations the diameter of the reconstituted vesicles was independent of the amount of protein used for the reconstitution (Table I). In studies of vesicles reconstituted with other proteins at higher protein lipid ratios than in our work, the diameter of the vesicles has been noted to increase with the amount of incorporated protein (27, 34).

The average diameter of concave fracture faces in all our preparations was smaller than the average diameter of the convex fracture faces. This difference is consistent with the explanation that the metal evaporated on the concave fracture surface (e.g., the outer half of the membrane) decreases the measured diameter of the concave fracture face and that metal

FIGURE 9 Transport rates for Na$^+$ and K$^+$ at 24°C in vesicles containing purified Na,K-ATPase. Sodium transport was measured with ^{22}Na and potassium transport with ^{42}K in a vesicle preparation containing 22 μg protein per mg lipid. After equilibration with isotope, ATP was added at time zero and transport was measured as described in Materials and Methods. ATP concentration was kept constant at 100 μM by adding 2 mM phosphoenol pyruvate and pyruvate kinase, 0.1 mg/ml. Transport was calculated from the difference in radioactivity between vesicles incubated with ATP and vesicles incubated without ATP.
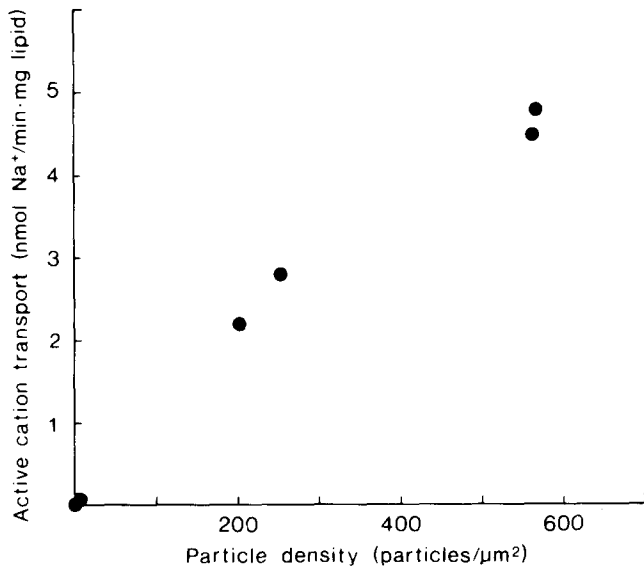
FIGURE 10 Correlation between frequency of intramembrane particles and active sodium transport in phospholipid vesicles reconstituted with Na,K-ATPase. Transport was measured as described in Materials and Methods, and active sodium transport was calculated from the difference in radioactivity between vesicles incubated with ATP and vesicles incubated without ATP.

The y-axis is labeled "Active cation transport (nmol Na$^+$/min·mg lipid)" and the x-axis is labeled "Particle density (particles/µm^2)".

26). A proportionality between the protein lipid ratio and the frequency of intramembrane particles in reconstituted membranes has been demonstrated by Yu and Branton (35) in recombinants of erythrocyte band 3 protein and lipid.

The diameter of the intramembrane particles in the reconstituted phospholipid vesicles was 90 Å which is quite similar to our previous values for intramembrane particles in preparations of purified Na,K-ATPase in membrane-bound form (6, 20). The similarity in dimensions indicates that the intramembrane particles in the reconstituted vesicles and the purified membranes represent the same protein unit. On the basis of dimensions and frequencies of intramembrane particles in purified Na,K-ATPase membranes of known composition, we have proposed that the intramembrane particles represent a protein unit with a mol wt of 280,000 ($\alpha_2\beta_2$) which contains two α-subunits with a mol wt close to 100,000 and two sialoglycoproteins (β-subunits) (6, 13, 15). When this unit is used in the calculation, the total number of particles per unit area of vesicle membranes in the present experiments also corresponds rather well with the calculated density of particles which was obtained from the protein lipid ratio (Fig. 8). As the rate of active coupled Na,K-transport in the reconstituted vesicles was shown to be proportional to the frequency of intramembrane particles over a range of 0.2–16 particles per vesicle, our data show that the intramembrane particles in the vesicle membrane correspond to Na,K-pump molecules. An additional inference from the relationship between the observed and calculated particle frequencies is that the reconstitution procedure is very efficient. Nearly all Na,K-ATPase molecules added to the recombinant appear as intramembrane particles in the membrane of the vesicles. It is noteworthy that the intramembrane particles almost exclusively appear as single particles and never as clusters. This demonstrates that active ion transport can be carried out by enzyme units which appear as single particles in the freeze-fracture images. It remains to be discussed to what extent conclusions from observations on the vesicles can be

extrapolated to the properties of the Na,K pump in the kidney cell membrane.

Inferences of structural similarities between the reconstituted Na,K pump and the native Na,K pump in the kidney cell membrane can only be drawn if it can be shown that the properties of the two pump systems are identical. The comparison of properties of the two pump systems is hampered by the lack of knowledge about the transport mode of the Na,K pump in the kidney cell. Also, the quantitative comparison is difficult as the turnover rate of the Na,K pump in the intact kidney cell is unknown. The turnover rate of the purified Na,K-ATPase relates well to the turnover rate of the Na,K pump in intact erythrocytes, HeLa and muscle cells (for reference 14). As the turnover rate of the Na,K pump reconstituted in the vesicles is 15% of the turnover rate of that in purified Na,K-ATPase, it seems that the turnover rate of the reconstituted Na,K pump is severalfold lower than the rate which prevails in intact cells. The reduced turnover rate of the reconstituted Na,K pump may in part be ascribed to detergent inactivation during the cholate dialysis procedure as a higher turnover rate has been reached in vesicles prepared by the sonication procedure (10). Different degrees of perturbation of the proteins of the pump or removal of lipids or other factors required for activity could also explain the reduction of the rate of active transport in the reconstituted systems.

Freeze-fracture of native membranes demonstrates an asymmetrical distribution of intramembrane particles (4). Also, isolated membranes from the sarcoplasmic reticulum (23) or Na,K-ATPase purified in membrane-bound form (6) reveal one particle-rich and one particle-poor fracture face. This asymmetry is evidence for specific orientation of the integral protein molecules in the membrane. In phospholipid vesicles reconstituted with Na,K-ATPase, the intramembrane particles are evenly distributed on the inner and the outer leaflet of the vesicle membrane. This loss of asymmetry suggests that the Na,K-ATPase molecules are inserted randomly into the vesicle membrane. This interpretation is consistent with observations on the Ca-ATPase reconstituted from sarcoplasmic reticulum (23, 28). Thus, only half of the Na,K-pump molecules expose their ATP site to the medium and catalyse active transport which is blocked by vanadate (2). The other half expose their extracellular aspects with the ouabain-binding sites to the medium, and ATP is prevented from access to the substrate site of these molecules.

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