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CHARACTERIZATION OF ($Na^+ + K^+$)-ATPase LIPOSOMES

I. EFFECT OF ENZYME CONCENTRATION AND MODIFICATION ON LIPOSOME SIZE, INTRAMEMBRANE PARTICLE FORMATION AND Na^+,K^+-TRANSPORT

BEATRICE M. ANNER [a], J. DAVID ROBERTSON [b] and H. PING TING-BEALL [b]

[a] *Department of Pharmacology, Centre Médical Universitaire, CH-1211 Geneva 4 (Switzerland) and* [b] *Department of Anatomy, Duke University Medical Center, Durham, NC 27710 (U.S.A.)*

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Rabbit renal ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) was purified and incorporated into phosphatidylcholine liposomes. Freeze-fracture analysis of the reconstituted system reveals intramembrane particles formed by ($Na^+ + K^+$)-ATPase molecules which are randomly distributed on concave and convex fracture faces. The reconstituted ($Na^+ + K^+$)-ATPase performs active Na^+,K^+-transport. The distribution of particles as well as the rate of active transport are directly proportional to the ($Na^+ + K^+$)-ATPase protein concentration used for reconstitution, while the total amount of sodium and potassium ions exchanged by ATP per volume vesicle suspension reaches maximum when each vesicle contains on the average more than two particles. ($Na^+ + K^+$)-ATPase pretreated with ouabain or vanadate yields the same particle density and vesicle size as control enzyme. However, detergent-denatured enzyme loses its ability to form intramembrane particles or to increase the vesicle size indicating that the lipids surrounding the protein part of the molecule are essential for the reconstitution process. The vesicle diameter increases as a function of the number of particles per vesicle. Histograms of the size distribution become wider with increasing intramembrane particle density and tend to show more than one maximum.

Introduction

The purified sodium pump (($Na^+ + K^+$)-ATPase) molecule of cell plasma membranes is a complex of protein (a 90000–120000 mol. wt. α subunit and a 35000–60000 mol wt. β subunit), phospholipids, and cholesterol [1–3]. The isolated pump molecule can be incorporated into artificial vesicular bilayers (liposomes) to study its transport and receptor function [4–7] or into planar bilayers via fusion of well characterized ($Na^+ + K^+$)-ATPase liposomes to analyze its channel activity [8].

Membrane model systems containing functional sodium pumps are becoming a useful tool for biochemical and pharmacological research. Therefore, we found it important to characterize different ($Na^+ + K^+$)-ATPase liposome preparations with regard to their structure, composition and function. We systematically determined the transport pattern of reconstituted native ($Na^+ + K^+$)-ATPase that has been chemically modified before reconstitution, measured the size distribution of the reconstituted vesicles and counted the number of incorporated enzyme molecules (intramembrane particles) per vesicle.

Materials and Methods

Purified ($Na^+ + K^+$)-ATPase was obtained from the outer medulla of rabbit kidneys by the

sodium dodecyl sulfate (SDS) procedure [9]. Purified egg phosphatidylcholine (PC) was purchased from Supelco. The other chemicals were analytical grade products from Merck.

Incorporation of ($Na^+ + K^+$)-ATPase into liposomes. The reconstitution procedures has been previously described [4–6] and a reproducible cholate-dialysis method was published elsewhere in detail [10]. Electron microscopy of the liposome suspension revealed a homogeneous population of single-walled vesicles [11,12]. For the present experiments, pure ($Na^+ + K^+$)-ATPase at concentrations ranging from 1 to 6 mg per ml was first treated with a 1% cholate solution to solubilize about 50% of the enzyme protein in active form. Only this $100\,000 \times g$ supernatant enzyme was used for reconstitution. Analysis of the supernatant enzyme by SDS-polyacrylamide gel electrophoresis showed that the subunits of the ($Na^+ + K^+$)-ATPase presented about 90% of the Coomassie blue stained polypeptides.

Transport measurements. Details of the transport measurements were essentially described in previous publications [13,14]. The externally added ATP activates the inside-out oriented ($Na^+ + K^+$)-ATPase molecules so that sodium ions are pumped into the vesicles and potassium ions are extruded [4,5]. These active as well as the passive ion movements were followed by using tracer, ^{22}Na or ^{86}Rb. It has been previously demonstrated that ^{86}Rb can be used as a convenient tracer for K^+ fluxes in this system [13,15–17]. The liposomes were prepared in a solution containing 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 30 mM imidazole, 1 mM EDTA, 1 mM cysteine, pH 7.10. The intravesicular radioactivity was determined by filtering the liposomes through 12 cm long, ice-cooled Sephadex G-50 colums. The protein concentration was determined according to the Lowry procedure [18].

Morphology. For electron microscopy, aliquots of the liposomes used for transport studies were transferred to 1.7 ml Eppendorf plastic tubes which were carefully sealed with several layers of Parafilm and were packed into a Thermo-Container packet (Dispolab, Regensdorf, Switzerland) kept cold without freezing during transit to Duke University arriving within 2 to 3 days. From transport studies we know that the ($Na^+ + K^+$)-ATPase liposomes are stable for several days even at room temperature.

For freeze-fracture experiments small samples (0.1 μl) of concentrated suspensions of vesicles were sandwiched between two copper strips and frozen in liquid propane at $-190°C$ using the dropping device developed by Costello and Corless [19]. Frozen samples were loaded in a specially designed hinged device [20] under liquid N_2 and then transferred to the precooled stage of a Balzers BA-360 freeze-fracture device. Samples were fractured at $-150°C$ and about 10^{-7} Torr and immediately replicated with Pt/C at a 45° angle and carbon from a 90° angle. Replicas were cleaned with 'Clorox' and picked up on uncoated 400 mesh copper grids. All specimens were examined in a Philips 301 electron microscope at 80 kV. We designate the convex and concave fracture faces, respectively, as convex and concave fracture faces. These notations are not used for living cells [21].

The vesicle diameter was measured perpendicularly to the shadowing direction. We are well aware that these measurements do not represent absolute vesicle diameters since the plane of fracture may occur asymmetrically. Hence the diameters will be underestimated if a simple average is taken. Nevertheless, in the present study we systematically measured the apparent average vesicle diameters and used these figures in a strictly comparative manner in order to quantify gross differences in size distribution of different liposome preparations. 200 concave and 200 convex fracture faces were analyzed for size at 75 000- to 102 000-fold magnification. The number of particles was counted on the same fracture faces. The vesicle diameter was measured in mm and converted to ångstrom units only after the statistical analysis in order to be able to calculate with a minimal number of positions.

Results

($Na^+ + K^+$)-ATPase concentration, intramembrane particles and vesicle size

Liposomes were reconstituted in the absence of ($Na^+ + K^+$)-ATPase protein or with protein concentrations ranging from 1 to 6 mg/ml. For convenience, the transport and the electron microscopic results were related to the initial protein

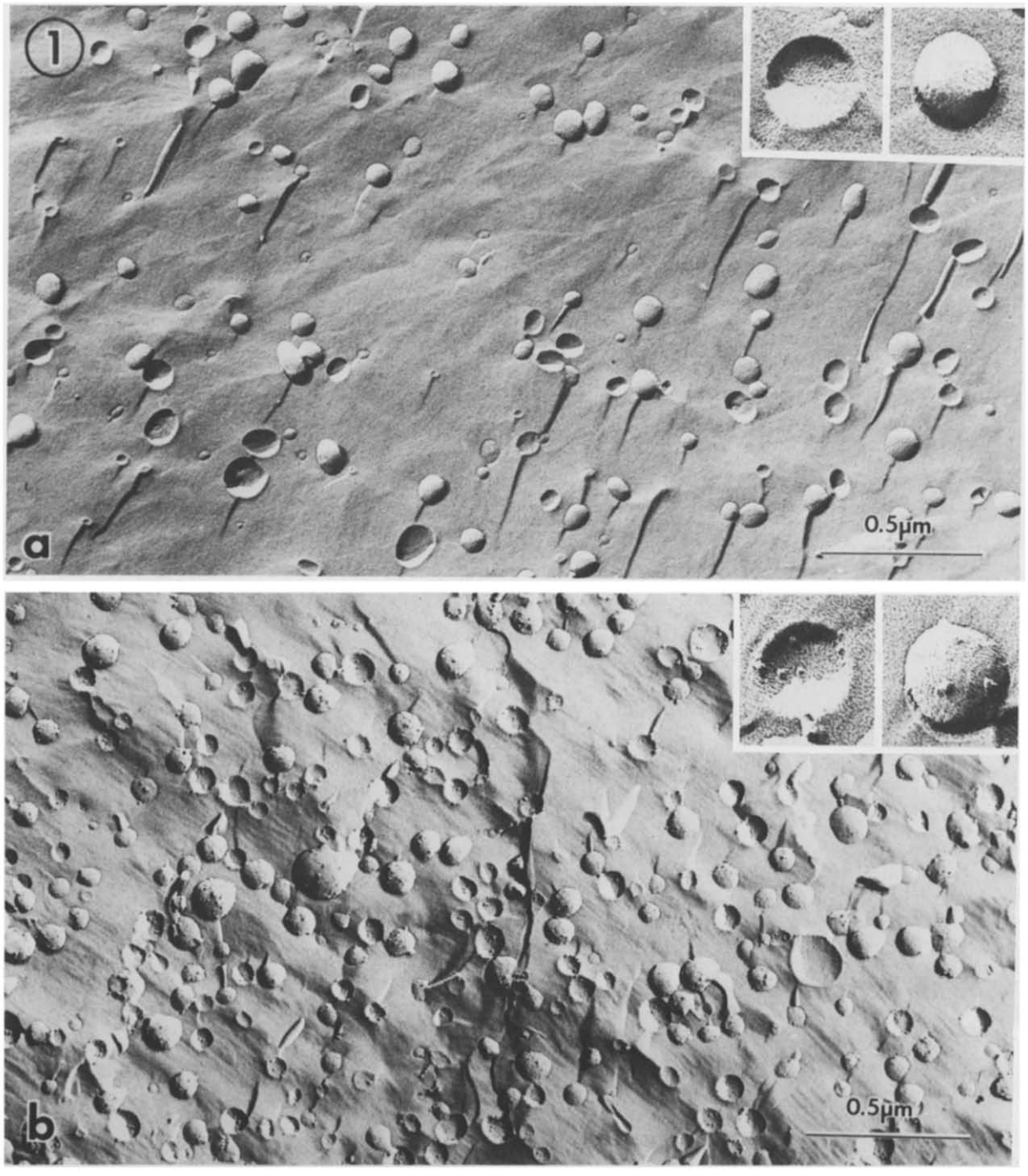

Fig. 1. Electron micrographs of freeze-fractured liposomes prepared (a) without or (b) with 3 mg/ml ($Na^+ + K^+$)-ATPase. The direction of shadowing is from below. Magnification ×75000. The insets were printed at 3× the magnification of the main figures showing the concave and convex fracture faces.

concentration used for reconstitution rather than to the protein concentration determined in the intermediary steps. It was previously shown that treatment of the $(Na^+ + K^+)$-ATPase in a 1% cholate solution reproducibly solubilized about 50% of the total protein [10]. Fig. 1a and Table I show that no particle are seen on freeze-fractured membranes when the liposomes were prepared in the absence of $(Na^+ + K^+)$-ATPase. In contrast, if the liposomes were prepared in the presence of $(Na^+ + K^+)$-ATPase, intramembrane particles become visible on both concave and convex faces of the fractured vesicle (Fig. 1b and Table I). Their number increases in proportion to the protein concentration used for reconstitution (Fig. 2). Their diameter is between 80 and 100 Å, in agreement with the intramembrane particles seen in previous work on the ultrastructure of $(Na^+ + K^+)$-ATPase liposomes [11]. In most preparations, the diameter of the convex fracture faces is up to 100 Å larger than the diameter of the concave fracture faces. The same observation was made by Skriver et al. [12]. We have no explanation for this finding but think it is not significant since in some preparations the reverse is true.

Table II lists the average vesicle diameters. These increase with increasing protein concentrations, being between 600 to 700 Å in protein-free vesicles and 900 to 1000 Å in vesicles containing 5 to 6 intramembrane particles. A simple calculation shows that the mere insertion of 5–6 ATPase molecules into a vesicle bilayer could not alone account for this increase in diameter. Another possibility is that the increase in vesicle diameter results from fusion during detergent removal by dialysis. We prepared histograms of the vesicle size distribution as well as of the particle distribution to test this alternative. In Fig. 3 it can be seen that

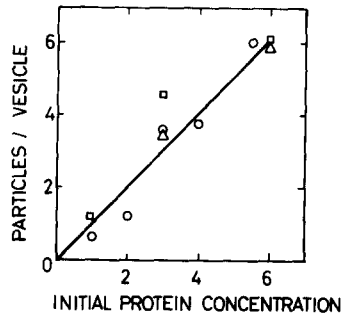

Fig. 2. Relationship between the $(Na^+ + K^+)$-ATPase concentration used for reconstitution and the number of particles per vesicle. Three (○, □, △) series of $(Na^+ + K^+)$-ATPase liposomes were prepared with increasing protein concentrations and the number of intramembrane particles was determined after freeze-fracturing the preparations as described under Materials and Methods.

TABLE I

EFFECT OF $(Na^+ + K^+)$-ATPase CONCENTRATION OF NUMBER OF INTRAMEMBRANE PARTICLES PER VESICLE

Liposomes were prepared in the absence and in the presence of increasing concentrations of $(Na^+ + K^+)$-ATPase protein as described under Materials and Methods. The vesicles were freeze-fractured. The morphological methods are described under Materials and Methods. The number of particles was counted on 200 concave (CC) and on 200 convex (CV) fracture faces of each preparation and the S.E. is indicated. The total number of particles per vesicle is obtained by adding the counts of the concave and convex faces. n.d., not determined.

Series No.	Fracture faces	Particle number; initial protein concentration (mg/ml)					
		0	1	2	3	4	6
83	CV	0	0.24±0.04	0.63±0.06	1.80±0.10	1.67±0.13	2.51±0.12
	CC	0	0.38±0.05	0.56±0.06	1.81±0.10	2.08±0.10	2.99±0.14
	CV+CC	0	0.62±0.05	1.19±0.06	3.61±0.10	3.75±0.12	5.50±0.13
97	CV	0	0.47±0.05	n.d.	2.52±0.13	n.d.	3.52±0.17
	CC	0	0.70±0.05		2.06±0.15		2.47±0.13
	CV+CC	0	1.17±0.05		4.58±0.14		6.09±0.15
98	CV	0			1.76±0.11	n.d.	3.04±0.15
	CC	0	n.d.	n.d.	1.69±0.10		2.93±0.11
	CV+CC	0			3.45±0.10		5.85±0.13

TABLE II

EFFECT OF $(Na^+ + K^+)$-ATPase CONCENTRATION ON VESICLE DIAMETER

The vesicle diameters were determined in the preparations listed in Table I on 200 concave (CC) and 200 convex (CV) fracture faces. See legend of Table I for details. Vesicle diameters are presented as means ± S.E.

Prep. no	Fracture faces	Vesicle diameter (Å); initial protein concentration (mg/ml)					
		0	1	2	3	4	6
83	CV	708±20	582± 9	831±15	867±22	1075±24	1053±19
	CC	706±18	555±10	813±17	820±18	978±23	992±19
97	CV	639±18	771±12		901±15		931±16
	CC	646±16	753±12	n.d.	847±13	n.d.	904±14
98	CV	691±14			778±12		787±13
	CC	622±11	n.d.	n.d.	768±14	n.d.	834±15

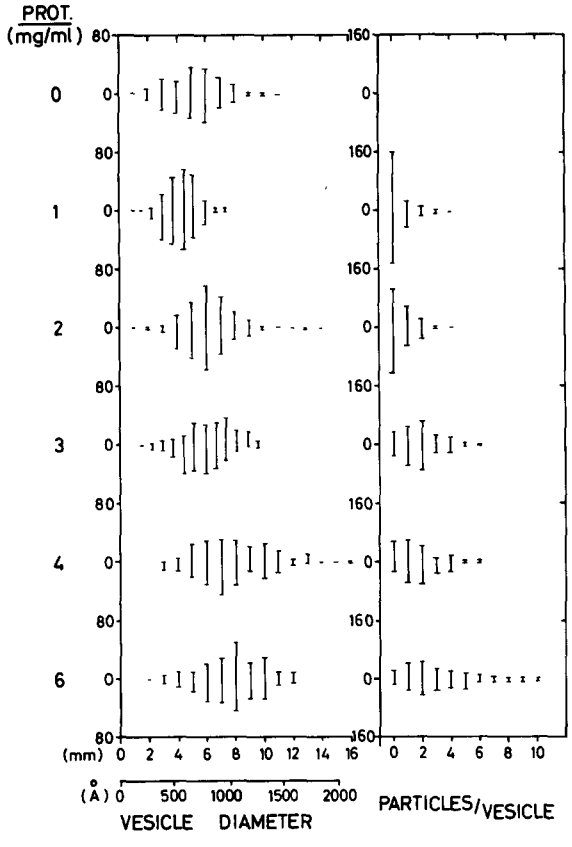

Fig. 3. Histograms of vesicle size and particle distribution as a function of the $(Na^+ + K^+)$-ATPase concentration used for reconstitution. Liposomes were prepared without protein or with $(Na^+ + K^+)$-ATPase concentrations ranging from 1 to 6 mg protein/ml (left ordinate column) and were freeze-fractured and vesicle sizes and particle numbers were measured as described under Materials and Methods. The numbers (0–80) of diameters measured are given in the second left ordinate column. The ordinate column to the right gives the number (0–160) of particles counted. In each preparation 200 convex (above zero line) and 200 concave (below zero line) fracture faces were analyzed with regard to their size and their particle content. In the absence of protein, no particles are present and there were therefore 200 vesicles with zero particles. The bar indicating this which belongs at the top right of the figure was omitted so as to avoid carrying a larger scale throughout the figure.

the vesicle size distribution is fairly symmetrical when the vesicles have been prepared in the absence of $(Na^+ + K^+)$-ATPase or in the presence of less than 2 mg protein/ml. In contrast, when the starting $(Na^+ + K^+)$-ATPase concentration was around 4 mg/ml, a second population with a diameter of about 1300 Å appears which is also seen in the preparations made up with 6 mg/ml protein. The appearance of additional larger vesicle populations with increasing protein concentrations may be an indication that the augmentation of the average vesicle diameter is caused indeed by a fusion process. It can be calculated that fusion of two identical vesicles would theoretically result in a vesicle with a diameter $\sqrt{2}$ or 1.41-fold larger than the original vesicles. If, for instance, two 700 Å vesicles fuse, the product would be a vesicle 990 Å in diameter.

Histograms of the particle distribution (Fig. 3) reveal that there is a predominance of empty vesicles when the vesicles are reconstituted with initial $(Na^+ + K^+)$-ATPase protein concentrations below 3 mg/ml. In contrast, when the initial protein concentration is above 3 mg/ml, the distribu-

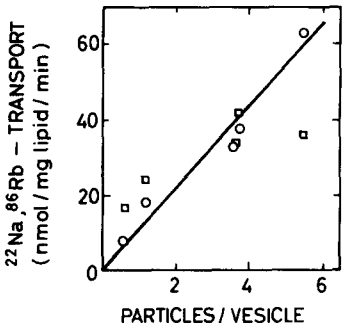

Fig. 4. Relationship between the rate of active ^{22}Na-transport (○) and ^{86}Rb [K] (□) and the number of intramembrane particles per vesicle. $(Na^+ + K^+)$-ATPase liposomes were reconstituted with increasing protein concentrations and the ATP-driver transport rates of Na^+-influx and K^+-efflux were determined as described under Materials and Methods. The transport rates were measured within 10 to 60 s after the addition of 5 mM ATP. The number of particles per vesicle were determined after freeze-fracturing the vesicles. Results from a representative experiment.

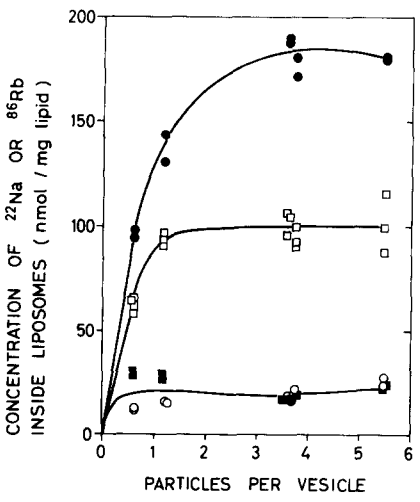

Fig. 5. Correlation between the number of intramembrane particles per vesicle and the transport capacity of $(Na^+ + K^+)$-ATPase liposomes. The ^{22}Na (●) and the ^{86}Rb[K] (■) ion concentrations that were reached inside the liposomes in the presence of ATP within 30 min (at transport equilibrium) were determined. The ^{22}Na ion concentration that was obtained in the absence of ATP was measured also at 30 min after the addition of external ^{22}Na (○), as this value served as a control for the active ^{22}Na uptake. The passive ^{86}Rb[K] transport (□) was measured after a 24 h incubation period in the presence of external ^{86}Rb, i.e., when isotope equilibrium in the $(Na^+ + K^+)$-ATPase containing liposomes was reached but not in the $(Na^+ + K^+)$-ATPase lacking liposomes. The internal isotope content was determined after passage of the isotope loaded liposomes across ice-cooled Sephadex G-50 columns. For methodological details see Materials and Methods and Ref. 10. Results from a representative experiment.

tion of the particles in the vesicle population becomes symmetrical.

Intramembrane particle distribution and Na^+,K^+-transport

Fig. 4. illustrates that the rates of active Na^+ and K^+ transport increase linearly with the number of particles per vesicle. Such a relationship has already been seen for active Na^+-transport in reconstituted vesicle [12] which were prepared by our reconstitution procedure [10,11].

Fig. 5 illustrates the effect of intramembrane particles on the cation permeability of liposomes. In the absence of $(Na^+ + K^+)$-ATPase protein (no intramembrane particle), the internal cation pools remain virtually unlabeled (Fig. 5) despite a 24 h incubation period in the presence of external ^{22}Na or ^{86}Rb. The pure lipid liposomes are known to display a very low a cation-permeability [14]. In contrast, when the liposomes contain on the average at least one intramembrane particle per vesicle, they incorporate external isotope (ascending curves in Fig. 5) via active or passive fluxes as specified in the legend to Fig. 5.

$(Na^+ + K^+)$-ATPase activity and intramembrane particle formation

Prior to reconstitution, the activity of the isolated $(Na^+ + K^+)$-ATPase was changed by three different means in order to test whether such changes altered the ability of the molecule to enter the lipid bilayer.

The first modifier was vanadate. It has been shown that vanadate blocks the active ion transport mediated by the reconstituted sodium pump [22,23]. Vanadate is thought to act as a transition state analogue [24] and to lock the enzyme in a conformation which favors phosphate transfer from the ATP molecule to the protein [25].

The second modifier chosen was ouabain. The cardiac glycosides are the most potent and specific inhibitors known for $(Na^+ + K^+)$-ATPase. They lock the enzyme into a conformation where it can no longer recognize or bind potassium ions [2].

The third modification consisted of heating

$(Na^+ + K^+)$-ATPase that had been solubilized in a 1% cholate solution for 30 s at 90°C. This treatment abolished about 95% of the enzyme activity.

These three types of modified enzymes were then reconstituted separately into liposomes in parallel to native control $(Na^+ + K^+)$-ATPase.

Fig. 6 shows the vesicle size and particle distribution of liposomes reconstituted with native $(Na^+ + K^+)$-ATPase or with enzyme that had been pretreated with vanadate or ouabain or with detergent-denatured enzyme. The vesicle size as well as the particle distribution of the first three preparations display similar patterns. When the $(Na^+ + K^+)$-ATPase activity has been reduced by treatment with vanadate or ouabain before reconstitution, the enzyme is still able to form intramembrane particles and to increase the vesicle diameter in the same manner as the control enzyme (Fig. 6). In contrast, the detergent-denatured preparation is clearly different: the vesicle size is

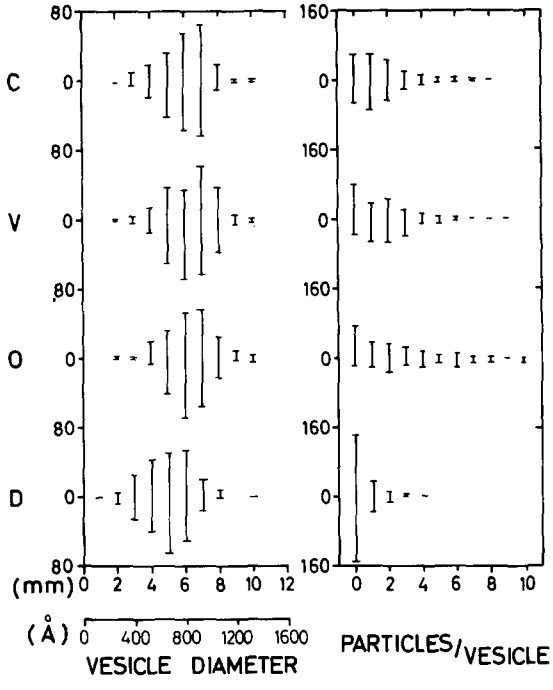

Fig. 6. Effect of $(Na^+ + K^+)$-ATPase configuration on vesicle diameter and number of particles per vesicle. The $(Na^+ + K^+)$-ATPase liposomes were prepared with 3 mg protein/ml in the presence of 1 mM vanadate (V), or 1 mM ouabain (O), or with enzyme that was denatured by heating (D) for 30 s in the 1% cholate solution used for solubilization (see Materials and Methods) or with native enzyme (C). The liposomes were freeze-fractured and the diameters and number of particles per vesicle were determined on 200 convex and 200 concave fracture faces per preparation. Above the zero line are histograms of the convex and below the zero line are histograms of concave fracture faces.

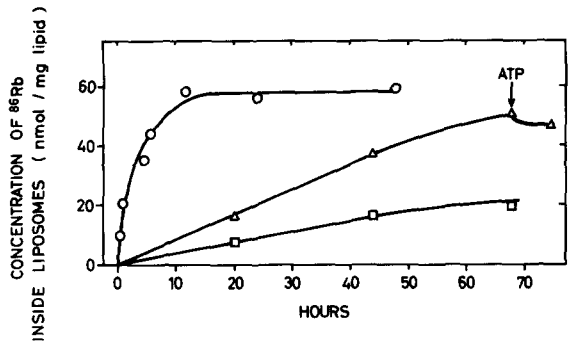

Fig. 7. Correlation of the passive $^{86}Rb[K]$-flux rate with the presence of reconstituted $(Na^+ + K^+)$-ATPase molecules. Liposomes were prepared in the presence of native (○) or cholate denatured (△) or in the absence (□) of $(Na^+ + K^+)$-ATPase protein. The detergent-denaturation was in 1% cholate for 30 s at 95°C. The flux rate was measured by adding ^{86}Rb to liposomes equilibrated with 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$ and the buffers indicated under Materials and Methods.

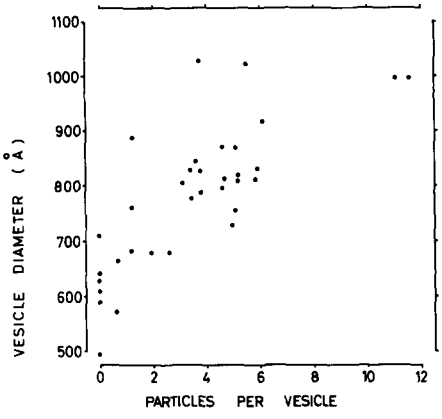

Fig. 8. Correlation between vesicle diameters and number of intramembrane particles per vesicle. The average vesicle diameters and the number of particles per vesicle were determined on 34 different freeze-fractured liposome preparations that had been prepared in the absence (no particles) and in the presence of $(Na^+ + K^+)$-ATPase concentrations ranging from 0.5 to 6 mg protein/ml. (See Fig. 1 for the correlation between the number of particles per vesicle and the initial protein concentration). The methods used for liposome preparation and analysis are described under Materials and Methods.

shifted to the left, i.e., to lower values, and a predominant fraction is devoid of particles. By comparing the vesicle size distribution with the one of protein-free liposomes (Fig. 3), there appears to be little difference. Apparently, detergent-denatured ($Na^+ + K^+$)-ATPase does not reconstitute nor does it influence the vesicle formation or size distribution. However, numerous single or aggregated particles of the same size as the ones that reconstitute are then seen in the background ice (not shown).

The kinetics of the transmembraneous K^+ fluxes shown in Fig. 7 illustrate also that the detergent-denatured ($Na^+ + K^+$)-ATPase interacts only very little with the lipid bilayer. In the liposomes reconstituted in the presence of detergent-denatured enzyme (middle curve), the K^+ flux rate is only about 2.5-times faster than the rate in protein-free liposomes (low curve). Conversely, in the presence of reconstituted control enzyme (upper curve), the potassium ions exchange through the liposome membrane in about 10 h. It was previously shown that the active ($Na^+ + K^+$)-ATPase forms a K^+-selective cation channel in the liposomes [14,26].

Discussion

Correlation between Na^+ and K^+ transport and intramembrane particle distribution

Since the K^+-pool inside protein-free liposomes remains virtually unlabelled after a 24 h incubation with external tracer, whereas the whole pool is labelled in liposomes containing at least one intramembrane particle per vesicle, we conclude that the permeability to K^+ is mediated by the reconstituted ($Na^+ + K^+$)-ATPase molecules. Similarly, maximal active-transport of Na^+ into the liposomes, that is, transport dependent on external ATP, requires an average particle density per vesicle above two (Fig. 5). This finding is in agreement with the notion that the ATPase molecules are incorporated in a random fashion. Only the liposomes containing at least one inside-out oriented molecule can hydrolyse the external ATP and transport the Na^+ into the liposomes. Thus both passive permeability to K^+ and the active uptake of Na^+ are saturating functions of the number of particles per vesicle.

Relationship between vesicle diameter and number of intramembrane particles

Fig. 8 shows that the vesicle diameter varies as a function of the number of intramembrane particles per vesicle. If each detergent solubilized enzyme molecule serves as a condensation center for the growth of lipid bilayers during vesicle formation by detergent-removal, it might be expected that when more than one molecule comes together during this bilayer growth period the extent of the resulting bilayer might be greater than when only one molecule is involved. This mechanism can be regarded as lateral fusion of incomplete, unstable bilayers during their formation. This is quite different from fusion in the usual sense between two completely formed liposome bilayers coming together and fusing to make a larger one. However, the net result on the diameter of the resulting liposome might well be the same. Either mechanism is compatible with our results but we tend to favor the former since we have no evidence that fully formed liposomes ever fuse under the conditions we have used.

($Na^+ + K^+$)-ATPase modification and intramembrane particle formation

($Na^+ + K^+$)-ATPase that has been inhibited with ouabain or vanadate reconstitutes equally well into the lipid bilayer as the native enzyme. Such inhibitor-induced changes could involve primarily the parts of the polypeptide exposed at the membrane surfaces without modifying the parameters that are critical for the reconstitution process.

In contrast, detergent-denaturation seems to involve more drastic changes. Intense detergent-treatment blocks the reconstitution process in terms of production of intramembrane particles. It is attractive to speculate that a shell of native lipid is decisive for reconstitution by facilitating the transfer of the protein into the artificial bilayer by fusion. Complete disruption of this lipid shell would then be expected to block reconstitution.

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