Activation and Inhibition of Na/K-ATPase by Filipin-Cholesterol Complexation. A Correlative Biochemical and Ultrastructural Study on the Microsomal and Purified Enzyme of the Avian Salt Gland *

Dieter Gassner and Hans Komnick

Institute of Cytology, University of Bonn, Ulrich-Haberland-Str. 61 A, D-5300 Bonn

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The Na/K-ATPase-rich microsomal fraction and purified Na/K-ATPase membranes of the salt-stressed avian salt gland were studied at defined filipin/cholesterol molar ratios (F/C) using enzyme assay and electron microscopy including negative staining, thin sectioning and freeze fracturing. Comparative examinations of detergent-treated microsomal fractions and the use of electron microscopic tracers revealed that F/C up to 2 activated latent Na/K-ATPase in sealed right-side-out vesicles by increasing membrane permeability without disrupting the vesicular membrane. Therefore, filipin offers an alternative to the detergents for the activation of latent vectorial membrane enzymes and a possible tool to examine their subcellular localization and sidedness in the membrane. The same F/C had no stimulatory effect on the microsomal anion-ATPase suggesting that the 2 ATPases are not located in the same membrane.

Increasing F/C applied to the unfixed Na/K-ATPase membranes caused an increase in the number of structural F–C-complexes and a progressive lateral displacement of the enzyme particles which finally led to a separation of the areal distribution of these structures at F/C = 10. Such displacements did not occur in unfixed microsomes and were prevented by glutaraldehyde fixation of the purified membranes.

F/C exceeding 2 progessively and temperature-dependently inhibited the Na/K-ATPase in its membrane-bound states, whereas the solubilized enzyme was rather insensitive. The structural and biochemical data suggest that inhibition results from the perturbation of the lipidic microenvironment of the enzyme caused by filipin-cholesterol complexation.

Introduction

Cholesterol plays an essential role in determining the flexibility, stability and fluidity of biological membranes [1, 2]. It changes phase transitions of phospholipids [3] and influences phospholipid-protein interactions, membrane permeability, and the distribution and function of membrane proteins [4–6].

The polyene antibiotic filipin binds specifically and stoichiometrically to cholesterol and related 3β-hydroxysterols [7–9]. After the demonstration that filipin-cholesterol interaction led to characteristic changes in membrane structure that were detectable by freeze fracture and negative staining [10, 11], this antibiotic drug was introduced into intramembrane cytochemistry as a cholesterol marker [12–15]. In the meanwhile a large body of literature has accumulated reporting on inhomogeneous cholesterol distributions and their relations to protein patterns in a variety of biological membranes [*e.g.* 16–25].

In contrast to the wide use of filipin as an ultrastructural probe for membrane sterols, the membrane-perturbing potency of the drug was rarely employed in the functional investigation of membrane-bound enzymes [*e.g.* 26–29] and has not yet been tested on the Na/K-ATPase. Therefore, we have examined whether filipin-cholesterol complexation modifies the activity and ultrastructure of Na/K-ATPase which is an integral and vectorial membrane protein mediating active counterport of Na^+ and K^+ ions across the cell membrane [30]. The basolateral plasma membranes of the principal cells of the salt-stressed avian salt gland are particularly rich in this enzyme [31–33]. From there it can be enriched,

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Reprint requests to Prof. Dr. H. Komnick.

Abbrevations: ATP, adenosine-5′-triphosphate; DAB, 3′3′-diaminobenzidine; DMSO, dimethylsulfoxide; DOC, deoxycholic acid; EDTA, ethylene diamine tetraacetic acid, disodium salt; EF, exoplasmic fracture face; F/C, filipin/cholesterol molar ratio; F/P, filipin/protein weight ratio; F–C, filipin-cholesterol; IMP, intramembrane particle; MW, molecular weight; Na/K-ATPase, ouabain-sensitive; ATP phosphohydrolase, EC 3.6.1.3.; PF, protoplasmic fracture face; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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purified and solubilized, and is accessible to functional and ultrastructural examination in these states [34, 35].

Materials and Methods

Reagents

Na_2-ATP, cytochrome C, myoglobin and bacitracin from Serva (Heidelberg, Germany). Sodium cholate and microperoxidase (MP-11) from Sigma (Munich, Germany). Filipin complex (lot nr. 8393-DEG-11-8, Upjohn, USA) was a gift of Prof. Dr. M. Höfer, Institute of Botany, University of Bonn. DAB from Fluka (Buchs, Switzerland). Crystalline g-strophantin (ouabain), deoxycholic acid, ruthenium red, sodium monovanadate and the other reagents used were mostly from Merck (Darmstadt, Germany) and of analytical grade.

Experimental animals

Domestic ducks (*Anas platyrhynchos*), about 3 months of age, were obtained from a commercial breeder and chronically stressed with 1% sodium chloride in the drinking water for at least 2 weeks.

Preparations of microsomes

Microsomal fractions of salt-stressed salt glands were prepared as the 48 000×*g* pellet of 2 combined supernatants. These resulted from each 2 consecutive 6000×*g* centrifugations of the crude homogenate and the first rehomogenized pellet. The microsomal pellet was resuspended in buffer A consisting of 0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.3, to a protein concentration of about 5 mg/ml, stored at 4 °C and used within three days.

Treatments of microsomes with DOC and SDS

Closed membrane vesicles can be opened by appropriate detergent treatments [36, 37]. Therefore, freshly prepared microsomal fractions were treated with DOC for 30 min at room temperature at the following final concentrations: 1 mg and 2.5 mg protein per ml, 0.06% DOC, 1 mM EDTA in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.1. The conditions of SDS treatment were 1.4 mg microsomal protein per ml, 3 mM Na_2ATP, 2 mM EDTA, 20 mM Tris-HCl, pH 7.4, 0.055% SDS, 30 min at room temperature. After detergent treatment the samples were diluted with 0.25 M sucrose to appropriate protein concentrations and aliquots immediately tested for enzyme activities.

Preparation of purified Na/K-ATPase membranes

Na/K-ATPase membranes were purified from SDS treated salt gland microsomes using the negative purification technique [35, 36] as previously described [34]. The resulting membrane preparations were tested for purity by SDS-PAGE. About 99% of their total ATPase was ouabain-sensitive amounting to $1000-1300\ \mu mol\ P_i \times mg\ protein^{-1} \times h^{-1}$. The purified enzyme preparations were resuspended in buffer A to a protein concentration of about 1.5 mg/ml, immediately used or stored at −25 °C.

Solubilization of Na/K-ATPase

The pellet of 1000 µg membrane-bound Na/K-ATPase protein (Beckman 65 rotor, 100 000×*g*, 30 min) was resuspended in a glass homogenizer at room temperature with 1000 µl of a solubilizing solution [38] containing 20 mM NaCl, 100 mM KCl, 1 mM cysteine, 1 mM EDTA, 5 mM $MgCl_2$, 30 mM imidazole, pH 7.4, and 0.5% sodium cholate (detergent/protein ratio of 5). Insoluble membrane material was pelleted, and the clear supernatants of 3 different solubilization experiments contained 330, 441 and 503 µg protein, so that up to 50% of the membrane-bound Na/K-ATPase were solubilized [39]. Buffer A supplemented with 0.5 or even 1% cholate was rather ineffective in enzyme solubilization, which stresses the salt requirements for the solubilization potency of bile salts [40, 41]. Aliquots of the supernatant were further processed for 4 purposes:

1) Determination of protein concentration;
2) measurements of Na/K-ATPase activity in the presence of increasing filipin concentrations;
3) lipid analysis;
4) formation of proteoliposomes by dialysis against detergent-free solubilization solution for 120 h at 4 °C.

The pellet of cholate-insoluble membrane material was also consumed for lipid analysis.

Lipid analysis

Total lipid was extracted from 4 different preparations (1) microsomes, 2) purified Na/K-ATPase membranes, 3) cholate insoluble membrane material, and 4) the supernatant of solubilized Na/K-

ATPase after dialytic cholate removal) by two consecutive washes with chloroform/methanol (2 : 1, v/v). The two lipid extracts were pooled, dried under nitrogen and redissolved in defined volumes of the above solution. The total lipid per mg protein was gravimetrically determined. Phospholipids, free fatty acids and neutral lipids were separated by one-dimensional thin-layer chromatography on silicagel plates by eluting the plates with 1) chloroform/methanol/H_2O (75 : 25 : 4), 2) chloroform and 3) *n*-hexane/chloroform (3 : 1). Lipid spots were visualized with H_2SO_4 and analysed with densitometric scans [42, 43].

SDS-PAGE

Samples of the microsomal fraction and the purified Na/K-ATPase membranes were dissolved in 2% SDS, 5% mercaptoethanol, 8 M urea, 0.0025% bromphenol blue, 62.5 mM Tris-HCl at pH 6.8 and incubated for 5 min at 100 °C. Electrophoresis was carried out in a discontinuous system [44] on slab gels of linear 8–20% acrylamide which were fixed and stained according to [45]. Molecular weight standards included myosin (MW 200 000), phosphorylase b (MW 94 000), bovine serum albumin (MW 68 000), catalase (MW 58 000), actin (MW 42 000), tropomyosin (MW 35 000), carboanhydrase (MW 30 000), soybean trypsin inhibitor (MW 21 000), myoglobin (MW 17 000), and cytochrome C (MW 12 500).

Enzyme assays

The incubation medium of the Na/K-ATPase assay contained 100 mM NaCl, 10 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 2.5 mM Na_2ATP, 50 mM Tris-HCl (pH 7.5) in the presence or absence of 0.2 mM ouabain. The anion-stimulated ATPase was measured with the use of 3 different activators, namely bicarbonate, chloride and sulfite. The reaction medium had the following composition: 20 mM histidine-Tris (pH 8.5), 0.5 mM Mg-acetate, 1 mM Na_2ATP, 0.2 mM ouabain, 25 mM of the activating anion in the presence or absence of 25 mM NaSCN [46]. Reaction was started by the addition of 300 µl samples of double-concentrated incubation media to final volumes of 600 µl alternatively containing 10 and 25 µg/ml microsomal protein, 2 µg/ml membrane-bound, 2 µg/ml solubilized and 2 µg/ml solubilized and dialysed enzyme protein at increasing concentrations of filipin (0–100 µg/ml). Stock solutions of filipin were freshly prepared for each experiment at concentrations of 0.015 or 0.03% (w/v) in 1.5% aqueous DMSO (v/v). The various enzyme preparations were preincubated with the adjusted concentrations of the antibiotic for 30 min at 25 and 37 °C. All experiments using filipin were performed in the dark whenever possible; for manipulations only minimal illumination was allowed.

Enzyme reactions were carried out in triplicate at 25 and 37 °C. Incubation times, within assured time linearity, were 5 to 15 min for Na/K-ATPase and 30 min for anion-ATPase. The reactions were terminated by adding ice-cold trichloroacetic acid to a final concentration of 6.7%. Inorganic phosphate (P_i) was determined according to [47], protein concentration according to [48] with bovine serum albumin as a standard. Na/K-ATPase was calculated as the ouabain-inhibitable fraction of total ATPase activity, while anion-stimulated ATPase was expressed as total activity in the presence of the respective activator anion and the residual activity in the additional presence of 25 mM NaSCN. Parallel to the filipin incubations, solvent controls were run using the same DMSO concentrations as contained in the corresponding filipin solutions. Further controls included appropriate blanks to determine spontaneous ATP hydrolysis under the various experimental conditions. Finally, the possible interference of the filipin solutions with phosphate determination was excluded by running appropriate controls with the phosphate standards. All data in Figs. 3, 4 and 5 were corrected for spontaneous ATP hydrolysis and the DMSO effect. The standard deviations were not included because they never exceeded ±5% of the mean.

Electron microscopy

All preparations were examined with the Philips EM 200 electron microscope at 60 KV accelerating voltage.

Fixation and embedding:

Fixative 1) 2.5% glutaraldehyde in 0.1 M phosphate buffer of pH 7.4.

Fixative 2) 2.5% glutaraldehyde in 0.1 M cacodylate buffer of pH 7.4.

Postfixation with 1% osmium tetroxide and rinsing in the respective buffer; dehydration in graded etha-

nol, embedding in Spurr's low viscosity medium, staining of thin sections with uranyl acetate and lead citrate.

Negative staining

Equal volumes of the samples and 15 mg% bacitracin as a surfactant [49] were mixed on formvar-coated and carbon-stabilized grids, and negatively stained for 10 s with 1% phosphotungstic acid/KOH of pH 7.2.

Freeze fracturing

The samples were cryoprotected by infiltration with 10, 20 and 30% glycerol in 0.1 M phosphate buffer and processed for freeze fracture with the use of the Leybold-Heraeus Bioetch 2005 under conditions described previously [34].

Filipin treatment

Filipin was applied in various solutions as specified later on, which consistently contained 0.03% (w/v) filipin and 1.5% (v/v) DMSO unless stated otherwise. The same solutions containing only 1.5% DMSO served as solvent control. Filipin solutions were prepared directly before use and applied in the dark.

Glandular tissue

Tissue slices of freshly excised salt glands were fixed with fixative 1 for 2 h. The slices were cut with the Sorvall tissue sectioner into small columns for 2 reasons: 1) to facilitate filipin penetration and 2) to fit the specimen holders of the Bioetch. Parts of the tissue columns were directly processed for freeze fracturing, parts of them were alternatively incubated in fixative 1 containing filipin and in fixative 1 containing only DMSO. Incubation was continued over night at 25 °C with permanent agitation of the solution. After washing with buffer, the specimens were cryoprotected and freeze fractured.

Microsomal fraction

Negative staining of the microsomal fraction included 4 different pretreatments: 1) untreated control; 2) filipin-treatment at F/P of 0.4 and 4 in buffer A for 30 min, both at 25 and 37 °C; 3) SDS-treatment; 4) DOC-treatment. Pellets of pretreatments 1 and 2 were also fixed with fixative 1 additionally containing 1% tannic acid to enhance membrane protein contrast [50] and processed for thin sectioning.

For freeze fracture a pellet (450 µg protein) of freshly prepared microsomes was fixed for 1 h by gentle resuspending in fixative 1. After centrifugation and washing, the pellet was cryoprotected and freeze fractured.

For permeability studies 4 samples (450 µg protein) of freshly prepared microsomes in buffer A were pretreated for 30 min at 25 °C 1) with filipin in buffer A at F/P = 0.4, and 2) with DMSO alone (untreated control). After pelleting the 2 pretreatments were gently resuspended with 1 ml of the following tracer solutions:

1) 0.8% ruthenium red in 0.1 M cacodylate buffer, pH 7.4;
2) 1% myoglobin in 0.1 M phosphate buffer, pH 7.4;
3) 3% cytochrome C in 0.1 M phosphate buffer, pH 7.4;
4) 1.5% microperoxidase in 0.1 M phosphate buffer, pH 7.4.

After 2 h of incubation at room temperature the 8 samples were centrifuged, the supernatant removed and the unwashed pellets processed according to the tracer used.

Tracer 1: Pellets were fixed over night at 4 °C with fixative 2, postfixed for 3 h at room temperature in the dark and rapidly dehydrated [51]. The 2 fixatives were supplemented with 0.8% ruthenium red [52].

Tracer 2 to 4: Pellets were fixed for 2 h at room temperature with fixative 1 and the tracers visualized on the basis of their peroxidatic activities by appropriate incubations with DAB/H_2O_2. Cytochrome C [53] and microperoxidase [54] were stained according to [55], myoglobin according to [56].

Na/K-ATPase membranes

For negative staining of unfixed Na/K-ATPase membranes aliquots of the membrane suspension (1.604 mg/ml enzyme protein in buffer A) were mixed with filipin in buffer A and with DMSO alone as solvent control. The final mixtures were adjusted to increasing F/P ratios (0.1; 0.2; 0.5; 1; 3; 6) at a constant protein concentration of 0.25 mg/ml except for the necessarily lower protein concentrations of 0.08 and 0.04 mg/ml in the respective cases of F/P = 3

and 6. After incubation at 25 °C and 37 °C for 20–40 min, the samples were negatively stained.

Fixation of Na/K-ATPase membranes and subsequent filipin treatment was performed in 2 ways. The first was based on dialysis, the second on centrifugation.

1) Aliquots of the purified membrane suspension in buffer A were diluted with 0.1 M phosphate buffer (pH 7.3) to a protein concentration of 0.1 mg/ml and dialyzed over night at 4 °C against the same buffer. This served as a preventive measure for the removal of Tris which interferes with the glutaraldehyde fixative resulting in pH decrease and membrane damage. Subsequent dialysis against fixative 1 for 12 h at 4 °C served the introduction of the fixative. Thereafter, the dialysate was divided into 2 portions. One portion was dialysed for 12 h at room temperature against filipin in phosphate buffer (F/P = 3) and the second against DMSO in buffer as solvent control. Samples of the final dialysates were washed on the grid with 2 changes of distilled water and negatively stained.

2) Pellets of purified Na/K-ATPase membranes containing 100 µg protein were fixed for 1 h at 4 °C in fixative 1. The unfixed control was treated with buffer alone. The fixative was removed by centrifugation and washing with buffer. Pellets of fixed and unfixed membranes were resuspended with 2 ml of 0.1 M phosphate buffer supplemented with 0.015% filipin and 1.5% DMSO (F/P = 3) and treated for 30 min at 25 °C. After pelleting the membranes were resuspended with buffer A to a protein concentration of about 0.25 mg/ml and used for negative staining.

Filipin-treated Na/K-ATPase membranes at F/P = 3 were prepared for comparative negative staining, thin sectioning and freeze fracturing according to the following protocol: An aliquot of the membrane suspension in buffer A containing 300 µg enzyme protein was diluted with buffer A to 3 ml. After pelleting, the membranes were resuspended in a Potter-Elvehjem-homogenizer with 3 ml 0.03% filipin in buffer A and incubated for 30 min at 25 °C. Samples of the suspension were negatively stained, the rest was pelleted at 100 000×*g* for 30 min. Part of the pellet was fixed for 3 h in fixative 1 supplement with 1% tannic acid and processed for thin sectioning. Another part was suspended with a few µl of buffer A and processed for freeze-fracturing without cryoprotection, using a prolonged etchtime of 5 min.

Vanadate-induced crystallization of Na/K-ATPase

A pellet of purified Na/K-ATPase membranes of the avian salt gland was resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 0.25 mM sodium monovanadate [57] at a concentration of 1 mg enzyme protein/ml. Aliquots of the suspension were negatively stained after storing at 4 °C for time periods of a few hours up to 4 weeks.

Results and Conclusions

Freeze fracture and filipin treatment of the glandular tissue

The knowledge of the ultrastructure of the principal cells of the salt-stressed avian salt gland accumulated since the early thin section studies on various species [58–61] facilitates the interpretation of freeze fracture images. Previous freeze fracture studies [62–64] have mainly focussed on the zonula occludens. The latter was first shown in thin sections of the herring gull salt gland to consist of an extremely narrow, in cross section punctate membrane junction, so that its sealing function was questioned [61]. The section image correlated well with the freeze fracture result showing a single-stranded junction in the herring gull [62]. In accordance with the figures given for the duck salt gland [63, 64] the zonula occludens of the principal cells observed in this study was always pauci-stranded (Fig. 1 a, b), consisting of up to 4, but mostly 2 junctional strands.

Like thin sections across the epithelium, freeze fractures of the subapical region show the abundance of basolateral plasma membranes in close association with mitochondria (Fig. 1 d). The amplification of the basolateral membrane area which is 3 orders of magnitude larger than the apical membrane area [61] results from intimate interlocking of slender cell processes. This is clearly illustrated by tangential fractures providing enface-views of the epithelial base from the blood side (Fig. 1 c) and by cross fractures of the epithelium showing numerous U-turns of the membranes, their more or less parallel alignment and basoapical orientation (Fig. 1 d).

The basolateral plasma membranes are endowed with the Na/K-ATPase [31]. This transmembrane protein is identified by freeze-fracture in purified plasma membranes as IMP [34, 65–68] and therefore, must be included in the dense particle population of the basolateral PF (Fig. 1 c, d, f). The

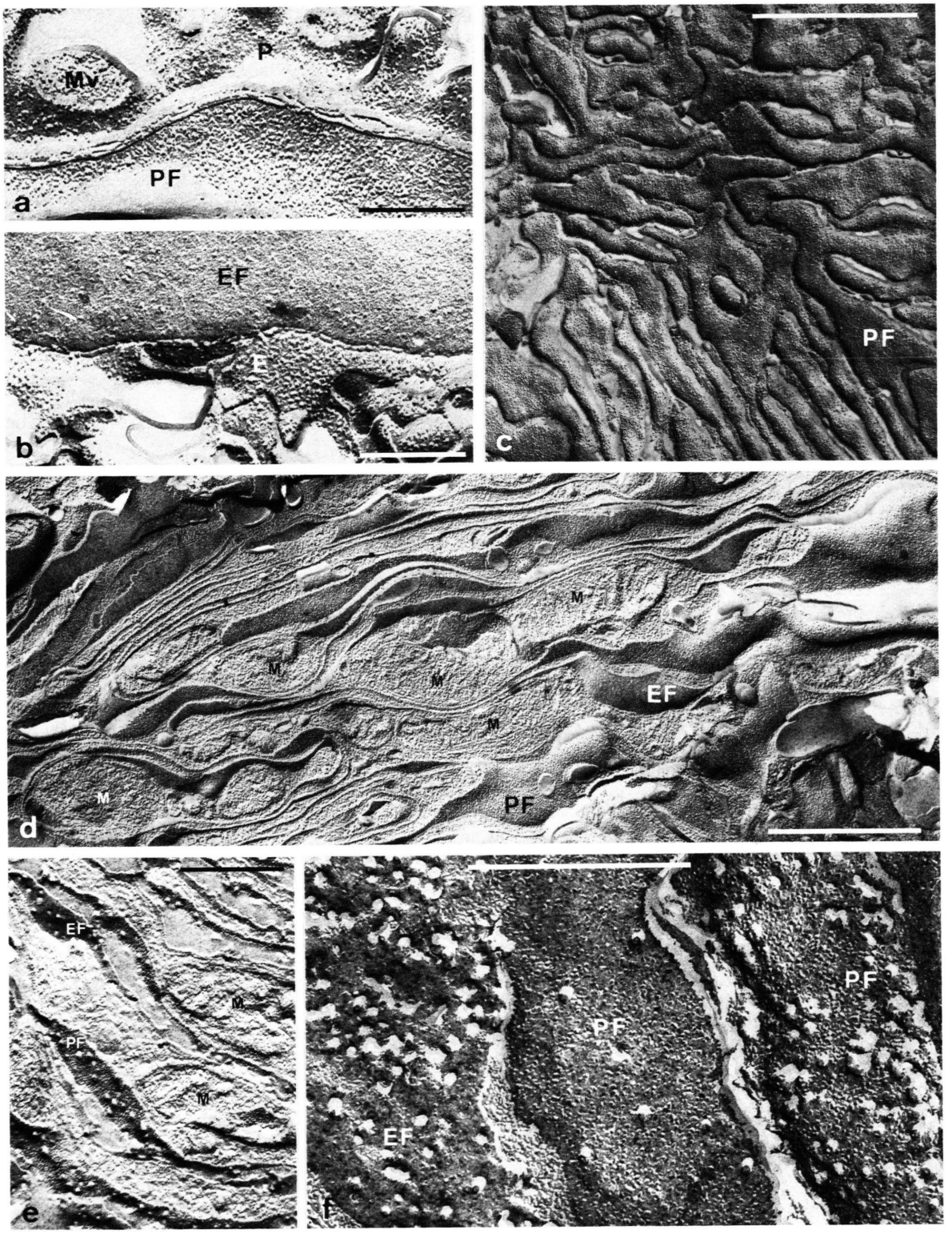
Mv
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Table I. Lipid charcteristics of various preparations from the avian salt gland.

Preparation	Total lipid	Phospholipid	Cholesterol	Cholesterol/ phospholipid [molar ratio][a]
	[µg per mg protein]			
1) microsomal fraction	574	415	111	0.52
2) purified Na/K-ATPase membranes	997	718	180	0.49
3) cholate-insoluble material	497	326	94	0.56
4) cholate-solubilized material	2012[b]	1515[b]	356[b]	0.46[b]
5) reconstituted material	502	350	43	0.24

[a] Based on molecular weights of 387 for cholesterol and 750 for phospholipids.
[b] Calculated from the data of the purified Na/K-ATPase membranes and the cholate-insoluble material.
Preparation 2 was obtained from preparation 1 by SDS treatment and subsequent sucrose gradient centrifugation. Preparation 3 and 4 were obtained from preparation 2 by cholate treatment and subsequent centrifugation. The pellet (preparation 3) yielded 67% and the supernatant (preparation 4) 33% recovery of enzyme protein. Preparation 5 was obtained from preparation 4 by dialytic cholate removal.

luminal and basolateral membranes differ in that the apical PF and EF are both rich in IMPs (Fig. 1 a, b) while the basolateral EF is poor of IMPs in contrast to the basolateral PF (Fig. 1 a, b, d).

Filipin treatment led to the formation of typical F−C complexes. These represent circular membrane protuberances 20−25 nm in diameter which appear mostly as crater-like elevations on PF and as pits on EF (Fig. 1 e, f). They are usually confined to the most peripheral portions of the tissue blocks as a result of poor filipin penetration even after prolonged treatment for up to 15 h. Inadequate penetration appears also to be responsible for different distributions in closely neighboured basolateral folds which may belong to the same or different cells (Fig. 1 f). Apart from such irregularities, normally the protuberances are rather evenly and densely distributed on the basolateral plasma membranes, thus indicating a high cholesterol content of these membranes.

Studies on the microsomal fraction

Lipid and protein composition: The total lipid content of the microsomal fraction was about half of the total protein content (574 µg lipid per mg protein) and consisted of 72% phospholipids and 28% neutral lipids and free fatty acids. About 70% of the last category were cholesterol resulting in a cholesterol/phospholipid molar ratio of 0.52 (Table I).

Gel patterns reveal the presence of numerous membrane peptides (approx. 30 bands) in the microsomal fraction. The 2 major bands correspond in position to the catalytic subunit of the purified Na/K-ATPase and to the skeletal muscle actin standard with molecular weights of 91 000 and 42 000, respectively (Fig. 2). The low molecular weight subunit of Na/K-ATPase yields a rather diffuse band which is accompanied by 2 sharp bands of minor peptides at nearly the same position [69]. SDS treatment and subsequent sucrose gradient centrifugation

Fig. 1. Freeze fractures of apical and basolateral plasma membranes of excretory tubule cells of the salt-stressed duck salt gland. a) The zonula occludens which separates the luminal and basolateral plasma membranes shows 2 to 3 junctional ridges when fractured from the basolateral PF (PF) to the luminal PF (P). Mv, microvilli. b) Corresponding furrows are visible when fractured from the luminal EF (E) to the basolateral EF (EF). Scales 0.25 µm. c) Tangential fracture of a glandular tubule providing enface-view from the blood side on the basal PF of interdigitated cell processes. d) Fracture of the sub-apical region showing EF and PF of the basolateral plasma membranes. M, mitochondria. Scales 1 µm. e, f) Similar regions as shown in (d) but after filipin treatment. The basolateral plasma membranes are labelled with F−C complexes mostly appearing as protrusion on PF and complementary pits on EF. Note the different labelling of neighboured cell processes in (f). M, mitochondria. Scales 0.5 µm.

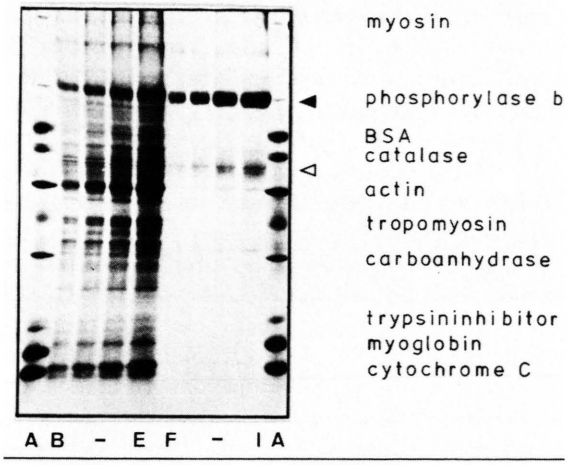


Fig. 2. SDS-PAGE patterns of the salt gland microsomal fraction and the purified Na/K-ATPase membranes. A. Molecular weight standards. B–E. Microsomal fraction: B 30 µg protein; C 40 µg protein; D 50 µg protein; E 70 µg protein. F–I. Na/K-ATPase membranes: F 7.5 µg protein; G 10 µg protein; M 15 µg protein; I 22.5 µg protein. Positions of the high (full arrowhead) and low (open arrowhead) molecular weight subunit of Na/K-ATPase.

removes most of the peptides, so that the purified Na/K-ATPase membranes yield only 2 substantial bands representing the 2 subunits of the enzyme with molecular weights of 56 000 and 91 000.

ATPase studies: Different microsomal preparations yielded specific Na/K-ATPase activities in the range of 100 to 200 µmol $P_i \times$ mg protein$^{-1} \times h^{-1}$. Filipin treatment of the microsomal fraction (final concentration of 25 µg protein/ml) resulted in a biphasic effect on the Na/K-ATPase activity. Low F/P caused a steep increase in Na/K-ATPase activity which peaked at 10 µg filipin/ml corresponding to F/P = 0.4 and F/C = 2 (Fig. 3a). The ouabain-insensitive Mg-ATPase of the microsomal fraction was not activated by filipin. F/P ratios exceeding 0.4 were inhibitory causing a relatively slow decline of Na/ K-ATPase activity. In contrast to the first activating phase which was independent of the 2 assay temperatures tested the second inhibitory phase showed a clear dependence on the temperature (Fig. 3a). The decline of activity was much steeper at 37 °C than at 25 °C. At 100 µg filipin/ml (F/P = 4, F/C = 21) only 5–10% of the filipin-induced maximal activity were left at 37 °C, whereas the high remainder of ca 65% of the maximal activity at 25 °C still exceeded the original activity before stimulation by filipin (Fig. 3a). The temperature dependence points to the possibility that the extent of inhibition by filipin depends on membrane fluidity.

The suspicion that the filipin-induced stimulation of the microsomal Na/K-ATPase represented an unmasking effect of latent enzyme molecules prompted comparative measurements on microsomal samples pretreated with SDS and DOC which are known to activate latent Na/K-ATPase molecules. These measurements were performed at 37 °C and with 10 µg/ml microsomal protein in order to simultaneously test a lower protein concentration. The control measurements on the samples which had not been pretreated with detergent, showed the same biphasic effect of filipin as described above. The lower protein concentration shifted the activation peak from 10 µg filipin/ml in Fig. 3a to 4 µg/ml in Fig. 3b. However, the F/P inducing maximal enzyme stimulation remained unchanged. This means that the optimal filipin concentration directly correlates with the final cholesterol concentration which in turn is a function of the protein concentration. Consequently, the optimal filipin concentration can be calculated for any protein concentration which falls in the range of the linearity of the ATPase.

Incubation of the microsomal fraction with the ionic detergent SDS leads to maximal enzyme activation [35] by the exposure of latent enzyme sites [37]. The exposure results from the morphologically detectable transformation of closed microsomal vesicles into open cup-shaped membranes [34, 65, 70] thus providing free access of substrate and activators. The strong activating effect of SDS on the microsomal Na/K-ATPase is also apparent in these experiments (compare curves A and D at zero filipin in Fig. 3b). The maximal activations by SDS and filipin differ only by about 10% and thus are quantitatively very similar. As clearly recognized from curve D in Fig. 3b filipin has no further stimulatory effect when the enzyme is fully activated by SDS treatment.

Deoxycholate was studied under two conditions which according to [37] cause maximal and submaximal activation of the microsomal Na/K-ATPase from the rabbit kidney. These conditions also cause respective, graded activations of Na/K-ATPase of the salt gland microsomes, which are

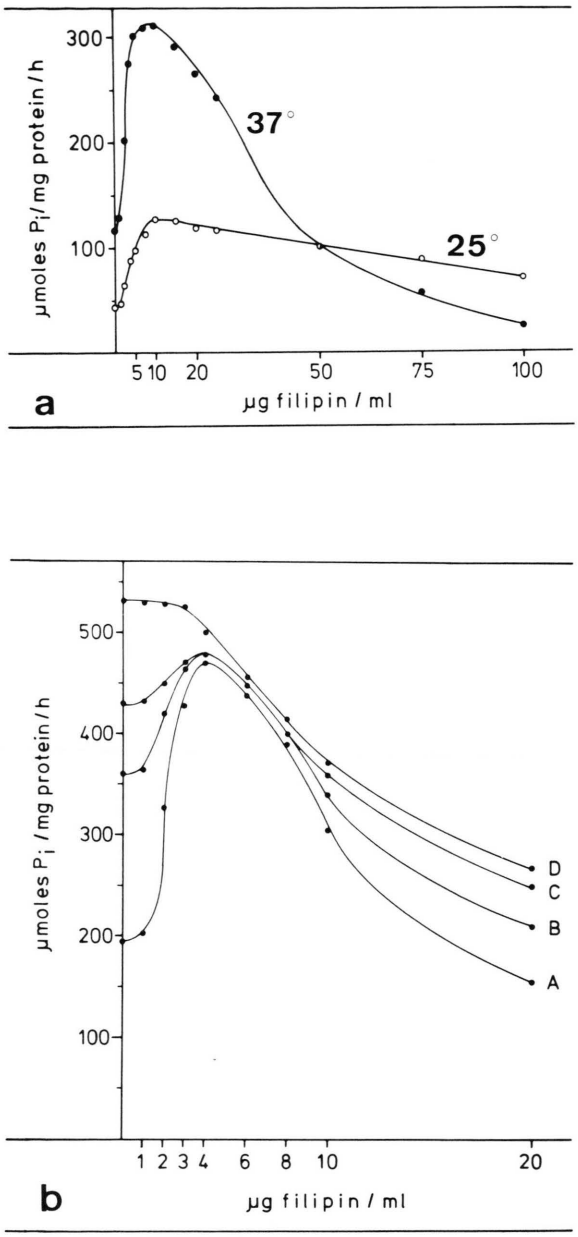


Fig. 3. a) Microsomal Na/K-ATPase activity (25 µg/ml final protein concentration) as a function of filipin concentration at 37 °C (closed points) and 25 °C (open points) assay temperature. Activation peaks at F/P = 0.4 (F/C = 2). Representative results of three different experiments. b) Microsomal Na/K-ATPase activity (10 µg/ml final protein concentration) as a function of filipin concentration at 37 °C assay temperature after different pretreatments. Curve A: untreated; Activation peak at F/P = 0.4 (F/C = 2). Curve B: DOC treated at submaximal activation conditions; Curve C: DOC treated at maximal (in fact slightly submaximal) activtion conditions; Curve D: SDS treated. Representative results of 3 different experiments.

smaller than the maximal activations by SDS and filipin. Filipin evokes a further stimulation at both submaximal and maximal DOC conditions, the accumulative effect of DOC and filipin peaking at practically the same level and same F/C as the stimulation by filipin alone (Fig. 3b, curves A, B and C). These data clearly suggest that stimulation by filipin depends on the same mechanism as stimulation by the 2 detergents, that means on the exposure of latent enzyme molecules.

The small additional stimulation by filipin at maximal DOC condition indicates that this condition is still slightly submaximal (about 90% of maximal activation) for the duck salt gland microsomes in contrast to the rabbit kidney microsomes [37]. Consequently, the two DOC conditions tested were actually submaximal to different extents, so that in both cases small fractions of microsomal vesicles remained closed and their still latent enzyme molecules were exposed by the subsequent filipin treatment.

As seen in Fig. 3b, maximal activation by SDS alone exceeds the 3 other maxima by about 10%. This raises the question whether an additional, but far less effective activation mechanism is involved [71]. SDS treatment does not only open the microsomal vesicles but also solubilizes about 80–90% of protein and 65% of lipid of the microsomal fraction. On the other hand, the slight decline of activity in curve D at 4 µg filipin/ml possibly indicates that the activation maxima in curves A to C actually result from the interference of counter-current activation and inhibition phases.

Fig. 3b further shows that the extent of inhibition by filipin is different in the 4 microsomal preparations. It seems to be directly correlated with the protein and lipid content of the membranes. SDS treatment, which is strongest in the solubilization of membrane protein and lipid, leads to the greatest reduction of enzyme inhibition by filipin. Detergent pretreatment implies a possible reduction of the effective filipin concentration by capture of solubilized membrane cholesterol and reduction of F–C complexation within the membrane as a consequence of partial cholesterol solubilization.

Since the activation of the microsomal Na/K-ATPase at low F/P obviously results from the exposure of latent enzyme, filipin treatment on the microsomal anion-ATPase [34] was used in order to probe whether or not both ATPases are bound to the

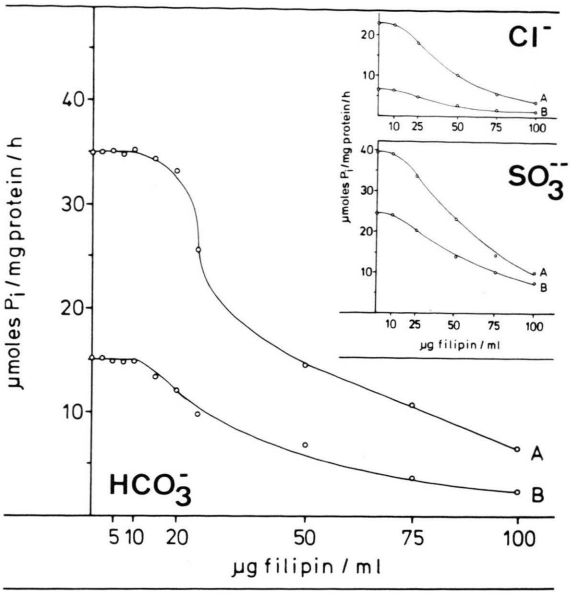


Fig. 4. Microsomal anion-stimulated ATPase activity (25 µg/ml final protein concentration) as a function of filipin concentration at 37 °C assay temperature. Curves A: Total ATPase activity in the presence of 25 mM of the respective activator anion. Curves B: Residual ATPase activity in the additional presence of 25 mM thiocyanate. Representative results of 2 different experiments.

same structural component of the microsomal fraction. However, in contrast to the microsomal Na/K-ATPase, no biphasic effect was observed regardless of whether bicarbonate, chloride or sulfite served as activator (Fig. 4). Instead, there was a monophasic effect. Both the total activities in the presence of activator and the residual activities in the presence of inhibitor remained constant at low F/C up to 2 and thereafter declined. The specific activities, *i.e.* the difference of the respective total and residual activities were also inhibited, because the reduction of total activities was greater than the drop of residual activities (Fig. 4). This finding agrees with the filipin-induced inhibition of bicarbonate-stimulated ATPase in brush border membranes isolated from rat kidney cortex which requires high F/C as estimated from the data given by [26]. The failure of filipin to activate the anion-ATPase under conditions which lead to the exposure of latent Na/K-ATPase molecules suggests that the 2 ATPases are not bound to the same membrane vesicles of the microsomal fraction. If in addition to the mitochondrial localization [72], avian salt gland anion-ATPase is located in the plasma membrane at all, only the apical plasma membrane can be taken into consideration. This possible enzyme site would agree with the demonstration of anion-ATPase in the brush borders of enterocytes and nephrocytes [26, 73] but disagrees with the exclusive mitochondrial localization favored by others [74].

Ultrastructural and tracer studies: The preponderant components of the microsomal fraction are vesicles with closed and broken membranes (Fig. 6a, d). Most of these are apparently derived from the basolateral plasma membranes which represent the most frequent membranes in the salt-stressed gland [61].

Tannic acid fixation reveals that the trilaminar structure of many vesicular membranes is highly asymmetric with a thicker, densely staining layer inside (Fig. 6b). Provided the vesicles were oriented right-side-out, the staining asymmetry is possibly related to the unidirectional alignment of the Na^+-pump protein similar to the sarcoplasmic reticulum Ca^{2+}-pump protein [75] or to the presence of a submembrane cytoskeletal network. The asymmetric staining of avian salt gland microsomes is opposite to mammalian kidney microsomes with the intensely stained layer outside [70].

Negatively stained microsomes are densely studded with surface particles arranged in clusters and strands (Fig. 6d). The faint appearance of these structural details results from membrane superposition of the collapsed vesicles. A large portion of the membrane particles certainly represents the Na/K-ATPase located in the basolateral plasma membranes. This can be deduced from the size and distribution of the particles which are very similar in the purified Na/K-ATPase membranes (Fig. 9a). The transformation of the vesicles into open cup-shaped membranes by the detergents and their deposition as even, single-layered membrane sheets lead to a clearer depiction of the particles (Fig. 7e, f). The surprising similarities of the 3 microsomal preparations (untreated, SDS and DOC-treated) which largely differ in their membrane protein content [36] suggest that only the transmembrane protein Na/K-ATPase is visualized by negative staining in these cases.

Freeze fracture further reveals that the particle-rich fracture face which obviously represents the particle-rich PF (Fig. 1d) is nearly exclusively correlated with the convex fracture face of the microsomal vesicles while the concave fracture faces

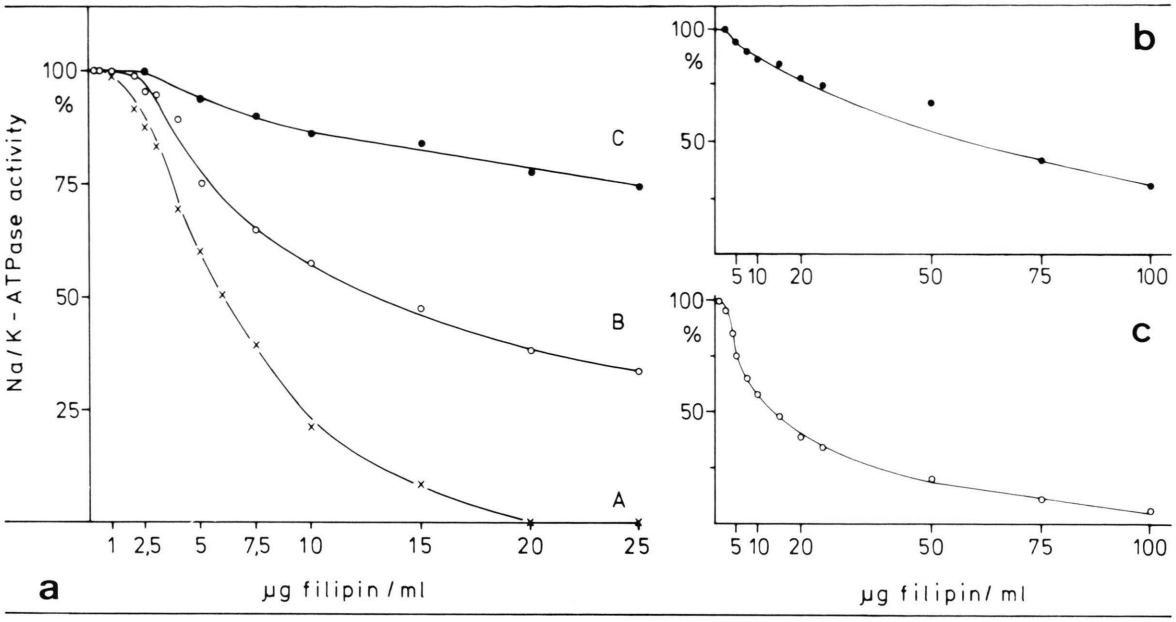
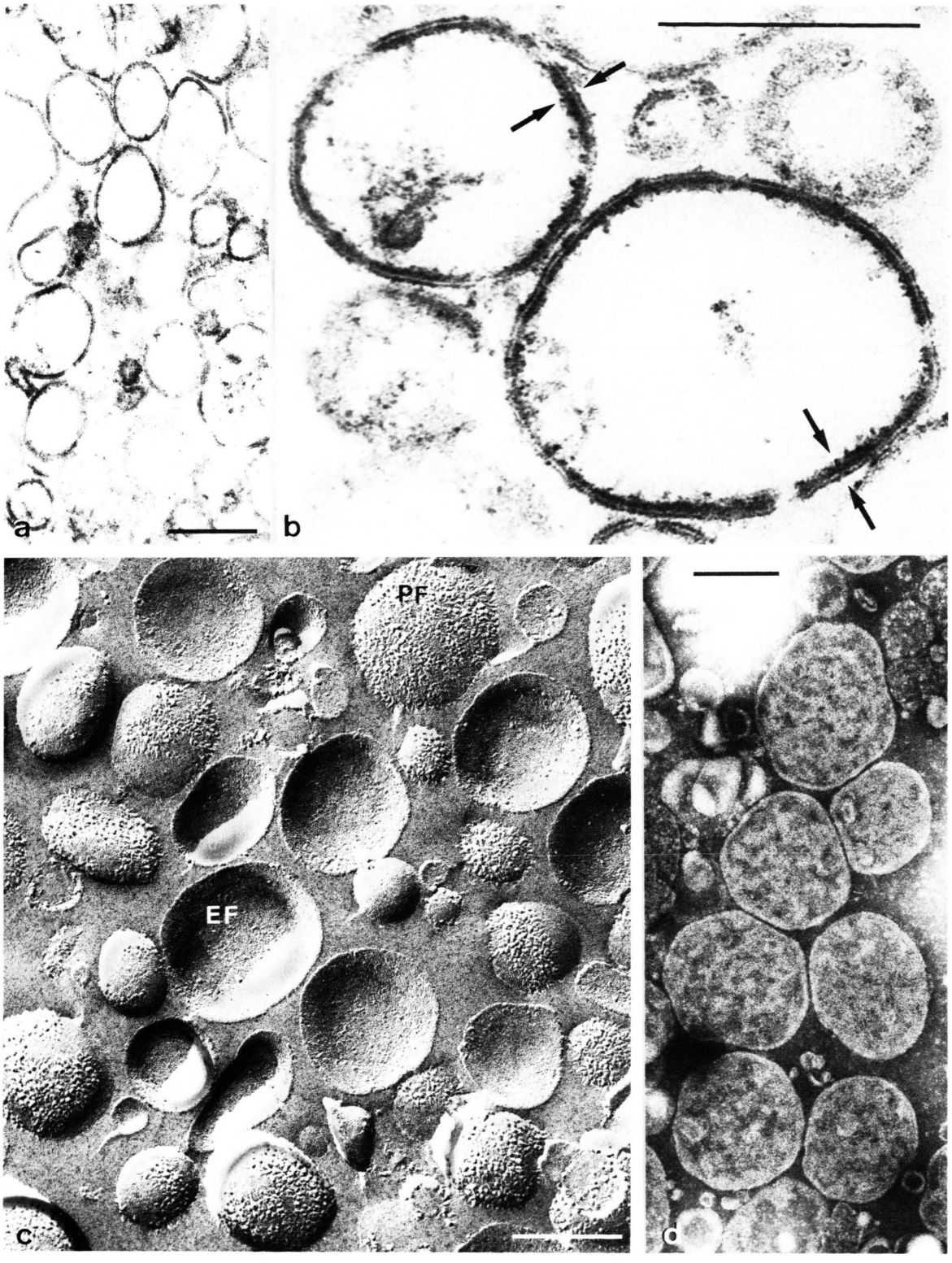


Fig. 5. a) Relative ATPase activity of purified Na/K-ATPase (2 µg/ml final enzyme protein concentration) as a function of filipin concentration (0–25 µg filipin/ml final concentration). Curve A: membrane-bound Na/K-ATPase, 37 °C assay temperature. Curve B: membrane-bound Na/K-ATPase, 25 °C assay temperature. Curve C: cholate solubilized Na/K-ATPase, 37 °C assay temperature. b) Curve C of Fig. 5a up to 100 µg filipin/ml. c) Curve B of Fig. 5a up to 100 µg filipin/ml. 100% values corresponds to a specific Na/K-ATPase activity of 1274 (A), 696 (B) and 458 (C) µmol $P_i \times$ mg protein$^{-1} \times h^{-1}$. Representative results of 3 to 6 different experiments.

are devoid of or poor in particles and therefore correspond to the cellular EF (Fig. 6c). This implies that most of these membrane vesicles derived from the basolateral plasma membranes during homogenisation are right-side-out vesicles with the ATP-binding site of the Na/K-ATPase facing inwards. Consequently, the Na/K-ATPase activity of these vesicles remains undetected unless provisions are made that enable ATP and activators of the incubation medium surrounding the vesicles to reach the internal binding sites of the latent enzyme molecules. Such provisions are the transformation of closed vesicles into open cup-shaped membranes, and the adequate increase in membrane permeability of the closed vesicles. SDS and DOC act the first way [34, 65, 70] and filipin the second.

Direct evidence that filipin-cholesterol complexation renders the membrane more leaky was obtained from correlative negative staining, thin sectioning and tracer experiments. Negative staining of the microsomal fraction preincubated at F/C = 2 revealed the presence of F–C complexes in the membrane domains which were free of particles (Fig. 7a). No differences in particle distribution and the amount of F–C complexes were observed in dependence on the incubation temperature of 25 °C and 37 °C, and all vesicles, without exception, were labelled. This result assures that interaction of filipin and membrane cholesterol had actually taken place under the F/C condition which was most effective in stimulating the microsomal Na/K-ATPase. The complexes show the same circular structure and size as those

Fig. 6. Electron microscopy of the microsomal fraction of the duck salt gland. a) Thin section after glutaraldehyde-tannic acid fixation showing membrane vesicles contaminated with other cell structures. b) High magnification showing membrane asymmetry of 2 vesicles (arrows). c) Freeze fracture showing concave fracture faces (EF) with few or no particles and convex particle-rich fracture faces (PF), both indicative of right-side-out vesicles. d) Negatively stained preparation showing collapsed membrane vesicles with a marginal bulge and the superimposed surface particles arranged into clusters and strands. Scales 0.25 µm. (Structure of the untreated microsomes at zero filipin used for activity measurements in Fig. 3b, curve A.)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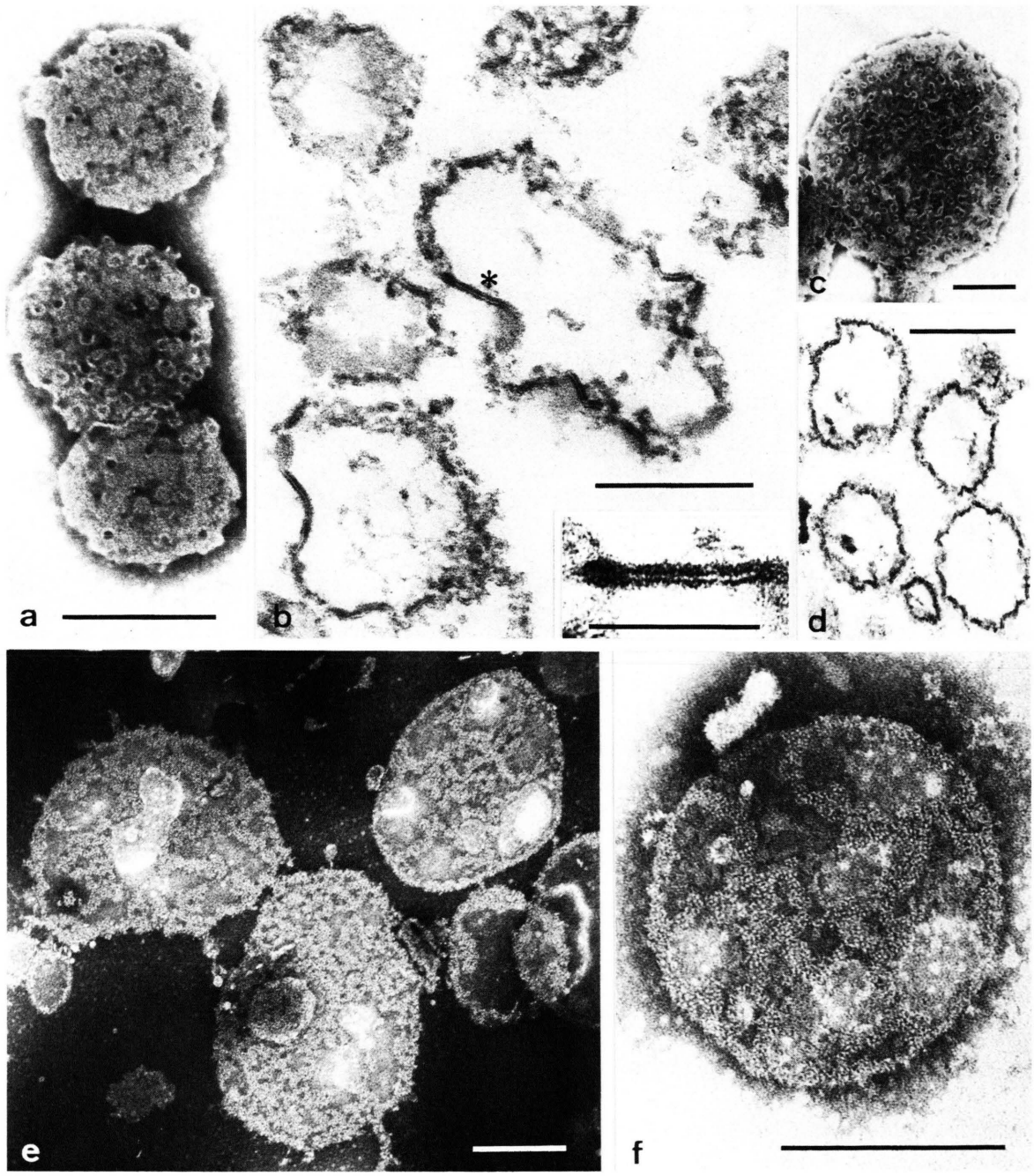


Fig. 7. Electron microscopy of microsomal vesicles after different pretreatments. a, b) Filipin pretreatment at F/P = 0.4 (F/C = 2) and (c, d) at F/P = 4 (F/C = 21); (a, c) negative staining; (b, d) glutaraldehyde-tannic-acid fixation, thin sections. Scales 0.25 μm. (Structure of the filipin-treated microsomes (a, b) causing activity peak of curve A in Fig. 3b.) Inset: High magnification of asymmetric membrane segment labelled in (b) with asterisk. Scale 0.1 μm. e, f) Negative staining of SDS (e) and DOC (f) treated microsomal vesicles. The opened, transformed vesicles appear as single-layered membrane sheets with surface particles arranged into clusters and strands. Scales 0.25 μm. (Structure of the detergent-treated microsomes at zero filipin used for the activity measurements in Fig. 3b, curves C and D.)

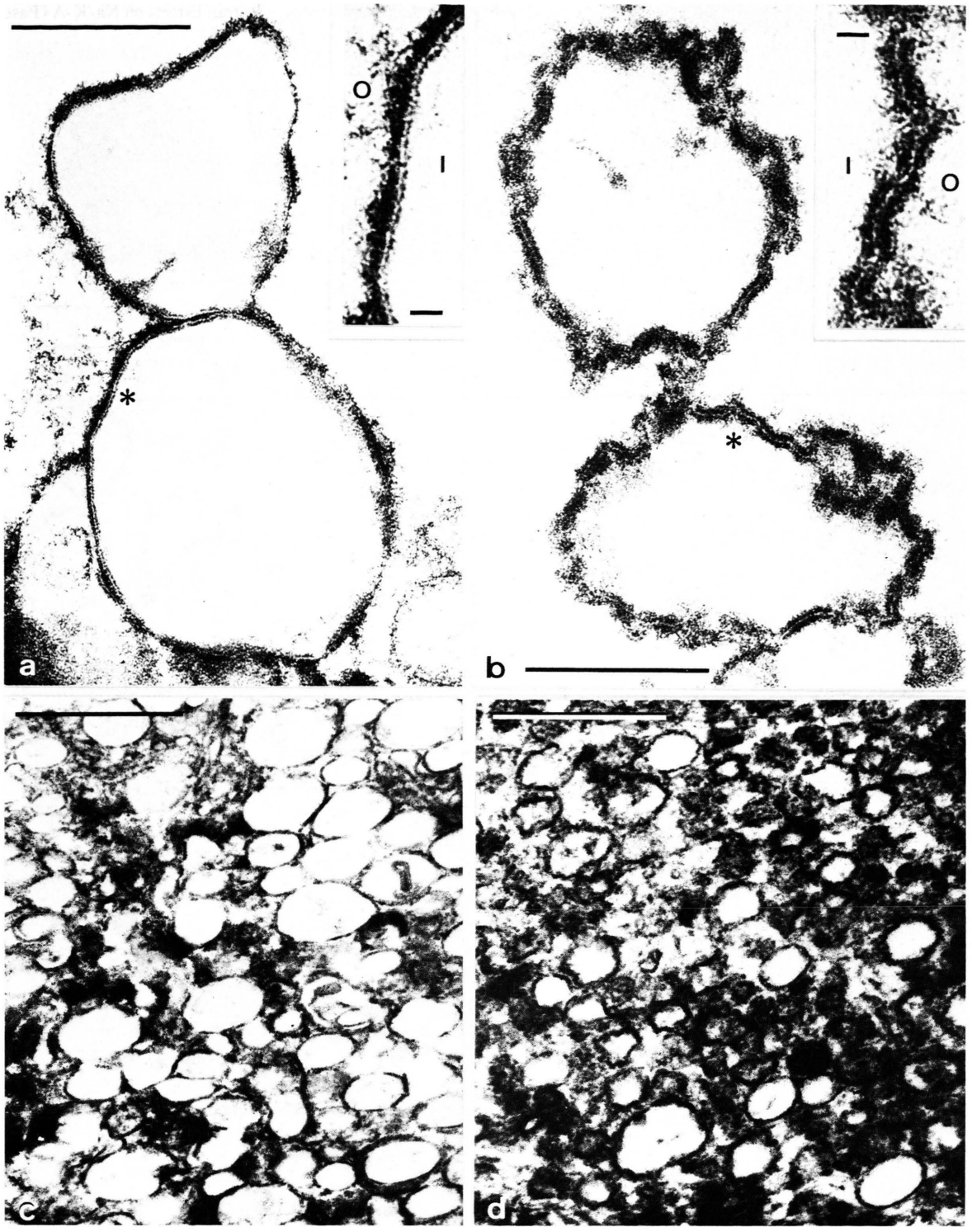


Fig. 8. Thin section electron microscopy of tracer experiments on the microsomal fraction. a, b) Application of the membrane marker ruthenium red on (a) the solvent control and (b) the filipin-pretreated vesicles (F/P = 0.4; F/C = 2). Scales 0.25 μm. Insets: High magnification of the respective membranes (asterisk) showing different label localization. I inside and O outside of vesicle. Scales 0.02 μm. c, d) Application of the volume marker microperoxidase on the (c) solvent control and (d) filipin-pretreated vesicles (F/P = 0.4; F/C = 2). Scales 1 μm.

described from negative staining of other natural and artificial membranes [10, 25, 76, 77, 78].

The clustering effect on unfixed plasma membranes *in situ* of cultured cells [79] allowed to expect a pronounced clustering of F – C complexes and particles in the unfixed microsomes. Such clustering was only occasionally observed, and it was hard to decide whether it actually resulted from filipin-treatment or merely represented extreme cases of the normal, clustered distribution. The large majoritiy of vesicles showed the same particle distribution as the untreated controls, and the F – C complexes fitted into the particle-free areas. Even at F/C = 21 the F – C complexes were rather evenly distributed (Fig. 7c). This points to some intrinsic constraint in particle positioning which impedes morphologically visible rearrangements within the isolated but otherwise still intact plasma membrane.

Thin sections showed that the formerly smooth membrane circumference (Fig. 6a, b) had changed into an irregularly pleated outline (Fig. 7b). The wavy membrane results from closely adjoining small membrane protuberances which are the thin section correlates of F – C interaction [13]. The interpersed segments of smooth membrane show the above-mentioned structural asymmetry after tannic fixation and apparently correspond to those membrane domains which are rich in particles. The structural continuity of the vesicular membranes is not interrupted by filipin treatment. The vesicular membrane profiles are still preserved even when the F/C has been raised for 1 order of magnitude to 21 (Fig. 7d).

The cationic membrane marker ruthenium red which binds to negatively charged structures but is unable to penetrate through intact plasma membranes [51, 80] heavily stains the external membrane surface of closed microsomal vesicles in the solvent control (Fig. 8a). However, both the external and internal membrane surfaces are heavily stained, when the vesicles were pretreated at optimal F/C = 2 (Fig. 8b). These different staining behaviors allow to conclude that the vesicular membrane is impermeable to ruthenium red (MW 860 [80]), but permeable after filipin treatment. This holds also true for ATP (MW 605) as judged from the filipin-induced activation of latent microsomal Na/K-ATPase. Accordingly, ruthenium red appears to mimic the exclusion of ATP from right-side-out vesicles and its free access to the internal catalytic sites after filipin treatment.

On the other hand, the volume markers myoglobin (MW 17 000), cytochrome C (MW 12 500) and microperoxidase (MW 1900) failed to penetrate through the vesicular membranes regardless of whether or not they were pretreated with filipin. Fig. 8c and d document the results obtained with microperoxidase, the smallest volume marker used. The black label is located in the interspaces between the vesicles of the solvent control and filipin-treated preparation. Inside-labelled vesicles observed in both preparations can be pretended by unfavorable cutting directions through actually open and hence infiltrated vesicles. They can also be pretended by tangential sections of actually closed vesicles which are free of internal label, but surrounded by label. The latter are more frequent after filipin-treatment due to the wavy outline of the vesicles. The clear exclusion of label from the lumen of many vesicles (Fig. 8d) indicates that the increase in membrane permeability caused by filipin treatment is sufficient for the penetration of the membrane marker with molecular weight of approx. 900 but insufficient for the penetration of the volume marker with molecular weight of about twice that value. These results do not reveal the structural and molecular basis of how filipin-cholesterol interaction leads to permeability increase but may be explained by the formation of a filipin-pore [81]. Enhancement of membrane permeability by filipin was also reported for other plasma membranes [82 – 87].

Studies on the purified membrane-bound and solubilized ATPase

The filipin effect was further studied on purified Na/K-ATPase membranes, because such preparation eliminates the problem of enzyme sidedness. It also allows direct reference of the enzyme protein and cholesterol content of the membranes, since the impurities of the microsomal fraction have been removed by SDS-treatment and discontinuous sucrose gradient centrifugation [34, 35]. Some comparative experiments were also performed on the solubilized enzyme, so that any interference with the membrane structure was excluded.

Lipid analysis: Purification leads to an increase in the total lipid content per mg protein of the resulting membrane preparation (Table I). This indicates that more protein than lipid was solubilized by the detergent. In agreement with the data obtained from the purified rabbit kidney membranes [36, 88], the

total lipid content of the purified salt gland membranes is almost identical with the total protein content. The membrane lipids consist for 72% of phospholipids and for 28% of neutral lipids and free fatty acids. Cholesterol amounts to 65% in the latter category *i.e.* 180 µg cholesterol per mg membrane protein. Since the cholesterol/phospholipid molar ratio of the purified membranes remains nearly unchanged when compared to the microsomal fraction both groups of lipid have been removed to about the same extent (65–70%) together with 80–90% of microsomal protein.

Enzyme solubilization from the purified Na/K-ATPase membranes by cholate followed by pelleting of the insoluble membrane material led to a supernatant containing twice as much lipid as protein with no substantial alteration of the cholesterol/phospholipid molar ratio (Table I). Subsequent dialytic cholate removal from the supernatant also removed about 75% of the total lipid and 90% of the cholesterol content of the solubilized enzyme preparation (Table I). Nevertheless, the remainder of lipid was still sufficient for the formation of proteoliposomes during dialytic cholate extraction. The proteoliposomes had incorporated about 50% of the solubilized enzyme, the other half remained in the supernatant after pelleting of the vesicles. The resulting vesicles with the reconstituted enzyme were, apart from some exceptions, 50–150 nm in diameter and studded with large numbers of surface particles representing the negative staining appearance of Na/K-ATPase (Fig. 10f). The high frequency of surface particles obviously resulted from the high protein/ lipid ratio of the dialysate. Freeze fractures of proteoliposomes formed from solubilized Na/K-ATPase in the presence of surplus phosphatidylcholine showed comparatively few particles [34, 38].

ATPase studies: Similar to the results with the microsomal fraction there is a temperature-dependent, filipin-induced inhibition of the purified membrane-bound Na/K-ATPase at F/C exceeding 2 (Fig. 5a, A, B). However in contrast to the microsomal fraction, no enzyme stimulation occurs at low F/C, since this purified enzyme preparation consists of open cup-shaped membranes. This result adds further support to the conclusion that enzyme stimulation of the microsomal fraction is a secondary effect, the primary effect being the filipin-induced increase in membrane permeability. At 37 °C 20 µg filipin/ml are sufficient for complete inhibition of the purified, membrane-bound Na/K-ATPase (Fig. 5a). This corresponds to 3×10^{-5} M filipin and indicates that under these conditions filipin is even potenter than the specific inhibitor ouabain [89].

The I_{50} values for 37 °C and 25 °C are 6 and 15 µg filipin/ml corresponding to F/C of 10 and 25. This temperature-dependence of enzyme inhibition favors the assumption that membrane fluidity and proper position of the enzyme relative to the membrane lipid are involved in the inhibitory effect. In order to test this assumption the membrane structure was eliminated by solubilizing the Na/K-ATPase with sodium cholate. The solubilized enzyme retained 40% of the activity of its membrane-bound state. Although the solubilized enzyme is still inhibited, its filipin-sensitivity is drastically reduced (Fig. 5a, b). At 37 °C the activity of the membrane-bound enzyme is completely abolished by 20 µg filipin/ml, whereas the solubilized form shows only about 20% inhibition under the same conditions. This comparison is based on the same F/P which is 10 in both cases. However, the F/C at F/P = 10 of the two preparations differ by the factor of 2, because the cholesterol per mg protein estimated for the solubilized prepartion is about twice the figure of the purified membrane preparation (Table I). Therefore, on the basis of equal F/C, curve A at 10 µg filipin/ml must be compared with curve C at 20 µg filipin/ml (Fig. 5a). Although the comparison on the basis of F/C may be questioned because of the different states of cholesterol in the 2 preparations, it equally shows a large difference in filipin-sensitivity of the membrane-bound and solubilized enzyme. Such difference is still apparent at 100 µg filipin/ml when inhibition of the membrane-bound enzyme at 25 °C is compared with the inhibition of the solubilized enzyme at 37 °C (Fig. 5b, c). The I_{50} values for the membrane-bound and solubilized froms both at 37 °C differ by about 1 order of magnitude (6 versus 60 µg filipin/ml). Removal of 75% of the total lipid and of 90% of the cholesterol content from the solubilized enzyme preparation which accompanies dialytic cholate extraction causes only 10% decrease in the Na/K-ATPase activity without changing the insensitivity to filipin (not shown) in comparison to the solubilized enzyme before dialysis (Fig. 5b).

Ultrastructural studies: Negative staining of unfixed purified Na/K-ATPase membranes of the avian salt gland reveal the enzyme as surface particles arrang-

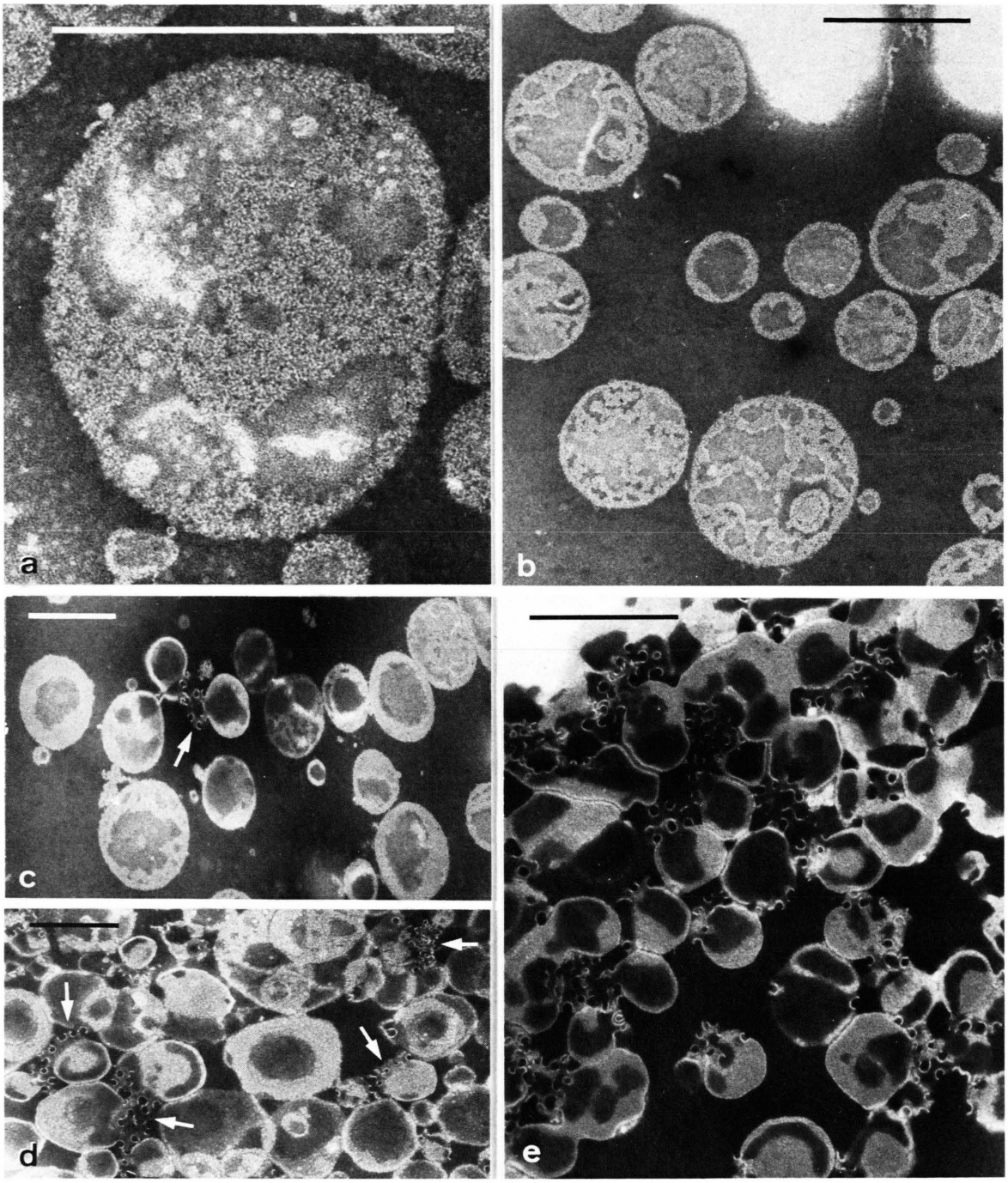
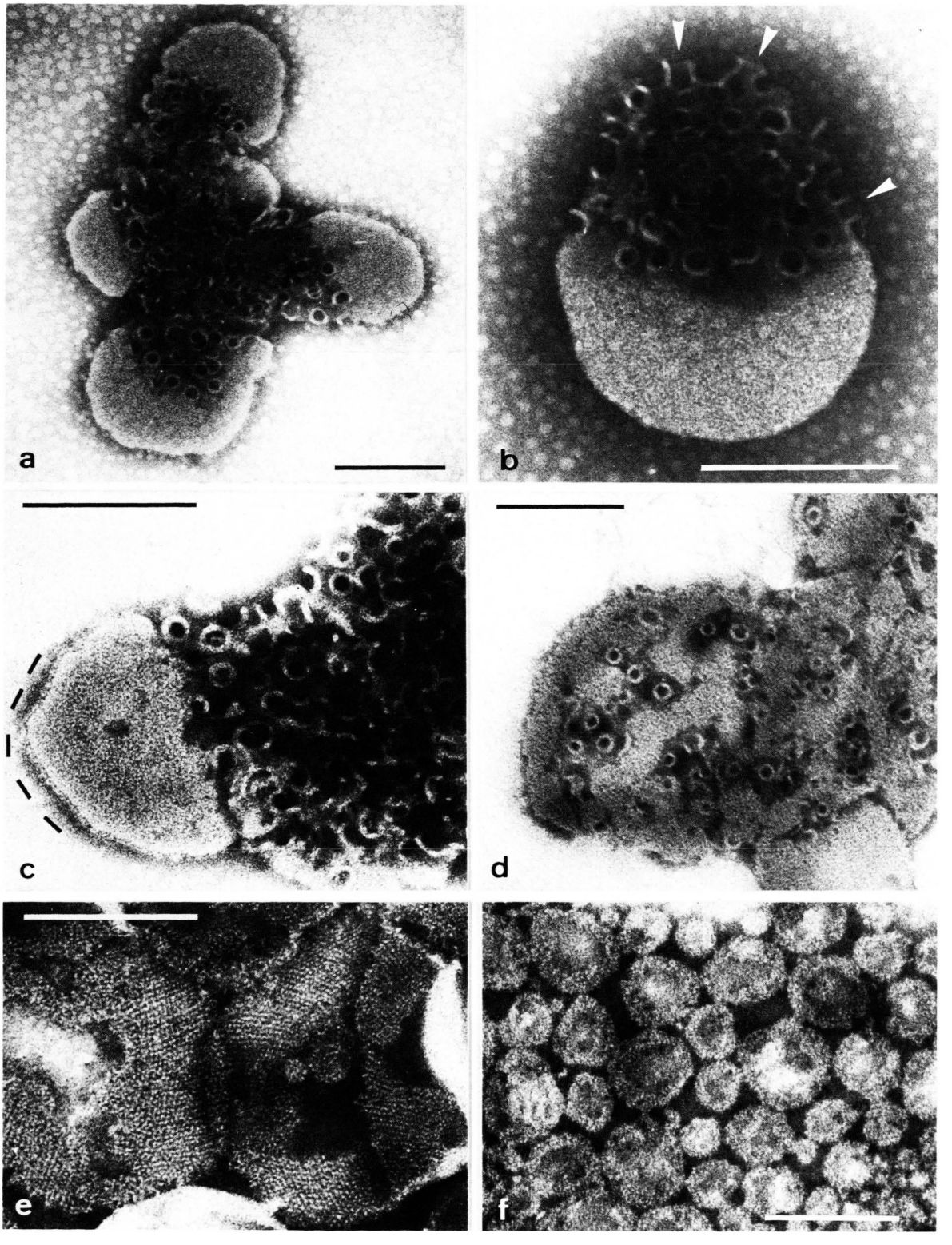


Fig. 9. Electron micrographs of negatively stained purified Na/K-ATPase membranes after different filipin pretreatments. a) Untreated control. b–e) Filipin-treatment at increasing F/P (F/C). b) F/P = 0.1 (F/C = 0.33). c) F/P = 0.2 (F/C = 0.66). d) F/P = 0.5 (F/C = 1.65) (e) F/P = 1 (F/C = 3.3). All scales 0.5 μm. Arrows in (c) and (d) label membranes showing structural F–C complexes.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ed into clusters and strands as previously described [34].

Incubation at F/P = 0.1 (F/C = 0.33) shows no structural difference to the untreated control (Fig. 9a, b). Since the stoichiometry of the chemical F−C complex is 1 : 1 [7], F/C = 0.33 offers the possibility for one third of the cholesterol to interact with filipin. However, no structural F−C complexes are visible. This is in line with the interpretation that the structural complexes represent aggregates of the chemical complexes or the structural implications of the aggregates [13, 25]. Consequently, the histochemical demonstration of cholesterol requires relatively high F/C. Structural F-C complexes become first visible in some of the purified membranes at F/P = 0.2 (Fig. 9c). Membrane labelling proceeds with the increase of F/P (Fig. 9d−e). Parallel with the increase in the number of F−C complexes a separation of enzyme particles and F−C complexes by lateral displacements becomes apparent, which is most pronounced at F/P = 3 (Fig. 10a−c) and indicates that cholesterol after complexation with filipin is no longer able to exert its membrane-stabilizing function. This capping effect with the enzyme particles on one side and the F−C complexes on the other appears to represent a final state, because doubling of F/P to 6 and increase in temperature from 25 ° to 37 °C had no further effect. At the margin of such membranes (Fig. 10c) the densely packed transmembrane enzyme particles are occasionally seen in side view [34, 66, 68]. Marginal side views also reveal the cylindrical shape of the F−C complexes (Fig. 10b).

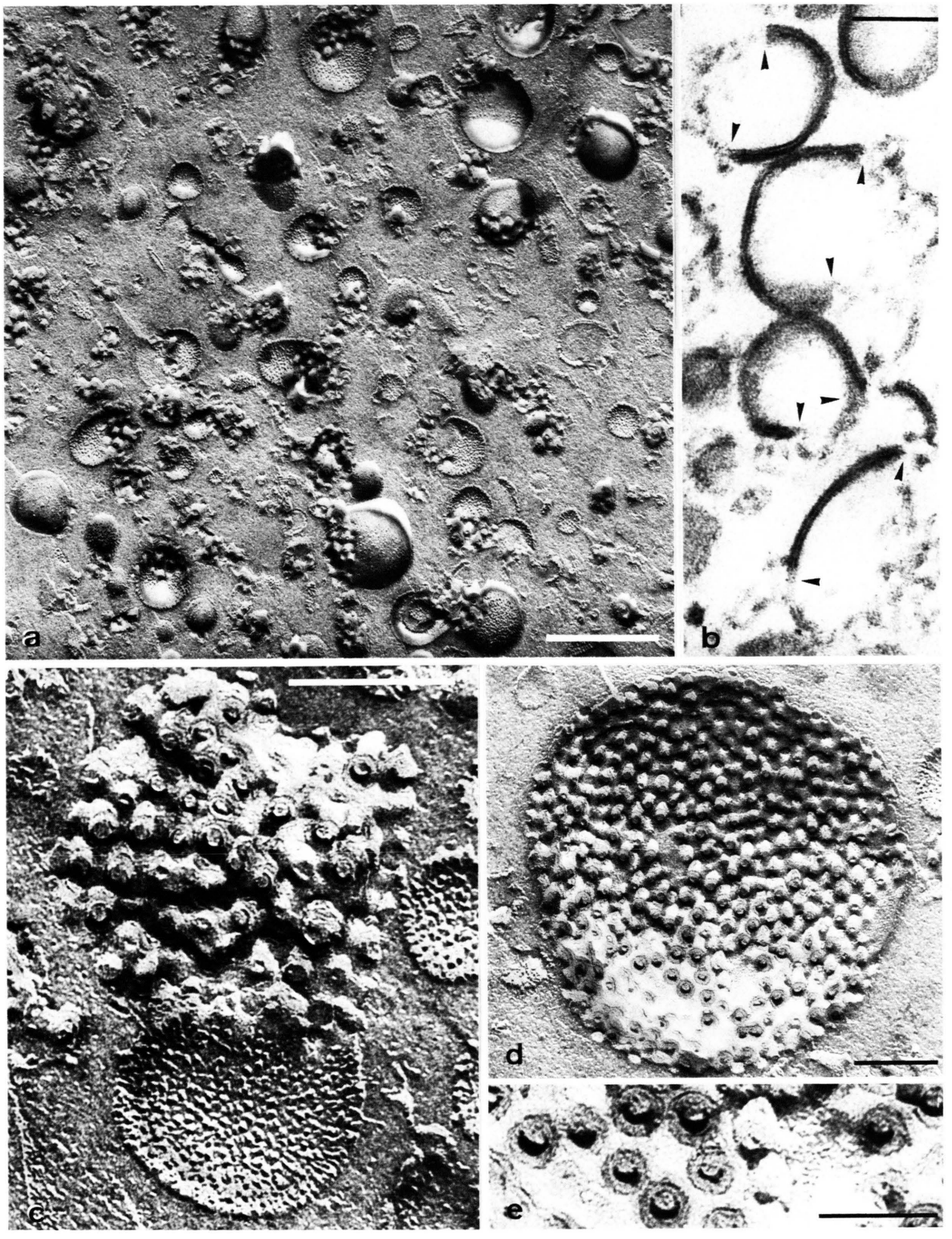
In contrast to the unfixed membranes, fixed membranes incubated at the same F/P = 3 showed the arrangement of enzyme particles characteristic of solvent controls regardless of whether or not these were fixed. The F−C complexes were located in the particle-free membrane domains (Fig. 10d). This clearly demonstrates that filipin failes to induce major lateral displacements of F−C complexes and integral membrane protein when the latter is anchored by glutaraldehyde fixation. The contradictory reports on the dependence [79] and independence [25] of clustering on the glutaraldehyde fixation appears to be related to the different cell membranes studied. The present study clearly shows that lateral displacements by filipin are prevented in the case of the microsomal membrane without fixation, whereas in the case of the purified membrane the prevention depends on fixation. Consequently, the component of the microsomal fraction responsible for the protein immobilization must have been removed by the purification process. The experiments performed on the erythrocyte plasma membrane [90] may shed some light on the possibility that a submembrane cytoskeletal protein such as spectrin could be involved in the anchorage of membrane particles. In this context, the finding deserves further attention that the 2 major bands in the gel pattern of the avian salt gland microsomes correspond to the positions of the catalytic subunit of the Na/K-ATPase and muscle actin.

Regardless of whether the membrane particles are naturally or artificially fixed in position, the filipin-labelling of the particle-free membrane areas indicates that these are rich in cholesterol and therefore more stabilized [5].

They seem to provide functionally more inert lipid areas of the membrane and are demarcated from interspersed membrane areas which are devoid of histochemically demonstrable cholesterol. These highly functional domains into which the Na/K-ATPase is embedded apparently facilitate conformational changes and molecular motions for enzyme function.

It should be mentioned, that the potency of artificial enzyme displacement in the purified Na/K-ATPase membranes is not unique to filipin. The vanadate-induced formation of two-dimensional crystals as first shown by Skriver *et al.* [57] for the purified renal membranes, also implies lateral diffusion of enzyme particles. Apart from the crystalline pattern, particle density of such enzyme crystals is

Fig. 10a−d. Negatively stained purified Na/K-ATPase membranes pretreated with filipin at a F/P = 3 (F/C = 10). a−c) Unfixed membranes showing a capping effect due to the sorting out of enzyme particles and F−C complexes by lateral displacements within the membrane. Arrowheads in (b) label side views of F−C complexes. Note the surface particles in side view at the margin of the membrane in (c) labelled with broken line. d) Membranes fixed prior to filipin treatment show an inplace anchoring of the enzyme particles, and F−C complexes exclusively located in the particle-free lipid areas. Note the particle-free halo around many F−C complexes. e) Negatively stained purified Na/K-ATPase membranes after 4 weeks exposure to vanadate, showing two-dimensional protein crystals. f) Negatively stained proteoliposomes formed by cholate dialysis and composed of Na/K-ATPase and lipid jointly solubilized from purified Na/K-ATPase membranes. Note the presence of numerous surface particles. All scales 0.2 μm.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similar to the filipin induced enzyme package (compare Fig. 10e and 10b). While filipin causes dramatic short-term displacements within few minutes, crystal formation induced by vanadate gradually continues over days and weeks.

Freeze fracture examination of unfixed purified Na/K-ATPase membranes treated with filipin at F/P = 3 confirms the results obtained with negative staining (compare Figs. 10b and 11c). The F−C complexes appear as 20−25 nm wide membrane protrusions and complementary depressions [10, 11, 13], which are jointly present on the same fracture face (Fig. 11d). The occurrence of complexes is neither related to the curvature nor the particle population of the fracture faces (Fig. 11a).

As a result of filipin-induced rearrangements within the cup-shaped membranes the IMPs are concentrated on one side and the F−C complexes on the other (Fig. 11c). While negative staining of the surfactant-treated membranes readily reveals this separated distribution of the two structures, their detection by freeze fracture depends on the coincidentally proper fracture plane. This fact explains the pretended disorder of structures seen in Fig. 11a, as well as the pretended vesicles exclusively showing either IMPs or F−C complexes, or none of both (Fig. 11a, d).

Under appropriate angles of shadowing the F−C complexes appear as 40 nm hat-like protrusions (Fig. 11c, d, e). The 10 nm wide brim (Fig. 11e) has no correlate in negative staining preparations. Since it is also depicted in the shadow it seems to represent a decoration effect.

Similar to freeze fractures, thin sections of Na/K-ATPase membranes treated under capping conditions showed a multitude of different membrane profiles resulting from the casual section geometry. Nevertheless, favorable places revealed the clear separation of a wavy, usually diffuse, faintly stained membrane region from a smooth, heavily stained membrane segment (Fig. 11b). The heavy staining after tannic acid fixation reflects the dense package of enzyme protein in the smooth membrane areas devoid of F−C complexes. Membrane asymmetry was not as apparent as in the filipin-treated microsomes (compare Figs. 11b and 6b).

Discussion

The avian salt gland is a highly specialized organ of extrarenal NaCl excretion and its glandular tubules represent one of the most effective and highest organized ion transporting epithelia [91]. Salt-stressing leads to the enhancement of Na/K-ATPase activity [32] which is accompanied by an extensive amplication of the lateral plasma membranes [59, 60]. These membranes are the exclusive seat of Na/K-ATPase [31].

Treatment of the salt gland microsomal fraction with detergent at critical micelle concentration causing rupture or lysis of the membrane [37] increases the Na/K-ATPase activity [34, 35]. This kind of enzyme activation also obtained with plasma membrane preparations of other sources was interpreted as a result of unmasking the catalytic sites of latent Na/K-ATPase which are located in tightly sealed right-side-out vesicles derived from the broken basolateral plasma membrane [70, 71, 92]. The orientation of the vesicular membrane and thus the sidedness of the Na/K-ATPase can be deduced from freeze fracture examination [70]. Most microsomal vesicles of the avian salt gland were right-side-out vesicles as judged from freeze fracture replicas.

The drastic increase in Na/K-ATPase activity by SDS, filipin and combined DOC-filipin treatments up to 300% indicates that about 2 thirds of the microsomal enzyme was located in closed right-side-out vesicles, the remainder third in inside-out vesicles or in vesicles with broken membranes. Filipin treatment at low F/C increased membrane permeability thereby providing access of the incubation medium to the interior of the right-side-out-vesicles for the activation of latent ATPase. The same activation mechanism was reported for the channel-

Fig. 11. Freeze fractures (a, c−e) and thin section (b) of purified Na/K-ATPase membranes which were treated with filipin at a F/P = 3 (F/C = 10) in the unfixed state. a) Survey freeze fracture replica showing the various structural aspects in dependence on the local fracture planes. Scale 0.5 μm. b) In thin section the membrane areas with densely packed enzyme particles are usually well depicted by their smooth outline and heavy staining, whereas the membrane areas with densely packed F−C complexes appear faintly stained and highly irregular in outline. Arrowheads label transitions of 2 different membrane areas. (Glutaraldehyde-tannic acid fixation.) Scale 0.1 μm. c) Favorable fracture plane clearly showing the separation of IMPs and F−C complexes. Scale 0.2 μm. d) Fracture plane showing only F−C complexes. Scale 0.2 μm. e) High magnification of the hat-like F−C complexes from Fig. 11d. Scale 0.1 μm.

forming ionophore alamethicin which parallely unmasked latent adenylate cyclase and Na/K-ATPase in right-side-out cardiac sarcolemmal vesicles [93].

Interaction of filipin and cholesterol can change membrane permeability [82–87] and cause structural distortions [77, 79] or even disruptions of the membrane [94] the extent of which depends on the F/C, exposure time and temperature [7]. Under the conditions applied in this study, structural disruptions by filipin of the vesicular membranes did not play a major – if any – role. The exclusion of microperoxidase from the interior of filipin-treated vesicles suggests that inspite of the increase in permeability the barrier function of the membrane still exists even for extremely small proteinaneous volume markers. Consequently, the leakiness created by filipin for the permeation of relatively small substances such as ATP and ruthenium red appears to be related to molecular rearrangements within the membrane rather than to structural interruptions. Hence, in addition to the ionophore alamethicin, filipin at appropriate F/C offers a true alternative to the detergents which along with membrane rupture remove high amounts of protein and lipid. Supplementary to its application in lipid histochemistry and membrane physiology, it may be a useful tool to study the sidedness and localization of vectorial enzymes making use of equal and different membrane cholesterol contents [26].

The inhibitory effect of filipin on the avian salt gland ATPase is intricate and more difficult to understand than the activation effect. The studies describing filipin interference with other membrane-bound enzymes offer no further explanation of the inhibition mechanism [26, 27, 95].

The low sensitivity of the solubilized enzyme allows to discard a putative filipin-protein interaction as the primary effect.

The extreme clustering of ATPase and F–C complexes may lead to the assumption that inhibition possibly results from the apparent phase separation [96] and/or from steric hindrance for conformational changes of the tightly packed enzyme. However this explanation does not hold for several reasons: 1) Enzyme inhibition by filipin is temperature dependent but filipin-induced protein capping is not. 2) The capping effect by F/C = 10 appears to represent a final stage, but enzyme inhibition by the same F/C is only 25% and 50% at 25 ° and 37 °C respectively, and proceeds with the increase in F/C. 3) F/C = 10 failes to induce enzyme capping in the microsomal vesicles but is inhibitory to the microsomal ATPase.

The suggestion [97] that alterations of membrane fluidity as caused by filipin-cholesterol interaction [29] may change enzyme activity appears to offer a more attractive explanation. The dramatic pertubation of membrane lipid in virtue of F–C complexation, which restricts cholesterol interaction with other lipids [98, 99], certainly influences the lipidic microenvironment of the enzyme and thus affects its activity. Another dramatic change of the lipidic environment is the solubilization of the enzyme from the membrane that leads to 60% inhibition. The solubilized, still active enzyme is presumably a component of mixed micellar structures [40] which can contain cholesterol (Table I). Therefore, filipin can still affect enzyme activity via cholesterol interaction depending on whether or not cholesterol and enzyme happen to be jointly present in those structures. Accordingly, the solubilized form is far less sensitive to filipin than the membrane-bound enzyme.

The unfixed purified Na/K-ATPase membranes of the avian salt gland showed a graded labelling by F–C complexes with the increase of F/C up to 10. This indicates that the formation of structural F–C complexes continues to a tenfold surplus of filipin for the possibility of chemical F–C complex formation. However, enzyme inhibition proceeds to F/C of 33 and 165 at 37 ° and 25 °C incubation temperature, respectively. Such high surplus of filipin and its inhibitory action on the solubilized enzyme lead to the question whether its chemical property to complex with cholesterol or an additional property causes invisible lipid perturbation at the molecular level and enzyme inhibition under these high F/C conditions. Although the complete extraction or oxydation of cholesterol plays no essential role for the activity of purified renal Na/K-ATPase [88], the primary filipin-cholesterol interaction possibly in conjunction with secondary effects severely deteriorates the lipidic environment of the enzyme [100] in the purified membranes of the avian salt gland and thus leads to complete inhibition.

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