

BASOLATERAL PLASMA MEMBRANE LOCALIZATION OF OUABAIN-SENSITIVE SODIUM TRANSPORT SITES IN THE SECRETORY EPITHELIUM OF THE AVIAN SALT GLAND

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ABSTRACT

The distribution of Na⁺ pump sites (Na⁺-K⁺-ATPase) in the secretory epithelium of the avian salt gland was demonstrated by freeze-dry autoradiographic analysis of [³H]ouabain binding sites. Kinetic studies indicated that near saturation of tissue binding sites occurred when slices of salt glands from salt-stressed ducks were exposed to 2.2 μM ouabain (containing 5 μCi/ml [³H]ouabain) for 90 min. Washing with label-free Ringer's solution for 90 min extracted only 10% of the inhibitor, an amount which corresponded to ouabain present in the tissue spaces labeled by [¹⁴C]inulin. Increasing the KCl concentration of the incubation medium reduced the rate of ouabain binding but not the maximal amount bound. In contrast to the low level of ouabain binding to salt glands of ducks maintained on a freshwater regimen, exposure to a salt water diet led to a more than threefold increase in binding within 9–11 days. This increase paralleled the similar increment in Na⁺-K⁺-ATPase activity described previously. [³H]ouabain binding sites were localized autoradiographically to the folded basolateral plasma membranes of the principal secretory cells. The luminal surfaces of these cells were unlabeled. Mitotically active peripheral cells were also unlabeled. The cell-specific pattern of [³H]ouabain binding to principal secretory cells and the membrane-specific localization of binding sites to the nonluminal surfaces of these cells were identical to the distribution of Na⁺-K⁺-ATPase as reflected by the cytochemical localization of ouabain-sensitive and K⁺-dependent nitrophenyl phosphatase activity. The relationship between the nonluminal localization of Na⁺-K⁺-ATPase and the possible role of the enzyme in NaCl secretion is considered in the light of physiological data on electrolyte transport in salt glands and other secretory epithelia.

KEY WORDS Na⁺ pump · Na⁺-K⁺-ATPase · [³H]ouabain · salt glands · salt secretion

A major function of many epithelia is the transepithelial transport of salt and water. Much of our current knowledge concerning electrolyte transport is derived from studies of reabsorptive epithe-

lia where the net direction of fluid flow is from a free (or luminal) surface to the epithelial surface facing the blood. Although several solute pumps have been described in the literature, Na⁺ pumps play a major role in solute-linked water movement. In this regard, the enzymatic basis for active Na⁺ transport appears to be mediated, at least in

part, by ouabain-sensitive $\text{Na}^+\text{-K}^+\text{-ATPase}$ (68). Physiological studies with reabsorptive epithelia generally support the concept that transepithelial salt and water flow is driven by ouabain-sensitive solute pumps situated at the basal pole of the cell, and this hypothesis has received direct morphological support from cytochemical and autoradiographic studies which show that ouabain-sensitive $\text{Na}^+\text{-K}^+\text{-ATPase}$ is restricted primarily to the basolateral plasma membrane (19, 40, 41, 48, 49, 57, 71, 72).

In contrast to reabsorptive epithelia, the relationship between $\text{Na}^+\text{-K}^+\text{-ATPase}$ and the transepithelial movement of NaCl from blood to lumen across secretory epithelia is poorly understood. It has been assumed that $\text{Na}^+\text{-K}^+\text{-ATPase}$ is localized predominantly along the luminal border in this type of epithelium, (e.g., references 7 and 45). Such an orientation would provide a concentrative extrusion step at the apical interface, despite the low intracellular Na^+ concentration which is common to most cells. The volume and tonicity of the resulting effluent would be a function, therefore, of factors such as rate of luminal pumping, permeability of the luminal membrane and intercellular junctions to ions and water, and the rate of Na^+ entry (presumably passive) across the basolateral plasma membrane to prime the apical pumps. This generalized model for Na^+ secretion has been explored experimentally in considerable depth in the avian salt gland (reviewed in detail by Peaker and Linzell [53]). These supraorbital nasal glands excrete an almost pure NaCl solution which is hypertonic to both plasma and seawater (62), thereby allowing salt-stressed birds to utilize salt water for the maintenance of a positive water balance. In contrast to the proposed apical concentrative mechanism in this tissue, however, biochemical and morphological studies of developing salt-stressed glands (20, 21) suggested indirectly that the transport ATPase might be associated with the basolateral rather than the apical plasmalemma. This hypothesis was supported by cytochemical results obtained with a new electron microscope procedure which showed that the $\text{Na}^+\text{-K}^+\text{-ATPase}$ enzyme complex was restricted to the basolateral cell surfaces of the secretory epithelium (17, 18). Since intracellular ion concentrations in salt gland slices were shown repeatedly to be low for Na^+ and high for K^+ (51, 61, 76), the data suggest that $\text{Na}^+\text{-K}^+\text{-ATPase}$ is oriented to extrude Na^+ and to accumulate K^+ across the basal pole of the cell. Accordingly, the

spatial orientation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in this secretory epithelium would appear, paradoxically, to be identical to that in reabsorptive epithelia despite the opposing direction of net salt and water flux in the two epithelial classes. Furthermore, the absence of cytochemically demonstrable $\text{Na}^+\text{-K}^+\text{-ATPase}$ along the apical plasmalemma suggests that ion concentration across this membrane involves a ouabain-insensitive pump or, alternatively, that some other mechanism which does not require luminal active transport functions in salt gland secretion.

Although the cytochemical procedure for the localization of $\text{Na}^+\text{-K}^+\text{-ATPase}$ provides the best evidence to date concerning the subcellular localization of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in this secretory epithelium, the use of an alternative method is clearly warranted in view of the apparent conflict between the observed basolateral membrane activity and the apical localization which might be expected on the basis of existing physiological data. Furthermore, current views concerning the mechanism of salt secretion in the avian salt gland (summarized by Peaker and Linzell [53]) are based, in part, on a single study which indicated that transepithelial duct potential (lumen positive) and NaCl secretion are abolished by a retrograde injection of ouabain (73), presumably by inhibition of a ouabain-sensitive luminal pump. Accordingly, the present investigation examines the light microscope distribution of [^3H]ouabain binding sites in avian salt gland by the freeze-dry autoradiographic procedure first described by Stirling (71, 72). Careful studies utilizing this autoradiographic procedure with other transporting epithelia (34, 48, 49, 57, 58, 71) have shown the method to be highly sensitive and specific for the localization of ouabain-sensitive transport sites. The results of the present investigation demonstrate that [^3H]ouabain binding sites are localized exclusively to the basolateral plasmalemma of salt gland cells. The observed pattern of localization is identical, therefore, to that demonstrated by the ultracytochemical method for the localization of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ enzyme complex (18) and raises questions as to the specific function of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in transepithelial secretion of NaCl .

A preliminary report of these studies was presented previously (22).

MATERIALS AND METHODS

Animals

Domestic ducklings (*Anas platyrhynchos*) received

several days after hatching were maintained on a regimen consisting of commercial chick starting mash and 1% NaCl drinking water. Previous studies with the avian salt gland showed that exposure to a 12-h salt water–12-h fresh water diet increased glandular Na⁺-K⁺-ATPase to maximal levels (21) and insured complete differentiation of the glandular epithelium (20). For convenience, most of the studies described below were conducted with birds maintained continuously on the 1% NaCl diet for at least 3 wk. However, in studies designed to relate days of salt water exposure to ouabain binding by salt gland slices, the 12-h salt water regimen was used. This allowed for direct comparison with the previous study (21) in which the relationship between exposure to this diet and glandular Na⁺-K⁺-ATPase levels was examined.

Tissue Preparation

Preliminary studies (see Results) indicated that conventional sectioning techniques, employing a Stadie-Riggs tissue slicer, produced sections which were too thick (500 μm or greater) to allow for saturation of salt gland ouabain binding sites within a reasonable period of incubation without excessive reduction in the specific activity of [³H]ouabain by the addition of unlabeled inhibitor. Satisfactory sections could be obtained, however, by using an Oxford Vibratome (Oxford Vibratome sectioning system, Model G, Oxford Laboratories, Foster City, Calif.) which is capable of producing relatively thin sections (100–150 μm) of unfixed and unfrozen tissue by subjecting tissue blocks to a laterally directed vibrating razor blade.

Freshly excised salt glands were transferred to cold Krebs-Ringer bicarbonate buffer (37) containing 6 mM glucose and saturated with 95% O₂ and 5% CO₂. Tissue blocks (1–3 mm thick) were prepared by slicing the gland transversely with a razor blade. The tough layer of connective tissue adhering to the gland was subsequently trimmed from the blocks. Tissue blocks were then glued to aluminum chucks by means of a tissue adhesive (Loctite Quick-set 404 adhesive, Loctite Corporation, Newington, Conn.). The chucks were clamped singly on the Vibratome specimen stage and covered with several drops of 6% agar (cooled to 40°C before application) to provide tissue support during sectioning. The specimen stage itself was mounted permanently in the base of a plastic petri dish which, in turn, was mounted in the Vibratome bath reservoir. After covering the tissue with agar, the petri dish was filled with ice-cold Ringer's solution thereby insuring that the tissue block was submerged during sectioning. The bath reservoir was filled with ice and water in order to maintain the contents of the petri dish at 1–4°C. Sections of salt gland were cut utilizing the minimal amplitude of razor blade vibration required to achieve sections of uniform thickness (100–150 μm). Sections were transferred to fresh cold Ringer's solution and carefully separated from any adhering agar.

[³H]Ouabain Binding Studies

In order to determine a suitable incubation period for equilibrium binding of ouabain at or near saturation of tissue binding sites, salt gland sections were incubated at 37°C for varying time periods in 1 milliliter of standard Na⁺-Ringer's solution containing 5 μCi of [³H]ouabain plus unlabeled inhibitor. Ouabain uptake as a function of loading time was analyzed by liquid scintillation counting at three ouabain concentrations: 0.44 μM, 2.2 μM, and 4.4 μM. In some of these experiments 5 μCi of [¹⁴C]inulin (sp act, 2.56 mCi/g) were included in the incubation medium as a marker for the extracellular space. In order to analyze the effect of elevated K⁺ concentration on ouabain uptake, salt gland slices were exposed for varying time periods to 2.2 μM ouabain in a Ringer's solution in which the KCl concentration was raised to 83 mM by replacement of NaCl with KCl (K⁺-Ringer's solution). To show that ouabain binding in salt gland slices is essentially irreversible, sections were exposed for 90 min to Na⁺-Ringer's solution containing 5 μCi of [³H]ouabain (final ouabain concentration was 2.2 μM) and then washed with one milliliter aliquots of ouabain-free Na⁺-Ringer's solution (4°C) for successive periods of 5, 10, and 15 min, followed by four 30-min rinses. The amount of label appearing in the rinses and in the tissue was then determined. In most of these experiments, 5 μCi of [¹⁴C]inulin was included in the incubation medium in order to provide comparative washout curves for the two labeled compounds. Each binding experiment was repeated two to six times with salt glands from different animals, and two to five replicates were assayed for each experimentally determined point within a given experiment. Morphological examination of tissue slices incubated and rinsed for the time periods described above generally showed no marked alteration in tissue fine structure except for some expansion of intercellular spaces.

Liquid Scintillation Counting

After incubation, tissue sections were dried overnight at 90°C on tared glassine weighing paper, and tissue dry weights were then determined with a Cahn Electrobalance (Cahn Instrument Div., Ventron Corp., Cerritos, Calif.). Dry weights for individual sections generally were in the range of 100–300 μg, which represent approx. 16% of tissue wet weight. Dried tissue samples were transferred to scintillation vials, moistened with 150 μl of distilled water, and then treated overnight at 45°C with one milliliter of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.). The resulting digest was mixed with 10 ml of Dimilume scintillation cocktail (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a Packard Tri-carb scintillation spectrometer (model 3320). Counts per minute were converted to disintegrations per minute after correction for quenching by the external standard method. Binding data are expressed as pmol ouabain bound per milligram

dry weight. Radioisotopes were obtained from New England Nuclear, Boston, Mass.

Effect of Salt Water Stress on Ouabain Binding

For these experiments, newly hatched ducklings were maintained on fresh water for several days and then divided into two groups. The experimental group received 1% NaCl drinking water for 12 h and tap water for the remainder of the day, whereas the control group received tap water exclusively. On various days, salt glands from two control and two experimental animals were removed for ouabain binding studies. Four Vibratome sections of several tissue blocks from the salt glands of each animal were assayed for ouabain binding after exposure to 2.2 μM ouabain containing 5 μCi [^3H]ouabain for 90 min at 37°C and rinses for 90 min at 4°C.

Autoradiographic Studies

Preparation of tissue for autoradiography is based on a procedure modified somewhat from the original method described by Stirling (71, 72). The methodology currently employed in our laboratory is described in detail elsewhere (49). After exposure to [^3H]ouabain and rinses in unlabeled Ringer's solution, individual sections were spread out on the surface of a piece of aluminum foil. Most of the adhering medium was then removed with filter paper before rapid freezing by immersion in Freon cooled to liquid nitrogen temperature. The frozen sections were then transferred to a Stumpf-Roth freeze-dry apparatus (Thermovac Industries Corp., Co-paigue, N. Y.). Subsequently, freeze-dried sections were vapor-fixed under vacuum with OsO_4 overnight (34) and embedded in the low viscosity Spurr resin (Electron Microscopy Sciences, Fort Washington, Pa.). Sections (1 μm thick) were coated with NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.), exposed for 7–38 days at 4°C, developed with Dektol (Eastman Kodak) and stained with methylene blue as described previously (49). Appropriate controls were performed to exclude chemographic effects. In some experiments in which the distribution of [^{14}C]inulin in washed and unwashed sections was examined autoradiographically, NTB-3 emulsion was employed.

Cytochemical Studies

For comparative purposes, the ultrastructural cytochemical method (17, 18) for the localization of the ouabain-sensitive, K^+ -dependent phosphatase component of the Na^+ - K^+ -ATPase complex, utilizing *p*-nitrophenylphosphate (NPP) as a substrate, was modified to show the distribution of the enzyme at the level of the light microscope. Cryostat sections (10 μm thick) of salt gland tissue fixed for 45 min with 3% formaldehyde prepared from paraformaldehyde (23) and Vibratome

sections (100 μm thick) fixed for 5 min with 1% formaldehyde-0.25% glutaraldehyde (19) were incubated at room temperature for 60–180 min, in the cytochemical medium described previously (18). Control sections were incubated either in the absence of KCl or in the presence of KCl plus 10 mM ouabain. After the incubation, strontium phosphate, precipitated at the site of hydrolysis of NPP, was converted to lead phosphate and then to visible lead sulfide by sequential treatment with 2% $\text{Pb}(\text{NO}_3)_2$ and 1% $(\text{NH}_4)_2\text{S}$ (19). Cryostat sections were mounted on glass slides for light microscope examination. Vibratome sections were embedded in Spurr resin, and 1- μm thick sections were examined by conventional and phase-contrast microscopy for deposits of lead sulfide and lead phosphate, respectively.

RESULTS

Ouabain Uptake as a Function of Incubation Time

Initial uptake studies were carried out with tissue sections prepared with a Stadie-Riggs tissue slicer and exposed to 0.44 μM ouabain for varying times. As is shown in Fig. 1, these comparatively thick sections (approx. 500 μm) contain so many binding sites that uptake is nearly linear over the entire course of the incubation. A double reciprocal plot of these data (not shown) approximates a half-maximal loading time of 210 min. As expected from these kinetic data, autoradiographs of Stadie-Riggs slices exposed to [^3H]ouabain for 90 min showed a non-uniform pattern of grain distribution with most of the binding sites localized to the peripheral portions of the tissue slice.

Considerably thinner sections (100 μm) were obtained with an Oxford Vibratome tissue sectioner. These sections allowed for rapid equilibrium binding at relatively low ouabain concentrations and were more suitable for the rapid freezing required for reasonable morphological preservation of tissues prepared for freeze-dry autoradiography. Fig. 1 shows the time-courses of ouabain uptake by Vibratome sections over a 10-fold range of ouabain concentrations. Uptake of inhibitor in normal Na^+ -Ringer's solution is rapid at all three concentrations of ouabain tested and, in the case of exposure to 2.2 and 4.4 μM ouabain, approaches equilibrium by 90 min. In contrast, uptake of the extracellular marker [^{14}C]inulin reaches equilibrium by 5–15 min (Fig. 1).

Fig. 2 shows double reciprocal plots of the ouabain uptake data presented in Fig. 1. The half-maximal loading time and the maximal uptake of ouabain at equilibrium, derived respectively from

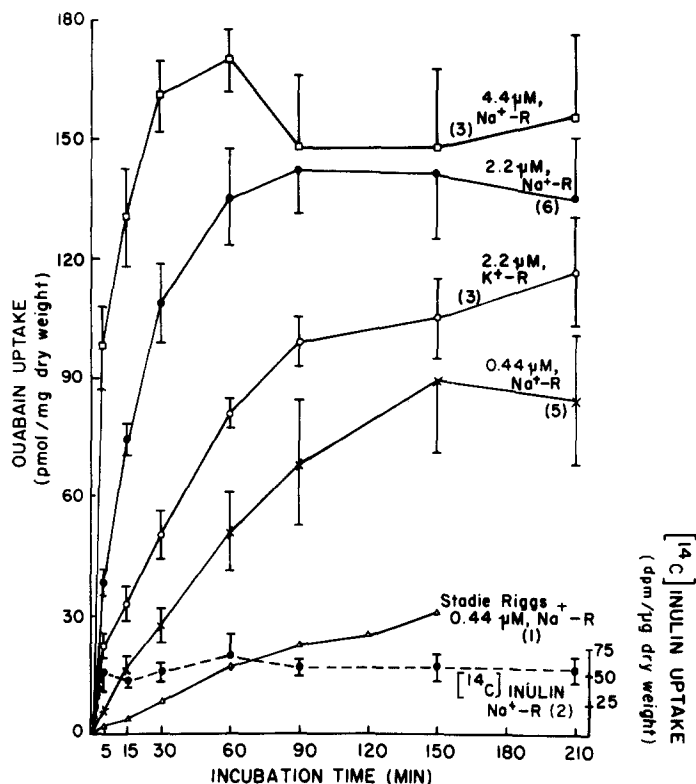


FIGURE 1 The effect of incubation time on the uptake of ouabain and inulin by salt gland slices. Slices (approx. 500 μm thick) prepared with the Stadie-Riggs tissue sectioner were exposed to 0.44 μM [^3H]ouabain (5 μCi) in normal Na^+ Ringer's solution ($\text{Na}^+\text{-R}$) (Δ — Δ). The other uptake curves were derived from exposure of Vibratome sections (approx. 100 μm thick) to 5 μCi of [^3H]ouabain in $\text{Na}^+\text{-R}$ containing final ouabain concentrations of 0.44 μM (\times — \times), 2.2 μM (\bullet — \bullet), and 4.4 μM (\square — \square), or in K^+ Ringer's solution ($\text{K}^+\text{-R}$) containing 2.2 μM ouabain (\circ — \circ). Uptake of [^{14}C]inulin (5 μCi , $\text{Na}^+\text{-R}$) by Vibratome sections (\bullet — \bullet) is expressed as $\text{dpm}/\mu\text{g}$ dry weight (right ordinate) and ouabain uptake is expressed as pmol/mg dry weight (left ordinate). Each point is the mean \pm SEM (vertical lines). n = number of animals and is given for each curve in parentheses.

the reciprocals of the X- and Y-intercepts for each curve, are compared in Table I. Although the interaction between ouabain and $\text{Na}^+\text{-K}^+\text{-ATPase}$ is bimolecular in nature and therefore follows second-order kinetics, less than 5% of the ouabain present in the medium was taken up by the tissue during the incubatory period. Consequently, since the medium ouabain concentration is, in effect, constant, the kinetics approximate first order and the constants determined from the double reciprocal plots are empirically accurate. Whereas 90 min is required to achieve 50% of binding equilibrium at 0.44 μM ouabain, a 5- or 10-fold increase in ouabain concentration reduces the half-maximal loading time to 14 min and 3 min, respectively. Total uptake of ouabain at equilibrium, i.e., bound plus unbound inhibitor, and equilibrium

binding to tissue sites (after correction for the ouabain present in the inulin space) were nearly the same for both 2.2 and 4.4 μM ouabain in the incubation medium (Table I). These data suggest that near saturation of binding sites occurs when binding equilibrium is obtained at the lower concentration of ouabain. Since a 90-min exposure to 2.2 μM ouabain achieved approx. 87% of maximal equilibrium uptake of inhibitor (calculated from the half-maximal loading time), these conditions were chosen for subsequent kinetic and autoradiographic experiments.

Effect of Elevated KCl Concentrations on Ouabain Binding

High K^+ concentrations are known to reduce the level of inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ by oua-

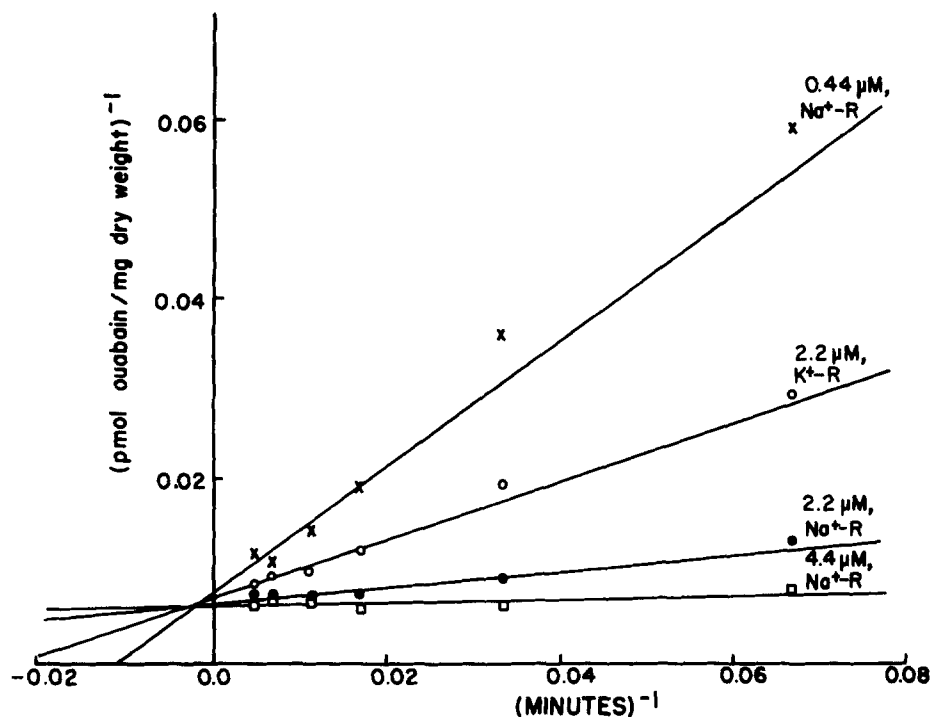


FIGURE 2 Double reciprocal plots of the data shown in Fig. 1. The data for ouabain uptake by Vibratome sections incubated in Na^+ -R or K^+ -R containing varying concentrations of ouabain (Fig. 1) were fitted to the equation for a hyperbola by the computer program described by Cleland (10). The symbols are the same as in Fig. 1. The reciprocals of the 5-min points are not shown but were used to determine the computed lines. The reciprocals of the X- and Y-intercepts for each line represent the half-maximal loading times and maximal levels of uptake at equilibrium, respectively, and are given in Table I.

TABLE I
Effect of Medium Ouabain Concentration on Half-Maximal Loading Time, Equilibrium Uptake, and Equilibrium-Binding of Ouabain in Salt Gland Slices

Ouabain concn	Ringer's solution	Half-maximal load time	Tissue uptake of ouabain at equilibrium	Tissue binding of ouabain at equilibrium*
μM		min	$\text{pmol/mg dry weight}$	$\text{pmol/mg dry weight}$
0.44	Na^+ -R	92.4 ± 24.5	131.3 ± 15.6	132.5 ± 18.3
4.40	Na^+ -R	3.0 ± 1.0	161.5 ± 6.4	140.1 ± 6.7
2.20	Na^+ -R	13.9 ± 2.6	155.7 ± 6.5	146.6 ± 7.7
2.20	K^+ -R	45.5 ± 7.7	140.7 ± 8.2	139.0 ± 9.4

The constants were derived from the computer program of Cleland (10) used to generate the lines shown in Fig. 2.

* Tissue binding of ouabain was determined from the uptake data shown in Fig. 1. Each of the ouabain uptake points shown in Fig. 1 was corrected for ouabain present in the extracellular space labeled by inulin. Tissue binding constants then were derived from the computer program of Cleland (10). Since inulin uptake appeared to reach equilibrium by 5 min (Fig. 1), a mean inulin space (57.9 ± 2.7 dpm/ μg dry weight) was determined as the average for all observations ($n = 56$).

bain in enzyme-rich microsomal fractions (68) and to reduce the amount of ouabain bound to intact cell preparations (4, 5, 48, 49, 57, 58). Accordingly, several autoradiographic studies of [^3H]ouabain binding to transporting epithelia

have employed high concentrations of this ligand as a test for binding specificity (48, 49, 57, 58). In the present study, the time-course of ouabain uptake in normal Na^+ -Ringer's solution (containing $2.2 \mu\text{M}$ ouabain) was compared to that in the

presence of 83 mM K⁺-Ringer's solution. As shown in Fig. 1, elevation of medium K⁺ decreases the rate of ouabain uptake but has little effect on the maximal amount bound. Thus, the difference in ouabain uptake in Na⁺-Ringer's vs. K⁺-Ringer's solution was significant at 5, 15, 30, 60, and 90 min of incubation ($P < 0.05$). At 150 min the difference in binding was less marked and at 210 min the mean values for the two conditions were not different ($P \geq 0.05$). A double reciprocal plot of these data (Fig. 2, Table I) shows that high K⁺ more than triples the half-maximal loading time with little effect on the maximal level of ouabain binding at equilibrium. These data are consistent with biochemical studies which indicate that K⁺ retards the rate of ouabain-enzyme interaction (2, 43, 63).

Effect of Washout on Ouabain Binding

For autoradiographic purposes it is necessary to show that the removal of unbound ouabain does not lead to significant extraction of bound inhibitor. In the dual label experiment shown in Fig. 3, salt gland slices were exposed to 5 μ Ci of [³H]ouabain (2.2 μ M) and 5 μ Ci of [¹⁴C]inulin for 90 min at 37°C and then rinsed at intervals up to 150 min with Na⁺-Ringer's solution (4°C). After 30 min and 150 min of washing, 95% and 97% of the inulin were removed, respectively. In contrast, ouabain was tightly bound to the tissue since washing for 30 min removed only 10% of the label (primarily unbound) and extending the rinse time an additional 120 min removed only 6% more ouabain.

Table II summarizes the binding data for washed and unwashed slices after a 90-min incubation in Na⁺- or K⁺-Ringer's solution containing 2.2 μ M ouabain. Rinsing for 90–150 min reduced the amount of tissue ouabain, taken up in the presence of Na⁺-Ringer's, from 142.4 \pm 10.9 pmol/mg dry weight to 130.0 \pm 13.3 pmol/mg. A similar decrease (approx. 10%) occurs with washed tissue after incubation in K⁺-Ringer's solution. For both washed and unwashed slices, incubation for 90 min in K⁺-Ringer's, rather than Na⁺-Ringer's, leads to a reduction in ouabain binding of approx. 30%. At earlier time intervals, e.g., 30 min, elevated K⁺ concentrations lead to more than a 50% reduction in ouabain uptake (Fig. 1). The ouabain extracted by washing, after incubation in Na⁺- or K⁺-Ringer's solution, is primarily unbound since the level of ouabain uptake at 90 min, after subtracting the amount of ouabain in the

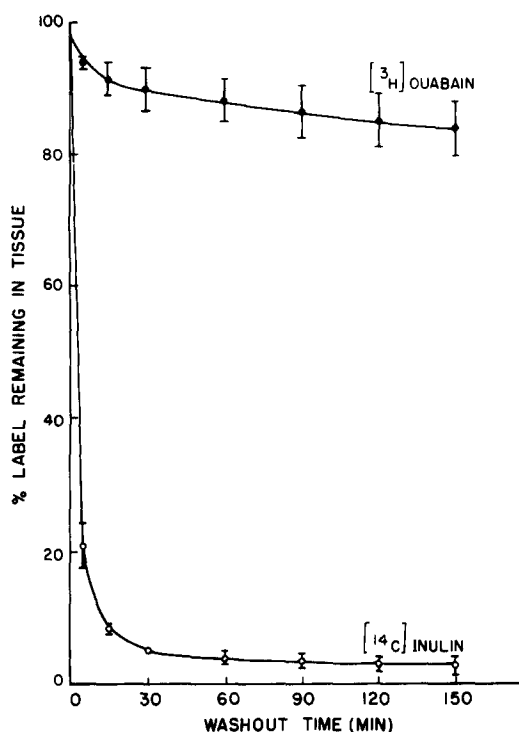


FIGURE 3 Effect of washout time on salt gland slices loaded with [³H]ouabain and [¹⁴C]inulin. Sections were incubated for 90 min in Na⁺-R containing 5 μ Ci of [³H]ouabain (final ouabain concentration of 2.2 μ M) and 5 μ Ci of [¹⁴C]inulin and then washed with unlabeled Na⁺-R for the times indicated. $n = 2$.

inulin space, approximates the observed value after washout (Table II). The data in Table II also indicate that exposure to 4.4 μ M ouabain in Na⁺-Ringer's solution produced nearly the same level of binding after washing (136.3 \pm 15.4 pmol/mg) as that obtained with 2.2 μ M ouabain (130.0 \pm 13.3 pmol/mg), suggesting that near saturation of tissue binding sites is approached at the lower concentration. Thus, increasing the medium ouabain concentration to 100 μ M effects only a 35% increase in tissue binding (Table II).

Effect of Salt Stress of Ducklings on Ouabain Binding to Salt Gland Slices

Previous studies (21) showed that ducklings exposed to a salt water diet (1% NaCl drinking water for 12 h, tap water for 12 h) rapidly increase their salt gland Na⁺-K⁺-ATPase, reaching maximal levels at 9–11 days of salt stress. The data presented in Fig. 4 show that this enzymatic response to days of salt stress is paralleled by similar

TABLE II
Effect of Washing on Ouabain Binding to Salt Gland Slices

Conditions	Ouabain concn μM	Mean tissue ouabain		Reduction %
		Na ⁺ -R	K ⁺ -R	
		<i>pmol/mg dry weight</i>		
90 min Load		142.4 ± 10.9 (6)	98.9 ± 6.2 (3)	30.5
“ less inulin space* “ , 90-150 min rinse	2.2	131.5 ± 10.9 (6)	88.0 ± 6.2 (3)	33.1
“ , 90-150 min rinse		130.0 ± 13.3 (4)	88.2 ± 10.0 (2)	32.2
“ less inulin space* “ , 90-150 min rinse	4.4	148.0 ± 18.1 (3)	126.3 ± 18.1 (3)	
“ , 90-150 min rinse		136.3 ± 15.4 (2)		
“ , 90 min rinse	100	182.9 ± 16.2 (2)		

* Tissue loading of ouabain after 90 min was corrected for ouabain present in the inulin space as discussed in the footnote to Table I.

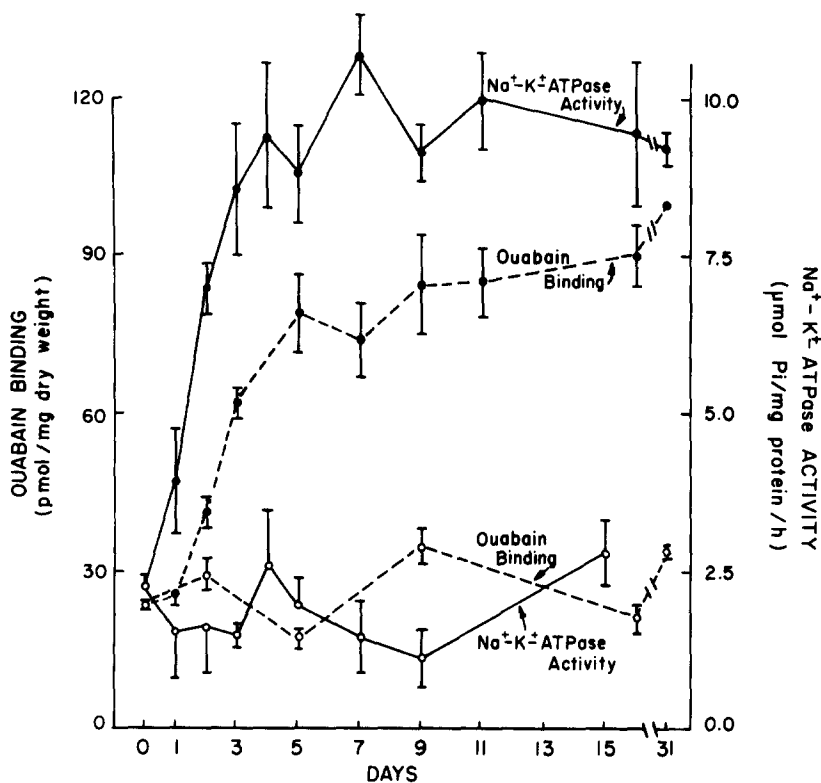


FIGURE 4 Effect of salt water and fresh water regimens on ouabain binding to salt gland Vibratome sections and on Na⁺-K⁺-ATPase activity of salt gland homogenates. Ouabain binding (dashed lines and left ordinate) to slices from salt glands of ducklings maintained on a salt water (solid circles) or a fresh water (open circles) regimen for various days was assayed after a 90-min exposure to 2.2 μM ouabain (containing 5 μCi [³H]ouabain) and four sequential washes with unlabeled Na⁺-R over an additional 90-min period. The data for Na⁺-K⁺-ATPase activity (solid lines and right ordinate) are taken from a previous publication (21).

increases in the levels of ouabain binding by salt gland slices. In contrast, ouabain binding to salt gland slices of fresh water ducklings and the specific activity of glandular $\text{Na}^+\text{-K}^+\text{-ATPase}$ remain at a baseline level over the entire time-course of the experiment. It should be noted also that salt glands of ducklings, stressed continuously with 1% NaCl drinking water, are capable of binding more ouabain (130.0 ± 13.3 pmol/mg dry weight, Table II) than glands from ducks maintained on only a 12-h salt water regimen (100.1 ± 10.0 pmol/mg at 31 days of salt stress, Fig. 4). The data shown in Fig. 4 emphasize, therefore, the close relationship between the specific activity of salt gland $\text{Na}^+\text{-K}^+\text{-ATPase}$, ouabain binding and osmotic stress.

Autoradiographic Localization of Ouabain Binding Sites

Histologically, the salt gland is comprised of discrete secretory lobules, each of which is composed of branched secretory tubules which radiate from a central canal to abut blindly on the interlobular connective tissue at the periphery of each lobule (20). At the ultrastructural level, the glandular epithelium of salt-stressed ducks is composed of two cell types (20). Relatively undifferentiated peripheral cells are found at the blind ends of the secretory tubules adjacent to the interlobular connective tissue. These cells have unfolded basolateral plasma membranes and have been shown to be mitotically active in developing glands from salt-stressed ducklings (13). In con-

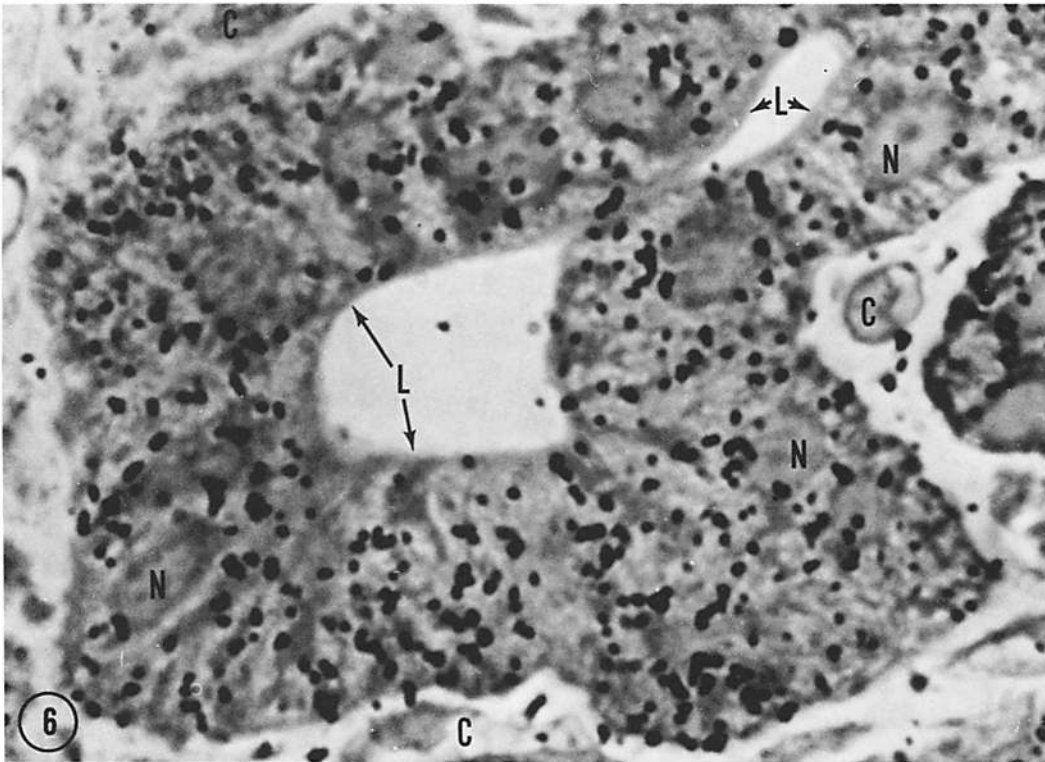
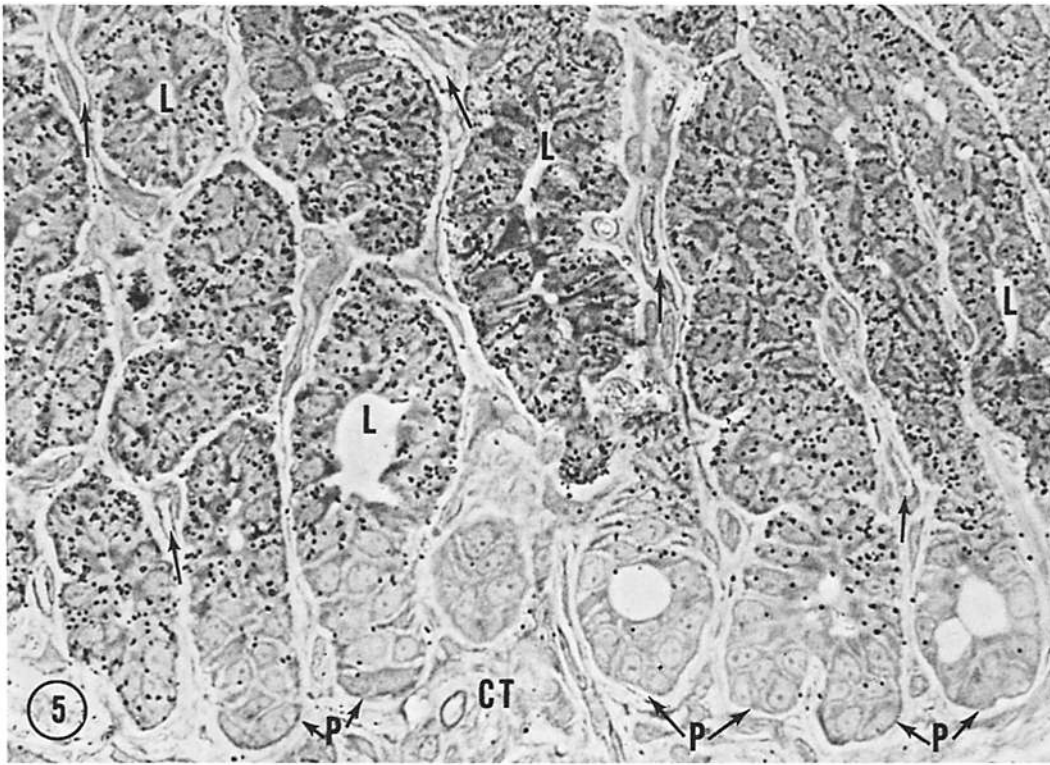
trast, the bulk of the glandular epithelium is composed of highly specialized secretory cells which are characterized by extensive folding of the basolateral plasmalemma to form extracellular spaces and intracellular compartments packed with mitochondria. Morphologically, this cytoarchitecture resembles that seen in distal nephrons of mammalian kidney tubules, and is a characteristic structural pattern common to most osmoregulatory epithelia.

The autoradiographic distribution of [^3H]-ouabain binding sites in salt gland epithelium is shown in Fig. 5. The highly specialized secretory cells are heavily labeled. In contrast, only a few grains are associated with the unspecialized peripheral cells. At higher magnification (Fig. 6), grains appear closely associated with the basolateral folds which are distorted somewhat by the marked expansion of the extracellular spaces which occurs during the rapid freezing of the tissue. The luminal spaces and apical surfaces were labeled only occasionally, the extent of which never exceeded background levels.

Because an apical localization of Na^+ pump sites in this secretory epithelium might be expected on physiological grounds, it was important to exclude the possibility (however unlikely with 100- μm sections) that the luminal compartments are sufficiently isolated from the incubation media to preclude significant exposure to [^3H]ouabain. In order to show that ouabain has access to the luminal space, slices were exposed for 90 min to 5–25 μCi of [^3H]ouabain in the presence of 10^{-4} M unla-

FIGURE 5 This autoradiograph demonstrates the distribution of [^3H]ouabain binding sites in a portion of a secretory lobule from a Vibratome section of the avian salt gland. The tissue was exposed for 90 min to 2.2 μM ouabain containing 5 μCi of [^3H]ouabain and then washed for 90 min. Unspecialized peripheral cells (*P*) which characterize the blind ends of the secretory tubules adjacent to the interlobular connective tissue (*CT*) are unlabeled. The remainder of the tubular epithelium is composed of highly specialized principal secretory cells which are heavily labeled by [^3H]ouabain. Note that the plasma membranes bordering the lumina (*L*) of these secretory tubules bear only occasional exposed silver grains. In contrast, the basolateral plasma membranes and associated intercellular spaces of principal secretory cells are rich in ouabain binding sites. Net transepithelial transport of NaCl is from peritubular blood vessels (arrows) to tubular lumina. One micrometer section of freeze-dried, plastic-embedded tissue, coated with NTB-2 emulsion, exposed for 18 days, and photographed with phase-contrast optics. $\times 900$.

FIGURE 6 Autoradiograph of salt gland tissue after exposure to [^3H]ouabain as in Fig. 5. Ouabain binding sites are restricted to the basolateral plasma membranes bordering the expanded intercellular spaces between these principal secretory cells. The luminal surfaces (*L*) are not labeled by ouabain. Note the absence of exposed silver grains over nuclei (*N*). Peritubular capillaries (*C*) are closely associated with the secretory epithelium. One micrometer section of freeze-dried, plastic-embedded tissue, coated with NTB-2 emulsion, exposed for 18 days, and photographed with conventional optics. $\times 2,600$.



beled inhibitor (to repress tissue binding of label) and then freeze-dried immediately without washing. Dextran (5%, mol wt 10,400) was included in the medium to provide a matrix to support unbound label during the freeze-drying procedure. After a 2–4-wk exposure, autoradiographs showed a modest number of grains distributed throughout the tissue. For the most part, these represented unbound label since, in parallel experiments in which the tissue was washed for 90 min, few grains above background could be detected. However, since the luminal spaces, as they appear in 1- μ m epoxy sections, represent an extremely small volume relative to the extensive intercellular space (see Fig. 5), the degree of luminal loading was not dramatic. Luminal loading could be visualized readily in autoradiographs, however, when [14 C]inulin was used to mark the extracellular space. Autoradiographic efficiency with this isotope is increased by as much as 10-fold over that obtained with tritium (60). Fig. 7 shows the random distribution of grains over the luminal, intercellular, and connective tissue spaces of slices loaded with 25 μ Ci of [14 C]inulin. As expected for an unbound extracellular marker, and consistent with the washout data of Fig. 3, rinsing these slices with unlabeled Ringer's solution produced autoradiographs devoid of exposed grains (Fig. 8). Since the molecular weight of inulin is more than 10 times greater than that of ouabain, it is clear that all extracellular compartments in these slices are accessible to ouabain during the normal 90-min incubation time.

Cytochemical Localization of Na⁺-K⁺-ATPase

The distribution of [3 H]ouabain binding sites visualized autoradiographically (Figs. 5 and 6) was compared to the staining pattern observed at the light microscope level with the cytochemical method for the localization of the K⁺-dependent phosphatase (K⁺-NPPase) component of the Na⁺-K⁺-ATPase enzyme complex (18). Fig. 9 illustrates the distribution of NPPase activity (in the presence of activating K⁺ ions) in cryostat sections of salt gland secretory lobules. Reaction product is restricted to the highly specialized secretory cells. In contrast, peripheral cells were unreactive. Addition of 10 mM ouabain to the medium abolishes the phosphatase activity of the principal secretory cells (Fig. 10). Similar results are obtained when K⁺ is deleted from the incubation medium. The cell specificity of the K⁺-dependent, ouabain-sen-

sitive reactivity observed with these cryostat sections (Figs. 9 and 10) could be resolved further in terms of membrane specificity by phase-contrast microscopy of 1 μ m sections from similarly incubated Vibratome slices. As seen in Fig. 11, reaction product is restricted to the expanded basolateral plasmalemma of the specialized secretory cells with little, if any, luminal reactivity. An identical conclusion was reached in an earlier ultrastructural study with this method (18). The pattern of staining observed with the cytochemical method (Fig. 9–11) is identical, therefore, to that seen with the autoradiographic technique (Figs. 5 and 6) and indicates a high degree of specificity for both procedures.

DISCUSSION

There seems to be little doubt in the literature that ouabain is a highly specific inhibitor of Na⁺-K⁺-ATPase (63, 68) and, consequently, of active transmembrane (31) and transepithelial (36) cation transport (see reviews by Baker [3] and Schwartz et al. [63]). At low concentration, ouabain has been shown repeatedly to bind to a finite number of high affinity sites in purified membrane fractions of Na⁺-K⁺-ATPase (47, 64, 65) as well as to the outside surface of plasma membranes of intact cells (30, 31). Recent studies have indicated, that the receptor for ouabain must be closely associated with Na⁺-K⁺-ATPase since in purified preparations of the enzyme, which consist of two polypeptide subunits (38, 39), the larger molecular weight chain specifically binds cardiac glycosides (59). Studies of glycoside binding to intact cells and epithelial plasma membranes have shown, moreover, a close correlation with inhibition of Na⁺ transport in these experimental systems (5, 48, 49). As expected from the biochemical and physiological data on cardiac glycoside interaction with the Na⁺ pump, autoradiographic studies of the localization of [3 H]ouabain binding sites have shown a similar high level of specificity on the basis of kinetic data on inhibitor uptake and saturation binding (49), concomitant inhibition of active cation transport (48, 49) and Na⁺-K⁺-ATPase activity (34) and the depression of binding induced by manipulation of various ligands (48, 49, 57, 58) which are known to affect ouabain binding to Na⁺-K⁺-ATPase in partially purified membrane fractions (47).

On the basis of many of these criteria, ouabain binding in the salt gland appears to be highly specific. Thus, kinetic studies show that binding of

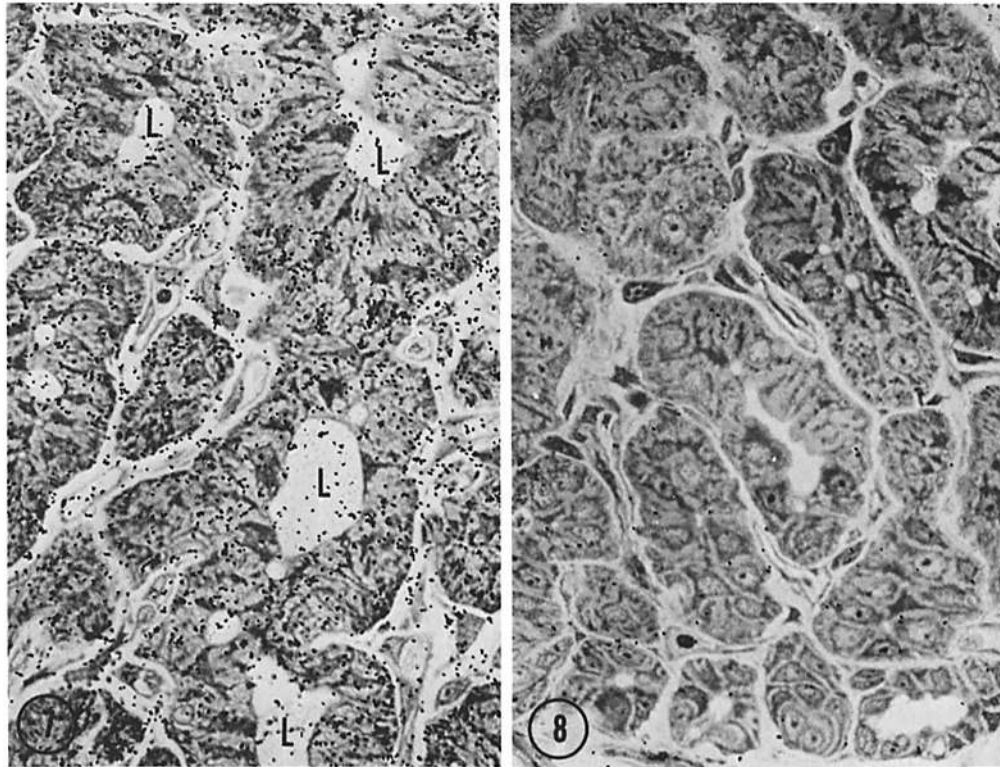
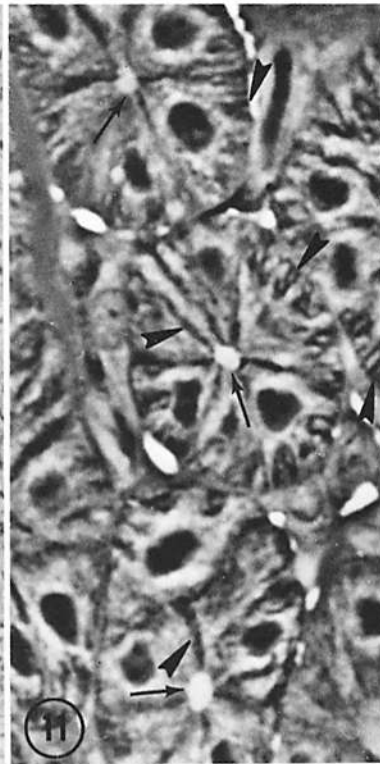
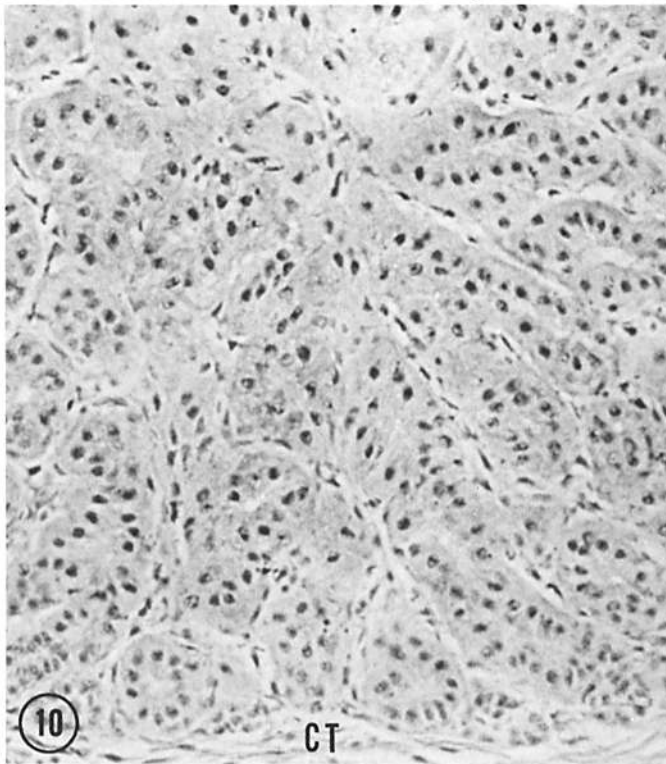
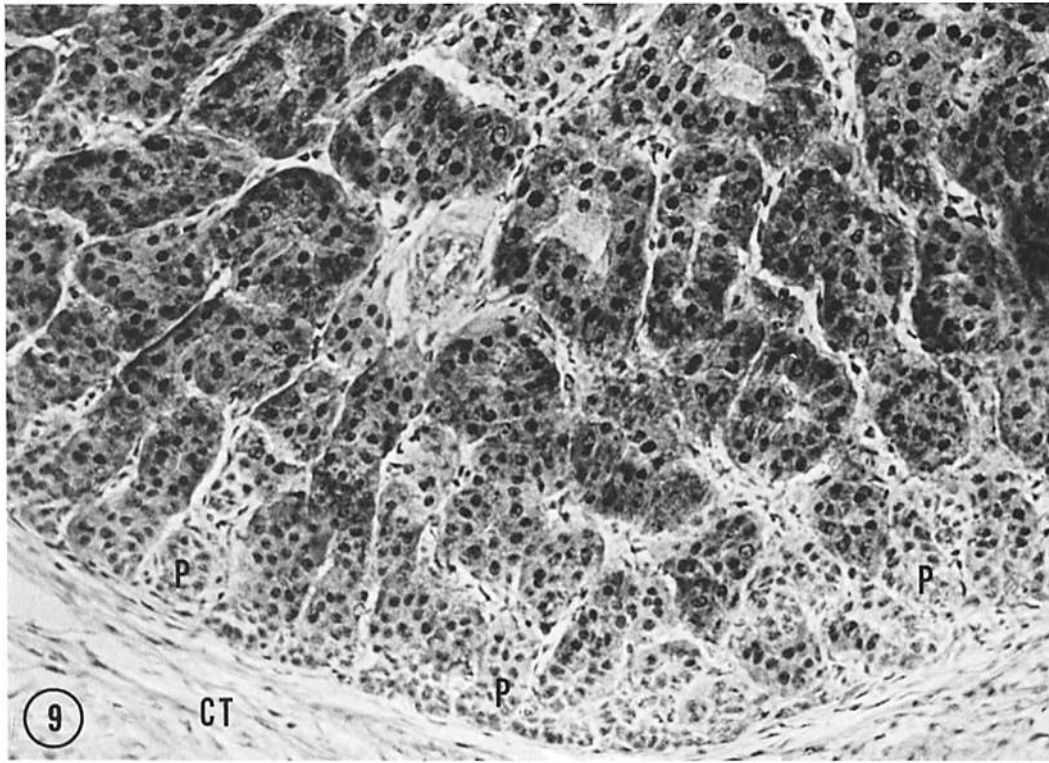


FIGURE 7 Autoradiographic distribution of [^{14}C]inulin in unwashed slices of avian salt gland. The tissue was exposed for 90 min to 25 $\mu\text{Ci/ml}$ of [^{14}C]inulin and then rapidly frozen. All extracellular spaces including the lumina (L) of secretory tubules are labeled by inulin. One micrometer section of freeze-dried, plastic-embedded tissue, coated with NTB-3 emulsion, exposed for 10 days, and photographed with conventional optics. $\times 825$.

FIGURE 8 Autoradiographic distribution of [^{14}C]inulin in washed slices of avian salt gland. The tissue was exposed to [^{14}C]inulin as in Fig. 7 and then washed with label-free Ringer's solution for 90 min before freezing. The marked reduction in the number of silver grains (cf. Fig. 7) indicates that inulin, unlike ouabain (Figs. 5 and 6), is extracted easily from the tissue by washing. One micrometer section of freeze-dried, plastic-embedded tissue coated with NTB-3 emulsion, exposed for 10 days, and photographed with conventional optics. $\times 825$.

ouabain approaches near-saturation at inhibitor concentrations of 2.2–4.4 μM (Tables I and II). Washout data demonstrate that most of the ouabain taken up in salt gland slices is bound tightly to tissue receptors, since the small fraction of tissue ouabain extracted by washing (10–15%) can be accounted for almost entirely as unbound ouabain occupying the extracellular space labeled by inulin (Fig. 3, Table II). Competition for a finite number of binding sites is indicated further by an approximate 99% reduction in binding of labeled ouabain (5 μCi , 0.44 μM) by addition of 100 μM unlabeled ouabain to the incubation medium (data not presented). It is of interest in this regard that

Baker and Willis (5) and Brading and Widdecombe (8) resolved a nonsaturable and nonspecific component of ouabain uptake at concentrations of ouabain greater than 1 μM , and that Mills et al. (49) demonstrated a similar nonspecific component in frog skin at concentrations above 10 μM . In the present study, similar ouabain uptake experiments, such as those shown in Fig. 1, were not conducted at inhibitor concentrations greater than 4.4 μM . However, in tissue exposed to ouabain for 90 min and then washed (Table II), increasing the total ouabain concentration more than 20-fold (from 4.4 to 100 μM) caused less than 1.5-fold increase in ouabain bound (from 130



to 183 pmol/mg dry weight). Such data are entirely consistent with a single population of specific and saturable binding sites.

Elevated K^+ concentrations in the incubation medium have been shown to reduce ouabain-inhibition of Na^+K^+ -ATPase (68) as well as to repress ouabain binding to partially purified Na^+K^+ -ATPase fractions (47, 74) and to intact cells (4, 5, 48, 49, 57, 58). Reduction in ouabain binding to intact cells is reflected by a quantitatively similar reduction in binding sites as revealed by autoradiography (48, 49, 72). Accordingly, ouabain binding studies at elevated K^+ concentrations are often employed as a test for binding specificity (5, 47, 48, 57, 58, 74). As indicated by Figs. 1 and 2, high K^+ concentrations also caused a reduction in the rate of ouabain uptake in salt gland slices. However, the effect of elevated K^+ is on the rate of binding rather than on the maximal amount of inhibitor bound (Fig. 2, Table I). Similar conclusions were derived from studies on ouabain binding to Na^+K^+ ATPase preparations (2, 63) and to intact cells (27, 42) and from kinetic and autoradiographic analyses of ouabain binding to frog skin (49). Caution must therefore be exercised in utilizing high K^+ media as a test for specificity in binding and autoradiographic experiments. For example, in salt gland slices, incuba-

tion times of 15–30 min in high K^+ -media reduce binding by 55%, whereas binding is depressed by only 30% after 90 min and by less than 15% (not statistically significant) at 210 min (Fig. 1). In addition, since the effects of K^+ are on rate of binding, residual binding of ouabain in high K^+ solutions should not be interpreted as nonspecific (49). Finally, it should be recognized that at least in autoradiographic studies, where very high K^+ concentrations (42–140 mM) have been used as a test for specificity, elevation of medium K^+ has been accomplished at the expense of medium Na^+ (48, 49, 57, 58). Accordingly, reduction in binding may be a response not simply to elevated K^+ but rather to a marked alteration in the Na^+K^+ ratio. It is this ratio which appears to play a critical role in the complex interaction between ouabain and Na^+K^+ -ATPase (47, 63, 64).

If most of the ouabain bound to washed salt gland slices is specific, as the discussion above would indicate, then increasing the specific activity of glandular Na^+K^+ -ATPase should increase the amount of ouabain bound in a parallel fashion. Maintenance of ducklings on salt water causes a marked increase in the specific activity of salt gland Na^+K^+ -ATPase over a 9–11-day period of stress (21), and this response is reflected in a similar increase in pmoles of ouabain bound per

FIGURE 9 Light microscope localization of NPPase activity in the secretory epithelium of the avian salt gland, fixed for 45 min with 3% paraformaldehyde. The portion of the secretory lobule shown here is from a 10- μ m cryostat section incubated for 60 min in the K^+ -containing phosphatase medium described by Ernst (18). After the incubation and washes, the sections were treated with 1% $(NH_4)_2S$ to visualize sites of phosphatase reactivity for conventional light microscopy. Reaction product (black) is restricted to the principal secretory cells with little stain deposition associated with the peripheral cells (*P*) which abut on the interlobular connective tissue (*CT*). The distribution of NPPase activity is identical to the distribution of [3H]ouabain binding sites (see Fig. 5). The nuclear deposits are artifacts of the technique (see Fig. 10). $\times 350$.

FIGURE 10 This light micrograph shows a portion of a secretory lobule processed exactly as in Fig. 9 except that 10 mM ouabain was present in the incubation medium. The reaction product deposition in the highly reactive principal secretory cells shown in Fig. 9 is abolished by ouabain. The localization of ouabain-sensitive K^+ -NPPase activity to the principal secretory cells mirrors the distribution of [3H]ouabain binding sites shown in Fig. 5. Note that the nuclear deposits seen in Fig. 9 are not eliminated by the addition of ouabain. $\times 400$.

FIGURE 11 Phase-contrast light microscope localization of ouabain-sensitive K^+ -NPPase activity in avian salt gland, fixed for 5 min with 0.25% glutaraldehyde-1% paraformaldehyde. The secretory tubules shown here are from 1 μ m sections of Vibratome slices incubated for 90 min in the K^+ -containing phosphatase medium. Reaction product (black) is deposited along the basolateral plasma membranes (arrowheads) of these principal secretory cells. The luminal surfaces (arrows) are unreactive. The membrane-specific localization of K^+ -NPPase activity in these secretory cells parallels the distribution of [3H]ouabain binding sites in Figs. 5 and 6. $\times 1,500$.

milligram of dry weight (Fig. 4). The original observation relating the level of specific activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ to osmotic stress (21) suggested, but did not prove, salt stress-induced *de novo* synthesis of the enzyme. Recently, Stewart et al. (70) confirmed this by showing that the increase in salt gland $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity paralleled a concomitant increase in the amount of large molecular weight catalytic subunit of the enzyme. Such data correlate closely with the identical increase in ouabain binding reported here and, when considered in the light of studies showing that ouabain binds specifically to the catalytic subunit (59), lend substantial support to a high degree of specificity for ouabain binding to salt gland slices.

Specificity of binding also is indicated by the grain distribution in autoradiographs. Differential binding to the basolateral plasma membranes of principal secretory cells with little labeling of peripheral cells (Fig. 5) shows that ouabain binding is cell-specific, and the absence of a luminal localization in these secretory tubules (Fig. 6) demonstrates membrane specificity as well. The validity of the autoradiographic procedure is substantiated further by comparing the distribution of ouabain binding sites (Figs. 5 and 6) to the cytochemical localization of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (Figs. 9-11). With both techniques, Na^+ pump sites are restricted to basolateral membranes of the principal secretory cells.

It is of interest that the gradient of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity (Fig. 9) and of ouabain binding sites (Fig. 5) along the secretory tubule parallels a similar gradient of cell specialization (20) with the more central cells exhibiting marked membrane amplification and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in comparison to peripheral cells. This relationship lends support to the hypothesis made by Staalend (69) and elaborated by Hanwell et al. (29) and by Peaker and Linzell (53) to account for the inverse relationship between the rate of secretion and the NaCl concentration of the secreted fluid which is observed in individual salt-stressed ducks. In their view, peripheral cells could form a primary secretion low in NaCl which would then be modified along the length of the tubule by addition of a more highly concentrated effluent. Accordingly, at low flow rates, the concentration of NaCl in the secretion would be higher because of longer exposure to the more active secretory cells which we have shown to be rich in $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity.

It has been suggested by some investigators (7,

51-53) that Na^+ secretion by the avian salt gland is brought about by a $\text{Na}^+\text{-K}^+\text{-ATPase}$ situated on the luminal border of the secretory cell. This proposal is based largely on the following lines of evidence: (a) ouabain-sensitive transport of Na^+ and K^+ has been demonstrated in slices of the gland (51, 76); (b) a ouabain-sensitive $\text{Na}^+\text{-K}^+\text{-ATPase}$ is present (7) which, moreover, increases in activity upon adaptation of the bird to salt water (reference 21; see also Fig. 4); (c) chemical analyses (51, 76) and electrophysiological studies (73) indicate that the movement of Na^+ across the apical surface of the cells may be against an electrochemical gradient; (d) a single study of retrograde injection of ouabain into the secretory duct (73) has been interpreted to indicate ouabain sensitivity of the Na^+ movements at the luminal surface of the cells (51-53). However, on the basis of combined morphological and biochemical studies, we suggested (20) that the basolateral membranes of salt gland epithelial cells are the sites of active Na^+ transport since it is these surfaces, in contrast to the apical membrane, which undergo vast surface extension in response to osmotic stress. Moreover, under conditions of osmotic stress, membrane amplification could be correlated temporally with a fourfold increase in the specific activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (20, 21) which, in turn, is directly proportional to an increased capacity of the gland to concentrate and eliminate a salt load (25). The cytochemical localization of salt gland $\text{Na}^+\text{-K}^+\text{-ATPase}$ at the ultrastructural level (17, 18) provided direct support for this hypothesis by showing that ouabain-sensitive activity was restricted to the basolateral, but not apical, plasmalemma.¹ Since these data are

¹ On the basis of light microscope studies with the Wachstein-Meisel procedure (77) for the localization of $\text{Mg}^{2+}\text{-ATPase}$, Ballantyne and Wood (6) suggested that much of the salt gland ATPase was associated with the luminal surfaces of the secretory cells. Since ouabain produced an apparent reduction in activity, this study has been used to support the concept of a luminal $\text{Na}^+\text{-K}^+\text{-ATPase}$ (51, 52). However, on the basis of critical biochemical and cytochemical studies (see references 17, 18, 23, and 75 for pertinent discussion and references), it is unlikely that $\text{Na}^+\text{-K}^+\text{-ATPase}$ can be localized by the Wachstein-Meisel procedure, even when the incubation medium is modified by the addition of appropriate cations. In any case, since K^+ was apparently not added to the incubation medium in the experiments of Ballantyne and Wood (6), this would preclude a successful localization of $\text{Na}^+\text{-K}^+\text{-ATPase}$. Furthermore, ultrastructural

confirmed by the present study, we feel confident in restricting the localization of ouabain-sensitive Na^+ pumps to the basolateral membranes and suggest that the apparent sensitivity of the luminal surface to ouabain in the retrograde perfusion studies of Thesleff and Schmidt-Nielsen (73) may have resulted from a physical effect of the perfusion on intercellular junctions, thereby allowing ouabain to gain access to peritubular binding sites. In this regard, binding and autoradiographic experiments with irrigated fish gills (34) demonstrated localized permeation of the epithelium by external (mucosal) ouabain with subsequent occupation of serosal binding sites. The localization of ouabain-sensitive Na^+ transport sites to the basal surface is apparently common to other Na^+ secretory systems, since recent studies of K^+ -NPPase localization in iguana and in marine turtle salt glands and in elasmobranch rectal glands (14, 15, 28) and [^3H]ouabain localization in the branchial epithelium (chloride cells) of teleosts (34) and in the secretory portion of human sweat glands (57) all show an exclusive localization to the abluminal cell surface. In fact, the apical localization of ouabain binding sites in the choroid plexus (58) is the only example of luminal membrane localization of Na^+ pumps.

The unequivocal localization of Na^+ - K^+ -ATPase to the basal cell surface makes the obligatory role of ouabain-sensitive transport of Na^+ in salt secretion less clear. A direct role for the basal ouabain-sensitive Na^+ pump in the overall excretion of Na^+ by the salt gland would appear to require that the pump be oriented so as to remove Na^+ from the blood, i.e., that it transport Na^+ into the epithelial cell. Such an orientation was suggested on theoretical grounds by Diamond and Bossert (12) in order to account for hypertonic solute transport across secretory epithelia by means of local standing osmotic gradients. In this

studies with the Wachstein-Meisel technique showed that the major site of activity of Mg^{2+} -ATPase is the basolateral plasmalemma (1, 23) rather than the apical pole of the salt gland secretory cell as claimed in the light microscope study. It should be emphasized, moreover, that the localization of ouabain-insensitive Mg^{2+} -ATPase by the Wachstein-Meisel procedure does not bear any necessary relationship to Na^+ - K^+ -ATPase since, in addition to several kinetic (21) and cytochemical (18, 19, 23) dissimilarities between the two enzymes, almost all Mg^{2+} -ATPase activity is removed by purification of Na^+ - K^+ -ATPase (32). A similar conclusion was reached in a recent study utilizing avian salt glands (70).

context, the development of basal membrane compartmentalization in salt-stressed glands (20) would provide the necessary structural geometry for the maintenance of such standing gradients. However, the secretory cells, both in vivo and in vitro, maintain a relatively high K^+ content and low contents of Na^+ and Cl^- (51, 54, 55, 61, 76). As with other tissues, incubation of salt gland slices in the presence of ouabain leads to a loss of K^+ and a gain of Na^+ (51, 53, 76), and to an inhibition of the efflux of $^{24}\text{Na}^+$ (76). These results indicate that ouabain inhibits a coupled transport system for Na^+ and K^+ which is oriented in the direction usually found in other cells; i.e., to accumulate K^+ and extrude Na^+ . Such a pump orientation in secretory epithelia is supported further by our observation that phosphate hydrolyzed from NPP by Na^+ - K^+ -ATPase is precipitated on the cytoplasmic side of the plasma membrane in salt gland epithelial cells (18), whereas [^3H]ouabain presumably binds to the extracellular side of the plasmalemma. Thus, this asymmetry is identical to that observed in reabsorptive systems where internal hydrolysis of ATP and release of inorganic phosphate (66) is coupled to exchange of internal Na^+ for external K^+ (78) and is inhibited by external ouabain (9, 30, 31). It seems likely at present, therefore, that at least one possible role of the basal, ouabain-sensitive Na^+ - K^+ -ATPase system is to maintain the cellular Na^+ and K^+ composition (and, perhaps, cell volume) that is apparently required for the normal metabolic activity of almost all vertebrate cells. A similar suggestion was made by Quinton and Tormey (57) to explain the serosal localization of ouabain binding sites in the secretory portions of eccrine sweat glands. The correlation of increased activity of salt gland transport ATPase with the expansion of the basal membrane during osmotic adaptation (20, 21) might be viewed as a necessary response to maintain the intracellular composition in the face of the greater surface area of the developing membrane.

Since the orientation of Na^+ - K^+ -ATPase is apparently counter to the direction of net Na^+ secretion, an outward facing electrogenic apical pump, insensitive to ouabain, might form the essential step in the secretory process by transporting Na^+ against an electrochemical gradient across the luminal membrane. It is of interest in this regard that secretion by the gland is activated by cholinergic drugs (reviewed by Peaker and Linzell [53]). These agents stimulate the efflux of $^{24}\text{Na}^+$ from salt gland slices, and, consistent with the absence

of ouabain binding to the luminal membrane (Fig. 6), this stimulation is at least partially independent of the presence of K^+ in the medium (76). Studies by Peaker and Stockley (54, 55) on lithium metabolism by salt gland slices are also consistent with the existence of an apical Na^+ pump which is responsive to cholinergic stimulation. It is tempting to speculate that the luminal pump, if present, might be analogous to the ethacrynic acid-sensitive electrogenic Na^+ pump described in kidney cortex slices which is also K^+ - and ouabain-insensitive (56, 79). However, preliminary studies (G. D. V. Van Rossum and S. A. Ernst, unpublished observations) indicate that the inhibitory effects of ethacrynic acid on Na^+ extrusion in salt gland slices can be accounted for as a secondary consequence of inhibition of respiration. Similar conclusions were reached by MacKnight (44) and Epstein (16) in studies with kidney slices.

Although a hypothesis which includes the presence of an apical, ouabain-insensitive, ion pump provides a possible explanation for hypertonic salt transport by the avian salt gland, this model has the less appealing aspect of relegating the basolateral Na^+K^+ -ATPase to a rather indirect and paradoxical function in transepithelial electrolyte metabolism. The following discussion considers an alternative model, and proposes a second, both of which emphasize a central role for this enzyme in salt secretion.

In a discussion of salt and water transport, Kyte (41) pointed out that an epithelium such as the salt gland could secrete a hypertonic saline solution directly from intercellular space $NaCl$. In this model, basolateral Na^+K^+ -ATPase would function to maintain an intercellular space $NaCl$ solution hyperosmotic to that of the luminal compartment and salt could then diffuse down the concentration gradient to enter the luminal space via the tight junction. In order for this to be an efficient method of salt secretion, such a mechanism would require that the resistance to $NaCl$ permeation along the intercellular space and tight junction be less than that along the intercellular space and basement membrane. Otherwise, there would be a preferential movement of considerable quantities of $NaCl$ down a favorable chemical gradient toward the blood.

An alternative mechanism by which a basolateral Na^+ pump could function in salt secretion may be postulated on the basis of a series of studies of ion transport across irrigated gill preparations (35), isolated, perfused elasmobranch rectal gland

(67) and the isolated opercular epithelium of *Fundulus* (11, 33). In all of these secretory tissues, the potential difference is oriented so that the lumen, or seawater side, is negative. In addition, Kirschner et al. (35) found that the irrigated gill is more permeable to Na^+ than to Cl^- , while Karnaky et al. (33) and Degnan et al. (11) demonstrated that Cl^- was transported across the short-circuited opercular epithelium of seawater-adapted *Fundulus* without net movement of Na^+ . Thus, it appears that salt secretion by the chloride cells of euryhaline fish is achieved by "active" Cl^- transport coupled with a passive flux of Na^+ through a parallel leak pathway (35).² We suggest that the basolateral Na^+ pump, oriented to extrude Na^+ from the cell, could drive secretion in these cells, as well as in those of the salt gland, by acting to maintain the Na^+ gradient from blood to cell. This would allow Na^+ to enter the cell down a favorable electrochemical gradient and would provide the driving force necessary for the co-transport of Cl^- into the cell against an electrical gradient. Once these ions have entered the cell, Cl^- then would be free to diffuse from the cell across the luminal membrane without the intervention of an apical pump, provided the cell interior is sufficiently electronegative to the lumen. Na^+ , pumped into the intercellular space, would then follow through a shunt pathway, presumably across the tight junction. Although definitive data

² Since the results of these electrical studies are at variance with the lumen positive potential difference observed by Thessleff and Schmidt-Nielsen (73) in the salt gland, a re-examination of the salt gland data is clearly warranted. Moreover, in the salt gland, the potential difference was measured between the blood and the main excretory duct which lies outside the body of the gland. Accordingly, the observed potential difference may not necessarily reflect the actual electrical events which occur across the secretory epithelium at some distance from the point of measurement. In addition, the suggestion that salt secretion is blocked by the retrograde-induced presence of ouabain in the luminal space (51-53, 73) contrasts with the absence of ouabain binding sites (Fig. 6) and Na^+K^+ -ATPase activity (reference 18, and Fig. 11) along this cell surface in avian salt glands as well as in other secretory epithelia (14, 15, 28, 34, 57). Rather, the basolateral cell membrane localization of Na^+K^+ -ATPase at least in the teleost gill (34) and elasmobranch rectal gland (28) appears to be intimately associated with the transepithelial potential difference (lumen negative) and (active) chloride efflux across these epithelia (11, 33, 35, 67), since all of these parameters are inhibited by serosal ouabain.

supporting the existence of a coupled NaCl pump in salt glands are not presently available, Hanwell et al. (29) and Peaker (52) have suggested that the high efficiency of NaCl extraction from the blood argues against a simple diffusion across the basal membrane and in favor of some form of a carrier-mediated process. Because inhibitors of carbonic anhydrase block secretion by the gland in vivo (24, 50), Peaker (52) proposed that linked movement of Na⁺ and Cl⁻ into the cell could occur via carrier-mediated neutral exchanges with intracellular H⁺ and HCO₃⁻ (see Peaker and Linzell [53] for supporting data and pertinent discussion). Alternatively, Na⁺ and Cl⁻ could enter as a consequence of a neutral coupled carrier-mediated mechanism similar to that proposed by Frizzell et al. (26) to account for net NaCl transport across the rabbit gall bladder.

In summary, the results presented here indicate that, like Na⁺-K⁺-ATPase activity, ouabain binding sites are restricted to the basolateral cell surfaces of the principal secretory cells of the avian salt gland. As suggested by other investigators (51, 57, 76), a basal Na⁺-K⁺-ATPase may function primarily to maintain the normal intracellular ion content common to most cells. However, in our view, the close association between days of salt stress and the level of glandular Na⁺-K⁺-ATPase (21), basal membrane amplification (20), and, importantly, the concentration of NaCl in the secretory effluent and the rate of its formation (25) argue for a central role for this enzyme in the mechanism of salt secretion. In this regard, of the various models proposed to account for electrolyte secretion by salt glands, a mechanism involving paracellular movement of Na⁺ is attractive since intracellular ion content, facilitated diffusion of NaCl into the cell and transepithelial NaCl secretion all could follow as a consequence of a basal ouabain-sensitive pump oriented to extrude Na⁺ from the cell. Moreover, such a model accounts for the striking similarities in cytoarchitecture between reabsorptive and secretory cells in the form of extensive basolateral cell membrane expansion and compartmentalization which, in view of the opposite polarity of net ion transport in the two classes of epithelia, has perplexed morphologists for years.

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