LOCALIZATION OF Na PUMPS IN THE TRACHEAL EPITHELIUM OF THE DOG

J. H. WIDDICOMBE, C. B. BASBAUM, and J. Y. YEE

From the Cardiovascular Research Institute, University of California Medical School, San Francisco, California 94143

ABSTRACT

Binding of [^{3}H]ouabain by the dog's tracheal epithelium shows a nonspecific component depending linearly on ouabain concentration, and a specific saturable component with a K_m of 10^{-7} M. Control experiments showed that the tracer taken up was not trapped within the extracellular space nor bound to tissue collagen. Inhibition of the saturable uptake by high K, metabolic inhibition, low Na, and low temperature indicated that binding was to Na/K ATPase. One-sided exposure of tissue sheets to tracer showed that the submucosal side took up 10 × as much tracer as the luminal. Autoradiography localized tracer uptake under all conditions to the cells' basolateral membranes.

KEY WORDS [^{3}H]ouabain binding · dog tracheal epithelium · Na pumps

The dog's tracheal epithelium actively transports Cl towards the lumen, and Na towards the submucosa. When mounted in Ussing chambers, these two ion movements account for the short circuit current (S.C.C.), the Cl movement being the greater of the two (28, 29, 34). Recently, we have shown that ouabain (2×10^{-4} M) reduces S.C.C. to zero in ~30 min when present on the submucosal side of the tissue, but has little effect when present on the luminal side (50). K-free medium shows the same one-sided effectiveness (Widdicombe, unpublished material). This suggests that active submucosal Na pumps in this tissue are essential for both active Na absorption and Cl secretion.

Na/K ATPase is believed to be essential for active salt and fluid movement in epithelia. Thus, ouabain and other cardiac glycosides have been shown to inhibit active transport in both Cl^--secreting (8, 10, 11, 13, 14, 19, 21, 23, 39, 43, 50) and other epithelia (4, 15, 24, 40). In addition, Bönting and his co-workers have demonstrated a close correlation between the inhibition of tissue Na/K ATPase and the inhibition of salt and fluid transport (3).

In a number of preparations, ouabain inhibits secretion only when present on the serosal side of the tissue (4, 8, 11, 15, 23, 40). Secretion by the choroid plexus, however, is inhibited only by ouabain on the luminal side (51). Localization of Na pumps by autoradiography using [^{3}H]ouabain or by immunoferritin staining (26, 27) has shown that Na pumps are confined almost exclusively to the basolateral cell membranes of most epithelia (9, 18, 22, 26, 27, 31–33, 36, 38, 41, 45). In the choroid plexus, however, they appear to be restricted to the apical cell membranes (37).

In light of the studies on other tissues, and because of the apparent importance of Na pumps to ion transport by the dog's tracheal epithelium, we have attempted to estimate the number and location of Na pumps within this tissue by studying the uptake of [^{3}H]ouabain. A crude estimate of the location of Na-pump sites was obtained by looking at the uptake of radioactivity when either

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/08/0380/11 $1.00
Volume 82 August 1979 380–390

one side or the other of epithelial sheets was exposed to [^{3}H]ouabain. The use of autoradiography allowed a more precise localization of the tissue's Na/K ATPase.

MATERIALS AND METHODS

Dissection

Mongrel dogs of either sex, weighing 20–25 kg, were anesthetized with sodium thiamylal (25 mg/kg i.v.), and their tracheas were removed and placed at room temperature in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution of the following composition (mM): Na^+, 144; K^+, 5.4; Ca^{2+}, 1.9; Mg^{2+}, 1.2; $H_2PO_4^-$, 1.2; HCO_3^-, 25; Cl^-, 127; SO_4^{2-}, 1.2; glucose, 5.5. A longitudinal cut extending the entire length of the trachea was made through the anterior cartilaginous portion. The trachea was then spread out in a dissecting tray. The trachealis muscle was next dissected away from the posterior cartilage-free part of the trachea. For experiments in which only one side of the tissue was to be exposed to ouabain, no further dissection was performed and sheets of tissue were mounted between Lucite half chambers, with an exposed area of 1.13 cm^2. These sheets had a cell layer 50 μm thick with a layer of collagen ~1 mm thick on their submucosal surface. Eight sheets were obtained from each trachea. For experiments in which both sides of the tissue were to be exposed to ouabain, much of the collagen was removed by blunt dissection with fine forceps and blunt-ended scissors. This reduced the thickness of the collagen layer from 1 to ~0.4 mm. The epithelium was then divided horizontally into strips, with up to 55 strips being obtained from any given trachea. These strips were ~1 cm long and 3 mm wide. They weighed 13.2 ± 6.6 mg (mean ± SD, n = 335).

The half chambers bearing the epithelial sheets were connected to perfusion chambers (MRA Corp., Clearwater, Fla.) of essentially the same type as used by Ussing and Zerahn (49), which provided the tissues with a constant circulation of fresh oxygenated Krebs-Henseleit solution at 37°C. The epithelial strips were placed in beakers of Krebs-Henseleit solution, kept at 37°C in a water bath, and oxygenated. When tracer was washed out into tracer-free medium, this was always maintained at 37°C.

Uptake of [^{3}H]Ouabain and [^{14}C]Sucrose

[^{3}H]ouabain was obtained from New England Nuclear (Boston, Mass.), dissolved in a mixture of ethanol:benzene (9:1). The solvent was evaporated over a stream of dry air, and the ouabain was redissolved in distilled water. A trace of nonradioactive ouabain was added to give a final stock [^{3}H]ouabain solution of 10^{-4} M and 1 mCi/ml. Aliquots of this solution were added to the appropriate uptake media. Concentrations of ouabain in the uptake solutions of $>10^{-7}$ M were obtained by adding nonradioactive ouabain.

Extracellular space (e.c.s.) was estimated with [^{14}C]-sucrose (Amersham Corp., Arlington Heights, Ill.). The concentration of sucrose used ($<10^{-7}$ M) had a negligible effect on the tonicities of the bathing media. When the e.c.s. was measured concurrently with [^{3}H]ouabain uptake, [^{14}C]sucrose was added to the uptake media at a sp act of 0.25 μCi/ml, to give a sucrose concentration of ~3 × 10^{-8} M. The sp act of [^{3}H]ouabain was 1 μCi/ml. Thus, during dual label counting of the tissue samples, spillover of counts from the carbon to the tritium channel was kept at a very low value compared to the counts from the tritium.

After uptake, the tissues were weighed on a Mettler HS1 balance (Mettler Instrument Corp., Hightstown, N.J.) to the nearest 0.1 mg and then dissolved overnight in 300 μl of either soluene (ICN Nutritional Biochemicals, Plainview, N.Y.) or protosol (New England Nuclear). Glacial acetic acid (50 or 100 μl) was then added to eliminate photo- and chemiluminescence, and the tissues were counted in aquasol scintillation fluid (New England Nuclear). Aliquots of the uptake media and blanks were treated in the same way as the tissues.

In some experiments, uptake was from a Na-free (high K) medium or from a Na-free (choline) medium. In both cases, the composition of the solutions was the same as that of the Krebs-Henseleit medium described above, except that all the Na was replaced by equivalent amounts of either K or choline.

All uptakes were calculated initially as

$$\mu l/g = \frac{\text{cpm/g wet wt}}{\text{cpm/}\mu\text{l uptake medium}}$$

By using the known concentration of ouabain in the uptake medium and Avagadro's number, this unit of uptake could then be converted into molecules/mg of tissue.

Autoradiography

Tissue samples were removed from the Lucite chambers and immersed for several seconds in Freon 22 cooled to the temperature of liquid nitrogen (−190°C). The tissues were then placed in aluminum cannisters and stored in liquid nitrogen for from 30 min to 7 d. They were quickly transferred from liquid nitrogen to the cooled (−190°C) stage of an Edwards freeze-drying apparatus (Edwards High Vacuum, Inc., Grand Island, N. Y.). The samples were left at −60°C in the freeze-drier at 10^{-7} torr for 4 d. On day 5, the temperature of the freeze-drier was gradually raised to 21°C. At this time, the bell jar of the freeze-drying apparatus was returned to atmospheric pressure and tissues were quickly transferred to a vacuum desiccator containing 1 g of crystalline osmium tetroxide and Drierite desiccant (W. A. Hammond Drierite Co., Xenia, Ohio). The desiccator was attached to a vacuum pump and a vacuum of 10^{-4} torr was established, which was maintained for 12–18 h. After this, tissues were placed in Araldite at room tem-

perature for 18–24 h. They were then transferred to fresh Araldite in aluminum dishes and embedded under vacuum at 60°C. 48 h were allowed for polymerization of the Araldite. The tissue blocks were trimmed, and 1 μm-thick sections were cut with glass knives and mounted on acid-cleaned glass slides. Slides were dipped into Kodak NTB-2 liquid emulsion diluted 1:1 with distilled water and stored in light-sealed plastic boxes with Drierite desiccant at 4°C for from 1 to 12 wk. The slides were developed for 3 min in D-19 (Kodak) at 17°C, washed in distilled water for 1 min, and fixed for 3 min at room temperature in Kodak Rapidfix. Slides were rinsed in seven changes of distilled water and the gelatin of the emulsion was fixed in 10% formalin before staining. Staining was performed by floating a Petri dish in a 65°C bath and heating slides in this dish for 15 s after applying a small drop of toluidine blue (0.5% in 0.5% borax) over the sections. The excess dye was then removed by washing in distilled water and destaining in a 0.5% solution of glacial acetic acid. Sections were air dried before coverslips were applied over a drop of immersion oil. Slides were then viewed and photographed in a Zeiss photomicroscope.

RESULTS

Extracellular Uptake and Uptake by Collagen

After removal of the trachealis muscle, there remains a substantial layer of collagen attached to the submucosal side of the epithelium. The thickness of this layer depends on the degree of stretch of the tissue; in tissues stretched out for use in Ussing chambers it is ~1 mm thick. To minimize potential trapping of tracer in this layer, we removed as much collagen as possible by blunt dissection. This reduced the thickness of the collagen layer to ~400 μm. Uptake of [^{3}H]ouabain by pieces of the dissected collagen was compared to uptake by the remaining tissue. The results of two typical experiments are shown in Table I. It is clear that the epithelial tissues took up significantly more label than the collagen. Furthermore, prolonging the washout period from 30 to 60 min reduced the ouabain activity in the collagen, but not in the epithelial strips. Thus, in the former the tracer may have been either loosely bound or trapped in crevices of the extracellular space between the collagen fibrils.

Because it was important to our binding studies to maximize the amount of ouabain bound to cells compared to extracellular ouabain, we determined the size of the e.c.s., and the rate at which extracellular markers could be washed out. Fig. 1 shows the time course of uptake and washout of [^{14}C]-

TABLE I

The Uptake of [^{3}H]Ouabain By Collagen and By Epithelial Tissue

Ouabain concentration	Uptake		Rinse duration
	Epithelial tissue	Collagen	
M	*μl/g*		*min*
10^{-7}	509 ± 31 (10)	145 ± 9 (12)	30
10^{-7}	565 ± 62 (9)	89 ± 9 (12)	60
2.5×10^{-8}	555 ± 75 (5)	94 ± 18 (10)	60

Uptake was for 2 h. After uptake, tracer was washed out into nonradioactive medium for either 30 or 60 min. Uptake is expressed as μl/g. Values are means ± SEM, number of observations in parentheses. The collagen pieces weighed 25.4 ± 12.6 mg (mean ± SD, n = 34); the epithelial pieces, 19.1 ± 7.8 mg (mean ± SD, n = 24).

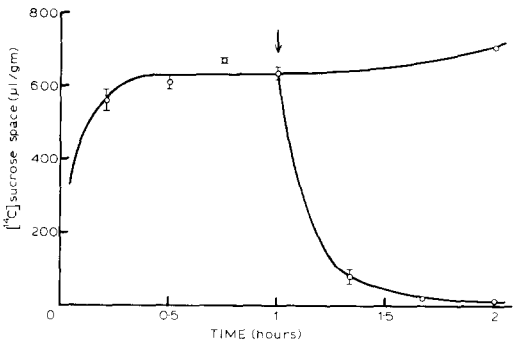


FIGURE 1 The uptake and loss of [^{14}C]sucrose by epithelial sheets. At the arrow, tissues were placed in a large volume of nonradioactive Krebs-Henseleit medium and the label was washed out. In some tissues, uptake was followed for another hour (uppermost line). Each point is the mean ± SEM of five tissues.

sucrose from tissues from one dog. Uptake was complete within 15–30 min and then remained constant for up to 60 min. Taking all the values at 30, 45, and 60 min, a value for the e.c.s. of 637 ± 11 μl/g (n = 15) is obtained. In another dog the [^{14}C]sucrose space after 1-h incubation was 617 ± 11 μl/g. We found no dependence of e.c.s. on tissue weight. After 120 min, the [^{14}C]sucrose space was 709 ± 4 μl/g (n = 5). This slow increase in the tracer volume may represent penetration of marker into the cells, as has been suggested for a similar finding in smooth muscle (5). At 60 min, most of the tissues were transferred to a large volume of Krebs' solution containing no [^{14}C]sucrose. Fig. 1 shows the time course of the washout of radioactivity. After 20 min the extracellular label had declined to 81 ± 20 μl/g (n = 5), after 40 min to 18 ± 3 μl/g (n = 5), and after 60 min to 14

± 3 μl/g (n = 5). Thus, after 20 min, only 13% of the extracellular marker remains, and after 40 min, only 3%. Small traces of sucrose (possibly intracellular) remain in the tissues for up to 1 h of washout. These results suggest that virtually complete washout of extracellular solutes from this tissue can be accomplished within about 30 min.

Uptake and Loss of [^{3}H]Ouabain

With a ouabain concentration of 2.5×10^{-7} M, uptake of tracer was followed over 2 h. After 2 h of uptake, tissues were placed in a large volume of nonradioactive Krebs-Henseleit solution and the washout of tracer was followed. The results are shown in Fig. 2. There was an initial rapid uptake of tracer, presumably into the extracellular space, which was followed by a slower component of uptake which appeared complete after 2 h. On washing out, there was a rapid loss of tracer which appeared complete after 30 min. The tissue ouabain content then remained more or less constant for a further 1½ h. The amount of tracer leaving the tissue during the first 30 min of washout corresponded to a ouabain space of 694 ± 44 μl/g tissue. This is not significantly different from the measured [^{14}C]sucrose space of 637 ± 11 μl/g, which also washes out within 30 min (Fig. 1).

Thus, a 2-h incubation followed by a 30-min wash would seem to produce equilibrium levels of cellular binding and nearly complete removal of extracellular tracer. Consequently, in most of the subsequent studies this was the uptake procedure adopted. In some experiments, 2-h washouts were used and this is mentioned in the text. However, given the undetectable loss of tracer between 30 and 120 min of washout (Fig. 2), this should not affect the results.

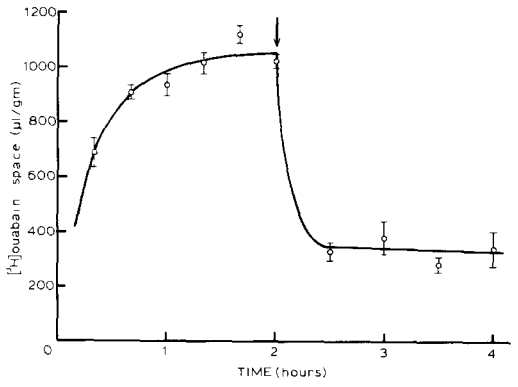


FIGURE 2 Uptake and washout of [^{3}H]ouabain by tissue sheets. Uptake was followed for 2 h. Concentration of ouabain = 2.5×10^{-7} M. At the arrow, tissues were placed in a large volume of nonradioactive Krebs-Henseleit solution and the loss of tracer was followed. Each point is the mean ± SEM of four to six tissues.

Concentration Dependence of Uptake

Using a 2-h incubation and a 30-min rinse, the concentration dependence of uptake over the range 5×10^{-9} M − 10^{-3} M was determined. The results are shown in Fig. 3. To display conveniently the wide range of concentrations and the correspondingly wide range of binding values, the data have been plotted with log/log axes. At ouabain concentrations greater than ~5×10^{-6} M, the uptake depends linearly on concentration. At concentrations <5×10^{-6} M, however, the uptake is greater than that predicted by the uptake at high concentrations. This reflects the presence of a high-affinity saturable component of binding. In Fig. 4, the results from Fig. 3 for low ouabain

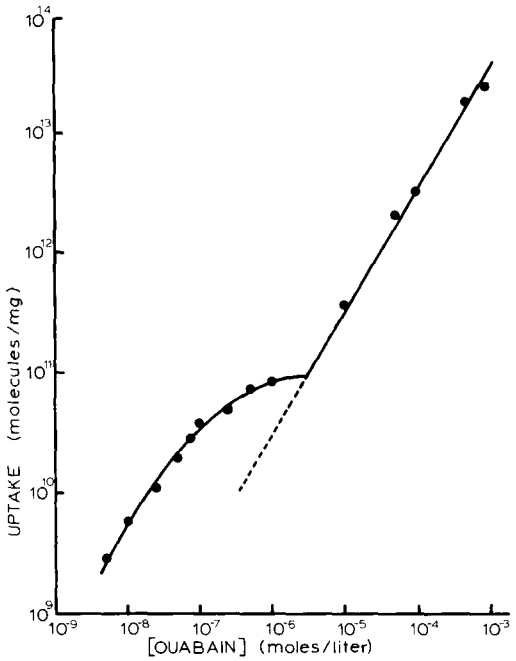


FIGURE 3 The concentration dependence of ouabain binding. Uptake of [^{3}H]ouabain was for 2 h followed by a 30-min washout in nonradioactive medium. Each point is the mean of 5–10 tissues, except when the concentration of ouabain was 5×10^{-9} M, where n = 2. The lines through the points have been drawn by eye. The dashed line is the extrapolated nonspecific component of binding taken from the uptake points for ouabain concentrations >10^{-5} M. Note that both ordinate and abscissa are logarithmic.

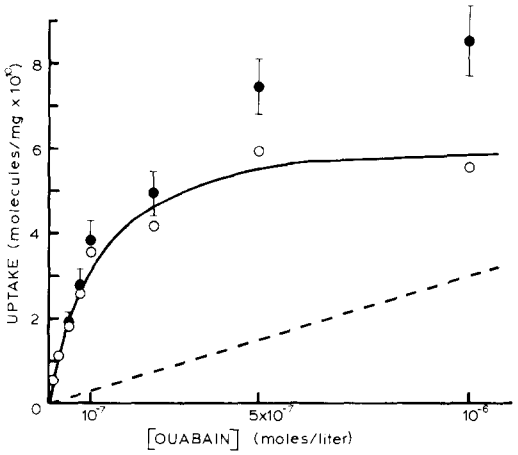


FIGURE 4 Ouabain uptake from concentrations $\leq 10^{-6}$ M. The solid circles show the means ± SEM of the measured [^{3}H]ouabain uptake, and correspond to the points in Fig. 3. The dashed line is the extrapolated value for nonspecific binding obtained from the uptake points for ouabain concentrations $>10^{-5}$ M (see Fig. 3). Subtraction of the estimated nonspecific uptake from the measured binding gives an estimate of the specific saturable component of binding (open circles). The line drawn through these points is a binding curve with a K_m of 10^{-7} M, and a maximal binding capacity of 6×10^{10} molecules/mg of tissue.

concentrations have been plotted with linear axes so as to more clearly differentiate the saturable (specific) and linear (nonspecific) components of ouabain binding. The dashed line is the extrapolated value for nonspecific binding at high concentrations of ouabain and corresponds to the dashed line on Fig. 3. If one subtracts estimates of nonspecific uptake taken from this line from the measured uptakes, values for the specific, saturable ouabain binding are obtained. Fig. 4 shows that these are well fitted by a binding curve with a $K_{0.5}$ of $\sim 10^{-7}$ M and a maximal binding capacity of 6×10^{10} molecules/mg.

In the binding experiments described below, the ouabain concentrations used ($\leq 10^{-6}$ M) were such that the nonspecific binding was small compared to the specific saturable component.

Variation Along the Trachea

In one experiment the epithelial strips were threaded onto fine silk thread in the order in which they were dissected, and the dependence of ouabain binding on the position along the trachea was determined. The concentration of ouabain was 10^{-7} M, and uptake was for 2 h followed by a 30-min rinse. The mean for binding for all samples was 5.21 ± 0.14 ($\times 10^{10}$) molecules/mg ($n = 46$). There was a slight increase in ouabain binding posteriorly (from a predicted 4.42×10^{10} molecules/mg in the most anterior strip to 6.00×10^{10} molecules/mg in the most posterior). However, the correlation was not significant ($R = 0.247$, $n = 46$, $P > 5\%$). The amount of ouabain bound declined with the size of the strip (−1.8%/mg). However, the correlation was, again, not significant ($R = 0.178$, $n = 46$, $P > 5\%$).

Submucosal vs. Luminal Uptake

Sheets of tissue were mounted between half chambers, and one of the two sides was exposed to 10^{-6} M tritiated ouabain. After 2-h uptake, the tissues were washed for a further 2 h in nonradioactive Krebs' solution, and the [^{3}H]ouabain con-

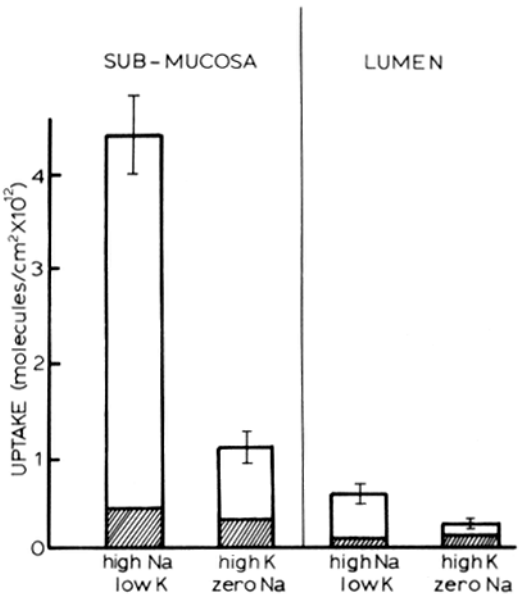


FIGURE 5 The binding of ouabain after exposure to tracer from one side only. Ouabain concentration = 10^{-6} M. The height of the columns represents the uptake of ouabain; the hatched area shows the ouabain expected in the simultaneously measured [^{14}C]sucrose space. The unhatched area of the columns, therefore, represents cellularly bound ouabain. The two left hand columns show uptake from the submucosal medium from normal Krebs-Henseleit (high Na, low K) or a K-substituted medium (high K, zero Na). The two right-hand columns show the corresponding uptakes after luminal exposure to tracer. The values are the means ± SEM of eight tissues from four animals. Uptake was for 2 h followed by a 2-h washout.

tent was determined. The uptake from normal Krebs-Henseleit for each side of the tissue was also compared with that from a Na-free (high K) medium. The [^{14}C]sucrose spaces were determined simultaneously on the same tissues, and the results are shown in Fig. 5.

Subtraction of the [^{14}C]sucrose space (converted here to molecules of ouabain/mg) from the total tissue ouabain gives a value for bound ouabain. This is 4.0 ± 0.4 (× 10^{12}) molecules/cm^2 when ouabain was added to the submucosal bath, and 0.4 ± 0.1 (× 10^{12}) molecules/cm^2 when it was added to the luminal bath. Replacement of Na by K in the solutions on both sides of the tissues reduced uptake to 0.4 ± 0.2 (× 10^{12}) molecules/cm^2 by the submucosal surface, and 0.14 ± 0.05 (× 10^{12}) molecules/cm^2 by the luminal surface. K is known to inhibit ouabain binding to Na/K ATPases under all conditions of binding (17, 30, 44, 48). Hence, the inhibition of binding by K in these experiments strongly suggests that the binding is to Na-pump sites.

TABLE II

Inhibition of Ouabain Binding

Ouabain concentration	Ouabain uptake	*n*	Treatment
M	μl/g		
10^{-7}	604 ± 77	9	control
	226 ± 19	8	Na-free (choline)
	152 ± 19	6	17°C
2.5×10^{-8}	555 ± 75	5	control
	153 ± 15	5	anoxia (N_2)

The uptake values are means ± SEM. Uptakes were for 120 min followed by a 30-min (10^{-7} M) or 60-min (2.5×10^{-8} M) wash.

Specificity of Binding

In addition to the inhibition of binding by K, we decided to use other tests to see whether the binding of ouabain was to Na pumps. In isolated Na/K ATPases binding is supported by Mg^{2+}, Na^+, and ATP (30, 42, 44, 47), and is also temperature sensitive, with the rate of binding less at lower temperatures (17). In Table II the effects of Na-free (choline) medium, lowering the incubation temperature to 17°C, and hypoxia induced by bubbling with 95% N_2, 5% CO_2 are shown. This last treatment reduces ATP levels in other tissues,

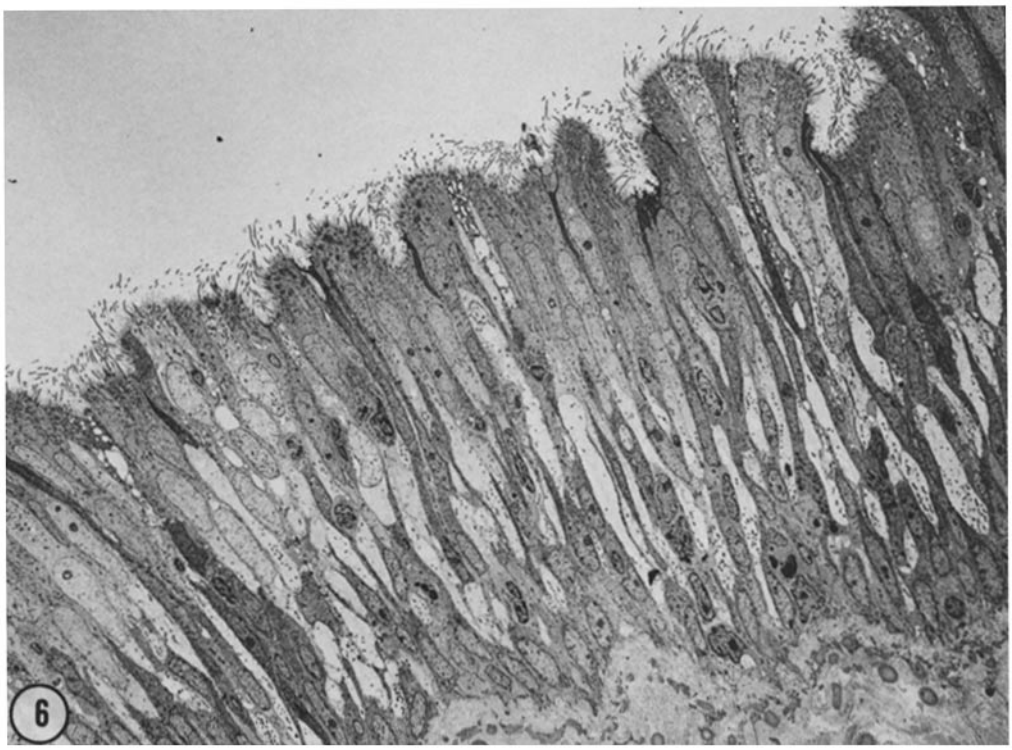
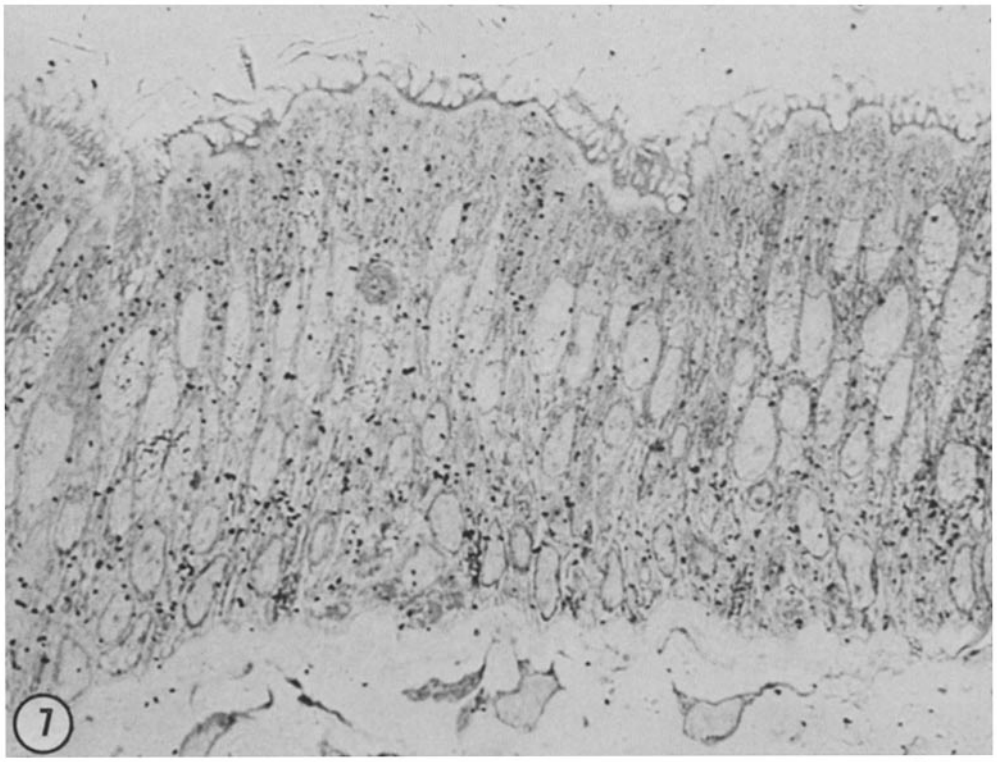
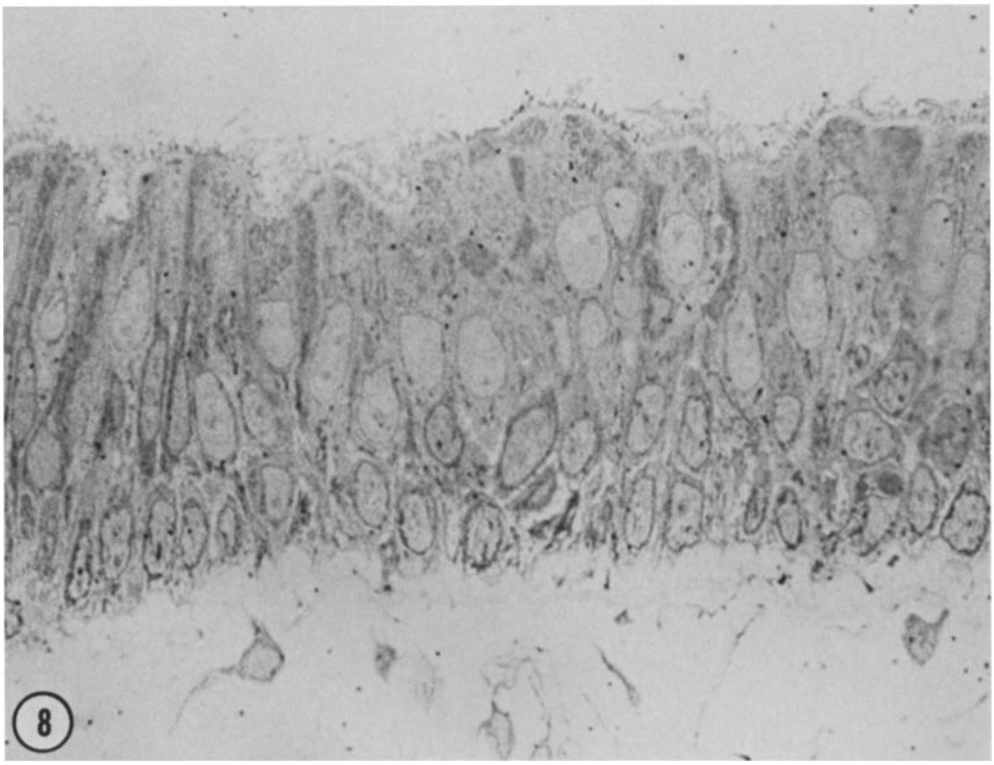
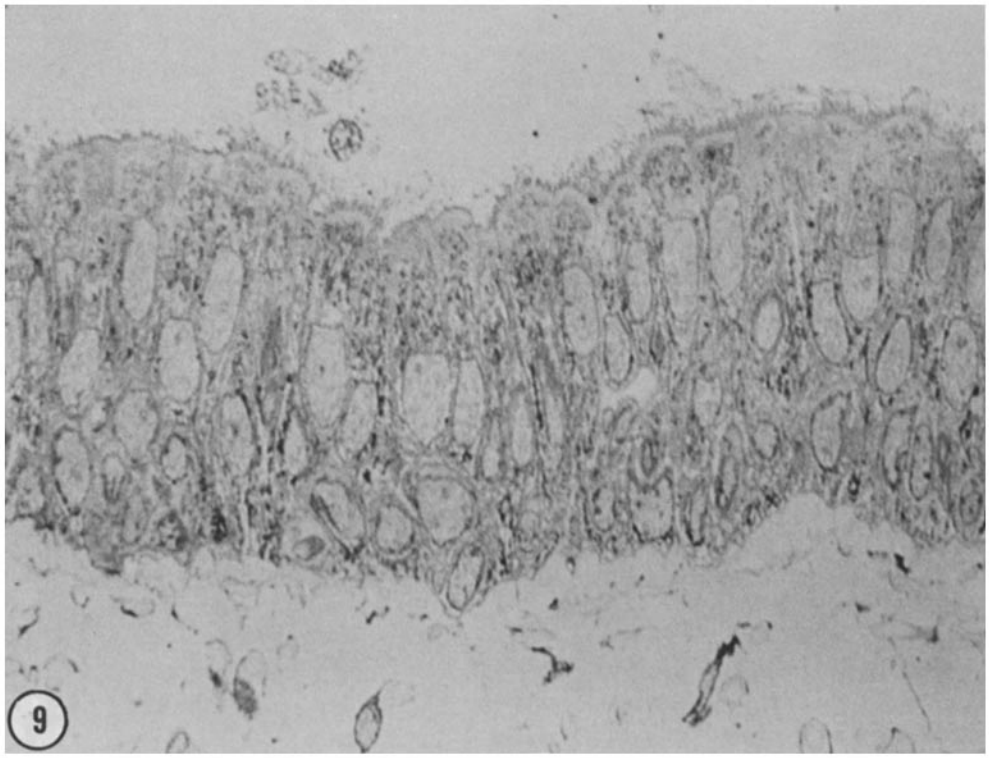


FIGURE 6 Low-power electron micrograph of dog tracheal epithelium. See text for description. × 1,150.

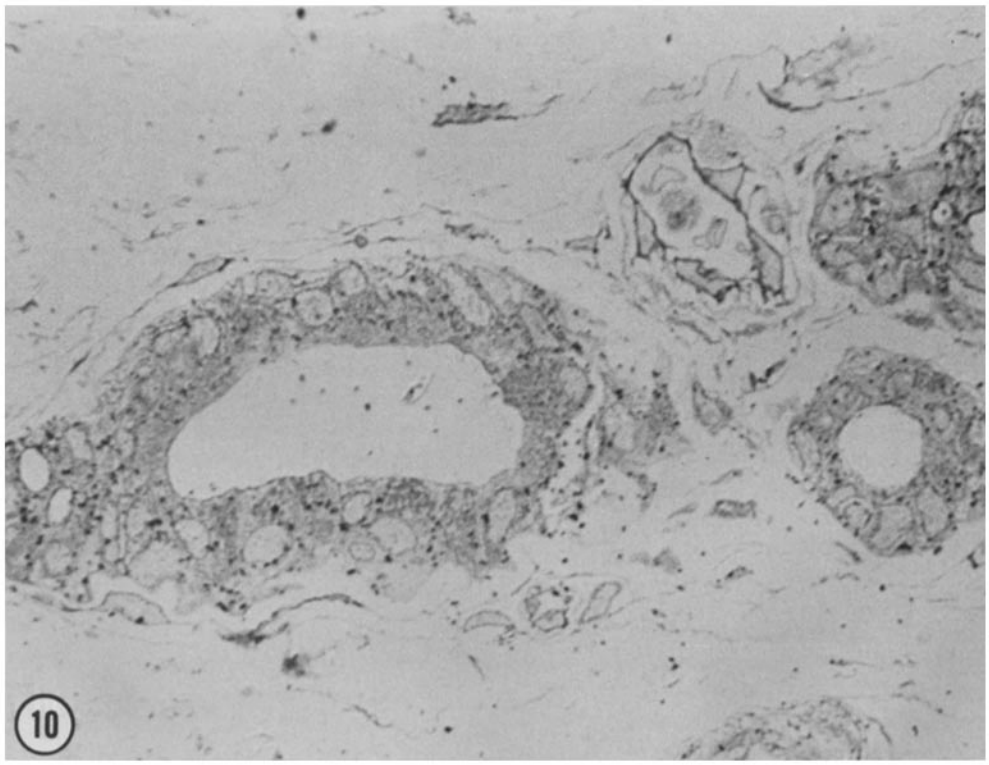




FIGURES 7–10 Autoradiographic localization of [^{3}H]ouabain. Tissues were exposed to tracer for 1 h followed by a 30-min rinse. The sp act of the ouabain was 10 μCi/ml throughout. All autoradiographs were exposed for 3 wk. Fig. 7: 10^{-6} M ouabain present on the submucosa. Fig. 8: 10^{-6} M ouabain in luminal bath. Fig. 9: 10^{-4} M ouabain on submucosa. Fig. 10: 10^{-6} M ouabain on submucosa. Figs. 7–9 show surface epithelium. Fig. 10 shows submucosal gland ducts. All figures × 1,040.



9



10

and is known to abolish S.C.C. in this tissue (34). All these treatments caused significant decreases in the amount of bound ouabain.

Autoradiography

Because of the comparatively poor resolution in freeze-dried preparations, we have included a low-power electron micrograph (Fig. 6) to aid the reader in his interpretation of the following autoradiographs. The dog's trachea has a pseudostratified columnar epithelium similar to tracheobronchial epithelium from other species (7). Ciliated cells extend the width of the epithelium from basement membrane to lumen. Between them lie basal and intermediate cells, which do not reach the lumen and are thought to be cells in the process of differentiation. A few goblet cells are also present, though not shown here.

Figs. 7 and 8 show two tissues from the same dog, both of which had been exposed to 10^{-6} M ouabain (10 μCi/ml) for 1 h followed by a 30-min rinse. In Fig. 7, however, ouabain was in the submucosal bath, while in Fig. 8 it was in the luminal bath. Tracer uptake is far less with luminal exposure than with submucosal exposure. In both cases, the label is located predominantly over the basolateral cell membranes. Cell nuclei show negligible labeling. The finding of many fewer grains binding to the tissue during exposure to [^{3}H]ouabain from the luminal than the submucosal side is in keeping with the results shown in Fig. 5. In both cases, no binding to the apical cell surfaces could be demonstrated.

The tissue shown in Fig. 9 was incubated in an uptake medium, which had the same specific activity of [^{3}H]ouabain as in the experiments illustrated in Figs. 7 and 8 (i.e. 10 μCi/ml). However, the ouabain concentration had been raised from 10^{-6} M to 10^{-4} M by addition of nonradioactive ouabain. Uptake was again for 60 min followed by a 30-min rinse. It is clear that addition of nonradioactive ouabain greatly reduces the binding of [^{3}H]ouabain. This indicates that binding is specific, and is on a limited number of sites from which nonradioactive ouabain can competitively displace tritiated ouabain.

Although it is difficult to provide precise cytological localization by light microscopy, the almost complete absence of grains over the cell nuclei suggests that the ouabain binding is to cell membranes. The absence of grains over the apical cytoplasm is also consistent with the idea that pump sites are confined to the basolateral membranes. We could not distinguish preferential binding to any particular epithelial cell type.

Of interest was the finding that mucus gland cells also showed considerable uptake of label after submucosal exposure to [^{3}H]ouabain (Fig. 10). Both surface epithelial cells and gland cells took up more label than nonepithelial cells (e.g., endothelial cells).

DISCUSSION

The uptake of ouabain appeared complete after 2 h. On washing with nonradioactive medium, there was an initial rapid loss of tracer, which was complete after 30 min; after this, tissue ouabain levels remained constant for up to 2 h of washing. The difference in counts between the uptake point at 120 min of uptake and that after 30 min of washing corresponds to 694 ± 44 μl/kg wet wt, and this is not significantly different from the [^{14}C]sucrose extracellular space. Thus, the remaining ouabain is presumably bound to the cells. The small amount of binding to isolated strips of collagen (Table I) and the autoradiographic evidence (Fig. 7) localizes this ouabain to the epithelial layer. This clearly has a very low dissociation constant (undetectable in our experiments), as has been described for Na/K ATPase preparations from other dog tissues (16, 46, 47).

The finding of a nonsaturable component of binding at concentrations $>10^{-6}$ M ouabain has been described before (2, 6, 20, 33). Subtraction of this component of binding from the total uptake of tracer gives values for the specific saturable component of uptake. This has a maximal capacity of 6×10^{10} molecules/mg and an estimated K_m of 10^{-7} M. This estimate of K_m is similar to values published for other tissues (1, 2, 6, 12, 35).

The binding of ouabain to the tracheal epithelium is saturable with a K_m similar to those published for binding to Na/K ATPases and the Na pump in other tissues. Further evidence that binding is to the Na pump comes from the inhibition of uptake by Na-free (choline) medium, by high K (Na-free) solution, low temperature, and metabolic inhibition (by anoxia). These procedures lead to reduced binding of ouabain to isolated Na/K ATPases (17, 30, 44).

When our tissues were exposed to [^{3}H]ouabain on one side only, submucosal exposure produced ouabain binding 10 × greater than that of luminal exposure (see Fig. 5), indicating that many more pump sites are accessible from the submucosal surface of the epithelium.

The autoradiographic studies confirmed the results obtained with tissue counts alone and provided a more precise localization of the pump sites. Whether the tissue was exposed to [^{3}H]ouabain from the luminal or the submucosal side, the silver grains were found almost entirely over the basolateral membranes. The number of grains over the apical cell membranes was no more than that predicted from the background levels. There are comparatively few grains over the cell nuclei, and it is quite possible that those represent the nonspecific binding, which at 10^{-6} M ouabain should account for ~25% of the total binding (see Fig. 5). The number of grains present over the epithelium was reduced by adding nonradioactive ouabain. This finding suggests that the uptake is specific, representing binding to a restricted number of sites. The reduction in the numbers of counts produced by high K, Na-free (choline), anoxia, and low temperature indicate that this specific binding is to Na pumps.

Our results agree with those of other studies in that the side of the epithelium which has the most Na pumps is the side on which ouabain has an inhibitory action on ion transport. We have reported that luminal ouabain has a small effect on ion transport (50). However, the location of grains in tissues exposed to luminal ouabain is on the basolateral membranes and it seems that luminal ouabain is crossing the tissue through some unknown route (possibly areas of edge damage), and causing reduced ion transport by inhibition of basolateral Na pumps.

Tissues exposed to submucosal [^{3}H]ouabain show a somewhat higher density of silver grains in the lower third of the epithelium than elsewhere (see Fig. 7). We feel that this probably does not have a functional significance, but merely reflects the fact that the surface area of cell membrane in this region is increased by the presence of basal and intermediate cells (see Fig. 6).

The autoradiographic localization of ouabain in other secretory epithelia revealed that, with the exception of the choroid plexus (37), the pumps are located on the basolateral membranes. In absorptive epithelia, this has generally been taken to support the view that the tissues move Na in the fashion originally proposed by Koefoed-Johnsen and Ussing (25), whereby Na passes passively into the cells across one membrane to be pumped actively out of the cells across the opposite membrane, on which the majority of Na pumps are located. The finding in secretory epithelia (18, 22, 36) that Na pumps were also located predominantly on the basolateral membranes led several authors to conclude that the Na pump played no direct role in the secretory processes, serving merely to maintain the necessary internal milieu (10, 36).

Recently, however, Silva et al. (43), for the shark's rectal gland, and Ernst and Mills (18), for the avian salt gland, have independently proposed a model for active ion transport which explains the predominantly basolateral location of the Na pumps in Cl-secreting epithelia. They propose that there is a linked entry of Na and Cl across the basolateral membranes, the energy stored in the transmembrane Na gradient providing for active accumulation of Cl. This Cl now diffuses down its electrochemical gradient across the luminal cell surfaces. The Na entering with the Cl is returned by the basolateral Na pumps. Under open circuit conditions, Na presumably follows the secreted Cl by passing down an electrical gradient. This passage is presumably between the cells, for the basolateral location of Na pumps would hinder its passage through the cells. That a similar system is responsible for Cl secretion in the canine tracheal epithelium is supported by the fact that under short circuit conditions, the net Cl movement is abolished by replacing the Na in the medium with choline or by submucosal ouabain (Widdicombe, unpublished results and reference 50).

In the short-circuited dog tracheal epithelium, there is a net movement of Na towards the submucosa (28, 29, 34). This is abolished by submucosal ouabain (Widdicombe, unpublished material). The basolateral location of Na pumps in the tissue suggests that there may be passive entry of Na across the luminal surfaces of the cells followed by active pumping of the Na across the basolateral membranes.

In conclusion, Na pumps in the dog's tracheal epithelium are located almost entirely on the basolateral membranes. This location is consistent with the active Cl secretion and Na absorption seen in this tissue, and with the fact that submucosal ouabain is a potent inhibitor of active transport, whereas luminal ouabain is relatively ineffective (50).

We thank Dr. J. A. Nadel for his support and interest, Ms. B. Cost for her excellent typing, and Iris Ueki for technical assistance.

This work was supported in part by United States Public Health Service Program Project grant HL-06285.

J. H. Widdicombe was supported by the Julius H. Comroe, Jr. Research Fellowship.

Received for publication 16 October 1978, and in revised form 27 March 1979.

REFERENCES

1. BAKER, P. F., and J. S. WILLIS. 1970. Potassium ions and the binding of cardiac glycosides to mammalian cells. *Nature (Lond.).* **226:**521–523.
2. BAKER, P. F., and J. S. WILLIS. 1972. Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol.* **224:**441–462.
3. BÖNTING, S. L. 1970. Sodium-potassium activated adenosine triphosphatase and cation transport. *In* Membranes and Ion Transport, Vol I. E. E. Bittar, editor. John Wiley & Sons Ltd., Chichester, Sussex, England. 257–363.
4. BÖNTING, S. L., and M. R. CANADY. 1964. Na-K activated adenosine triphosphatase and sodium transport in toad bladder. *Am. J. Physiol.* **207:**1005–1009.
5. BRADING, A. F., and A. W. JONES. 1969. Distribution and kinetics of Co-EDTA in smooth muscle, and its use as an extracellular marker. *J. Physiol.* **200:**387–401.
6. BRADING, A. F., and J. H. WIDDICOMBE. 1974. An estimate of the sodium/potassium pump activity and the numbers of pump sites in the smooth muscle of the guinea-pig taenia coli, using [^{3}H]ouabain. *J. Physiol.* **238:**235–249.
7. BREEZE, R. G., and E. B. WHEELDON. 1977. The cells of the pulmonary airways. *Am. Rev. Respir. Dis.* **116:**705–777.
8. BRUNTON, W. J., and R. L. BRINSTER. 1971. Active chloride transport in the isolated rabbit oviduct. *Am. J. Physiol.* **221:**658–661.
9. BUNGAARD, M., M. MØLLER, and J. H. POULSEN. 1977. Localization of sodium pump sites in cat salivary glands. *J. Physiol.* **273:**339–353.
10. BURG, M. B., and N. GREEN. 1973. Function of the thick ascending limb of Henle's loop. *Am. J. Physiol.* **224:**659–668.
11. CANDIA, O. A. 1972. Ouabain and sodium effects on chloride fluxes across the isolated bullfrog cornea. *Am. J. Physiol.* **223:**1053–1057.
12. CLAUSEN, T., and O. HANSEN. 1974. Ouabain binding and Na^+-K^+ transport in rat muscle cells and adipocytes. *Biochim. Biophys. Acta.* **345:**387–404.
13. COOPERSTEIN, I. L. 1959. The inhibitory effect of strophanthidin on secretion by the isolated gastric mucosa. *J. Gen. Physiol.* **42:**1233–1239.
14. DEGNAN, K. J., K. J. KARNAKY, and J. A. ZADUNAISKY. 1977. Active chloride transport in the *in vitro* opercular skin of a teleost (*Fundulus heteroclitus*), a gill-like epithelium rich in chloride cells. *J. Physiol.* **271:** 155–191.
15. DIAMOND, J. M. 1962. The reabsorptive function of the gall bladder. *J. Physiol.* **161:**442–473.
16. ERDMANN, E., and W. SCHONER. 1973. Ouabain-receptor interactions in (Na^+ and K^+)-ATPase preparations from different tissues and species. *Biochim. Biophys. Acta.* **307:**386–398.
17. ERDMANN, E., and W. SCHONER. 1973. Ouabain-receptor interactions in (Na^+ and K^+)-ATPase preparations. II. Effect of cations and nucleotides on rate constants and dissociation constants. *Biochim. Biophys. Acta.* **330:**302–315.
18. ERNST, S. A., and J. W. MILLS. 1977. Basolateral plasma membrane localization of ouabain-sensitive sodium transport site in the secretory epithelium of the avian salt gland. *J. Cell. Biol.* **75:**74–94.
19. GATZY, J. T. 1967. Bioelectric properties of the isolated amphibian lung. *Am. J. Physiol.* **213:**425–431.
20. GODFRAIND, T., and M. LESNE. 1972. The uptake of cardiac glycosides in relation to their actions in isolated cardiac muscle. *Br. J. Pharmacol.* **46:**488–497.
21. JACOBSON, H. R., J. B. GROSS, S. KAWAMURA, J. D. WATERS, and J. P. KOKKO. 1976. Electrophysiological study of isolated perfused human collecting ducts. Ion dependency of the transepithelial potential difference. *J. Clin. Invest.* **58:**1233–1239.
22. KARNAKY, K. J., L. B. KINTER, W. B. KINTER, and C. E. STIRLING. 1976. Teleost chloride cell. II. Autoradiographic localization of gill Na, K-ATPase in Killifish *Fundulus heteroclitus* adapted to low and high salinity environments. *J. Cell Biol.* **70:**157–177.
23. KITAHARA, S. 1967. Active transport of Na^+ and Cl^- by in vitro nonsecreting cat gastric mucosa. *Am. J. Physiol.* **213:**819–823.
24. KOEFOED-JOHNSEN, V. 1957. The effect of G-strophanthin (ouabain) on the active transport of sodium through the isolated frog skin. *Acta. Physiol. Scand.* 42: (Suppl. 145) 87–88.
25. KOEFOED-JOHNSEN, V., and H. H. USSING. 1958. The nature of the frog skin potential. *Acta. Physiol. Scand.* **42:**298–308.
26. KYTE, J. 1976. Immunoferritin determination of the distribution of (Na^+ and K^+) ATPase over the plasma membranes of renal convoluted tubules. I. Distal segment. *J. Cell. Biol.* **68:**287–303.
27. KYTE, J. 1976. Immunoferritin determination of the distribution of (Na^+ and K^+) ATPase over the plasma membranes of renal convoluted tubules. II. Proximal segment. *J. Cell. Biol.* **68:**304–318.
28. MARIN, M. G., B. DAVIS, and J. A. NADEL. 1976. Effect of acetylcholine on Cl and Na^+ fluxes across dog tracheal epithelium *in vitro*. *Am J. Physiol.* **231:**1546–1549.
29. MARIN, M. G., B. DAVIS, and J. A. NADEL. 1977. Effect of histamine on electrical and ion transport properties of tracheal epithelium. *J. Appl. Physiol.* **42:**735–738.
30. MATSUI, H., and A. SCHWARTZ. 1968. Mechanism of cardiac glycoside inhibition of the (Na^+ and K^+)-dependent ATPase from cardiac tissue. *Biochim. Biophys. Acta.* **151:**655–663.
31. MILLS, J. W., and D. R. DIBONA. 1978. Distribution of Na^+ pump sites in the frog gallbladder. *Nature (Lond.).* **271:**273–275.
32. MILLS, J. W., and S. A. ERNST. 1975. Localization of sodium pump site in frog urinary bladder. *Biochim. Biophys. Acta.* **375:**268–273.
33. MILLS, J. W., S. A. ERNST, and D. R. DIBONA. 1977. Localization of Na^+ pump sites in frog skin. *J. Cell Biol.* **73:**88–110.
34. OLVER, R. E., B. DAVIS, M. G. MARIN, and J. A. NADEL. 1975. Active transport of Na^+ and Cl^- across the canine tracheal epithelium *in vitro*. *Am. Rev. Respir. Dis.* **112:**811–815.
35. QUASTEL, M. R., and J. G. KAPLAN. 1975. Ouabain binding to intact lymphocytes. Enhancement by phytohemagglutin and leucoagglutinin. *Exp. Cell Res.* **94:**351–362.
36. QUINTON, P. M. and J. MCD. TORMEY. 1976. Localization of Na/K-ATPase sites in the secretory and reabsorptive epithelia of perfused eccrine sweat glands: a question to the role of the enzyme in secretion. *J. Membr. Biol.* **29:**383–399.
37. QUINTON, P. M., E. M. WRIGHT, and J. MCD. TORMEY. 1973. Localization of sodium pumps in the choroid plexus epithelium. *J. Cell. Biol.* **58:**724–730.
38. RENFRO, J. L., D. S. MILLER, K. J. KARNAKY, and W. B. KINTER. 1977. Na-K-ATPase localization in teleost urinary bladder by [^{3}H]-ouabain autoradiography. *Am. J. Physiol.* **231:**1735–1743.
39. ROCHA, A. S., and J. P. KOKKO. 1973. Sodium chloride and water transport in the medullary thick ascending limb of Henle. Evidence for active chloride transport. *J. Clin. Invest.* **52:**612–622.
40. SCHULTZ, S. G., and R. ZALUSKY. 1964. Ion transport in isolated rabbit ileum. I. Short circuit current and Na fluxes. *J. Gen. Physiol.* **47:**567–584.
41. SHAVER, J. L. F., and C. STIRLING. 1978. Ouabain binding to renal tubules of the rabbit. *J. Cell. Biol.* **76:**278–292.
42. SIEGEL, G. L., and L. JOSEPHSON. 1972. Ouabain reaction with microsomal (Na and K)-activated ATPase. Characteristics of substrate and ion dependencies. *J. Biochem.* **25:**323–335.
43. SILVA, P., J. STOFF, M. FIELD, L. FINE, J. N. FORREST, and F. H. EPSTEIN. 1977. Mechanism of active chloride secretion by shark rectal gland: role of Na-K-ATPase in chloride transport. *Am. J. Physiol.* **233:** F298–F306.
44. SKOU, J. C., K. W. BUTLER, and O. HANSEN. 1971. The effect of magnesium, ATP, P_i, and sodium on the inhibition of the (Na^+ and K^+)-activated enzyme system by G-strophanthin. *Biochim. Biophys. Acta.* **241:**443–461.
45. STIRLING, C. E. 1972. Radioautographic localization of sodium pump sites in rabbit intestine. *J. Cell. Biol.* **53:**704–714.
46. TOBIN, T., and T. M. BRODY. 1972. Rates of dissociation of enzyme-ouabain complexes and $K_{0.5}$ values in (Na^+ and K^+) adenosine triphosphatases from different species. *Biochem. Pharmacol.* **21:**1553–1560.
47. TOBIN, T., R. HENDERSON, and A. K. SEN. 1972. Species and tissue differences in the rate of dissociation of ouabain from (Na^+ and K^+)-ATPase. *Biochim. Biophys. Acta.* **274:**551–555.
48. TOBIN, T., and A. K. SEN. 1970. Stability and ligand sensitivity of [^{3}H]ouabain binding to (Na^+ and K^+)-ATPase. *Biochim. Biophys. Acta.* **198:**120–131.
49. USSING, H. H., and K. ZERAHN. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23:**110–127.
50. WIDDICOMBE, J. H., J. Y. YEE, and J. A. NADEL. 1978. Site of Na pumps in dog tracheal epithelium. *Fed. Proc.* **37:**221 (Abstr.).
51. WRIGHT, E. M. 1972. Mechanisms of ion transport across the choroid plexus. *J. Physiol.* **226:**545–571.