

# New Methods Used to Investigate the Control of Mucus Secretion and Ion Transport in Airways

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Our group developed two *in vivo* methods to study secretions from submucosal glands in exposed tracheal epithelium. (1) The exposed mucosal surface was coated with powdered tantalum; accumulated secretions produced elevations (hillocks) in the tantalum layer under which the duct openings were located. The rate of formation of the hillocks was observed through a dissecting microscope, and recorded by television on a video tape recorder. (2) Micropipets were used to collect timed samples from individual gland duct openings. With these techniques, the innervation of the submucosal glands and the autonomic regulation of their secretions were studied.

We studied the control of ion movement across tracheal epithelium because active ion transport forms local osmotic gradients across epithelia which could regulate transepithelial water movement. We mounted pieces of the posterior wall of dog trachea in Ussing-type chambers and measured unidirectional fluxes of  $\text{Cl}^-$  and  $\text{Na}^+$  under short-circuit conditions with  $^{36}\text{Cl}$  and  $^{24}\text{Na}$ . We found active transport of  $\text{Cl}^-$  toward the lumen and  $\text{Na}^+$  toward the submucosa. With this technique we investigated the effect of parasympathomimetic drugs on ion movement.

With a new *in vitro* method we studied output of  $^{35}\text{S}$  bound to sulfated mucins and movement of ions in cat trachea. We mounted pieces of anterior tracheal wall in Ussing-type chambers, added sodium  $^{35}\text{S}$ -sulfate to the submucosal side and monitored secretion of bound  $^{35}\text{S}$  in samples from the luminal side after dialysis. The unidirectional fluxes of  $\text{Cl}^-$  and  $\text{Na}^+$  were measured with  $^{36}\text{Cl}$  and  $^{24}\text{Na}$ . With this method we examined the effect of  $\alpha$ -adrenergic and  $\beta$ -adrenergic agonists on mucin secretion and ion movement. Also with this preparation we studied the relationship between the permeability of the paracellular pathway to  $^{14}\text{C}$ -sucrose and the pattern of tight junction strands.

## Introduction

Mucins secreted from submucosal glands and surface epithelial cells combine with water to form the respiratory tract secretions. The secretions with trapped inhaled particles are moved up the airway to the mouth by the sweeping action of the cilia. This clearing process may be controlled by neurotransmitters or mediators acting on secretion of mucin from submucosal glands and from surface epithelial cells, on the composition or consistency of mucus, on the interaction of the cilia with the mucus layer, and on the rate of ciliary beating. These controls may be altered by environmental toxins, infections, in-

flammatory responses or abnormal biochemical pathways and cause disease.

Knowledge gained from previous studies of airway epithelium has recently been reviewed (1). In the past, studies of respiratory tract secretions in man depended on the collection of sputum (2-4) which is contaminated by saliva and nasal mucus, or on the collection of samples of mucus secretion via a tracheostomy (4). Knowledge of the action of drugs and mediators on mucus secretion was inferred from measurements of respiratory tract secretions in animals (5-7) or from changes in volume in a fluid-filled segment of trachea (8). These older methods do not localize the source of the secretions to submucosal glands or surface epithelial cells; therefore, they cannot be used to examine the control of secretion.

In this review we describe new methods for studying the control of mucus secretions that we developed using preparations of trachea *in vivo* and

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*in vitro*. Preliminary descriptions of some of these methods have been published previously (9-15). The methods provide direct evidence of secretion from the submucosal glands and allow us to study the functions of airway epithelium under well controlled conditions.

## **In Vivo Techniques**

To study the control of secretion of individual mucus glands, we developed techniques which will allow us to study the submucosal gland as a physiological subunit *in vivo*. We anesthetize dogs and ventilate the lungs artificially by using a Harvard pump. Both vagus nerves are dissected free and placed on stimulating electrodes. We make an incision in the anterior midline of the upper two-thirds of the trachea and pull the cut edges apart widely to expose the epithelial surface. A fine powder of inert metal, (tantalum) is then sprayed onto the epithelial

surface. Since the normal dispersion of secretions from the submucosal glands is prevented, elevations in the tantalum layer at the sites of the submucosal gland duct openings appear during secretory activity (Fig. 1). These elevations which are caused by submucosal gland secretion are called "hillocks." We have demonstrated that our new technique is suitable for neurophysiological studies. In each observation period, after the previously secreted mucus has been removed by wiping, the mucosal surface is coated with powdered tantalum and photographed through a dissecting microscope (magnification, 6×). We photograph the tantalum layer over the same area of epithelium at one-half minute intervals during baseline conditions and after stimulating either the superior laryngeal or recurrent laryngeal nerve electrically. The number of hillocks on each photograph is counted and divided by the area of epithelium to obtain the numbers of hillocks per square centimeter. These studies show that sub-

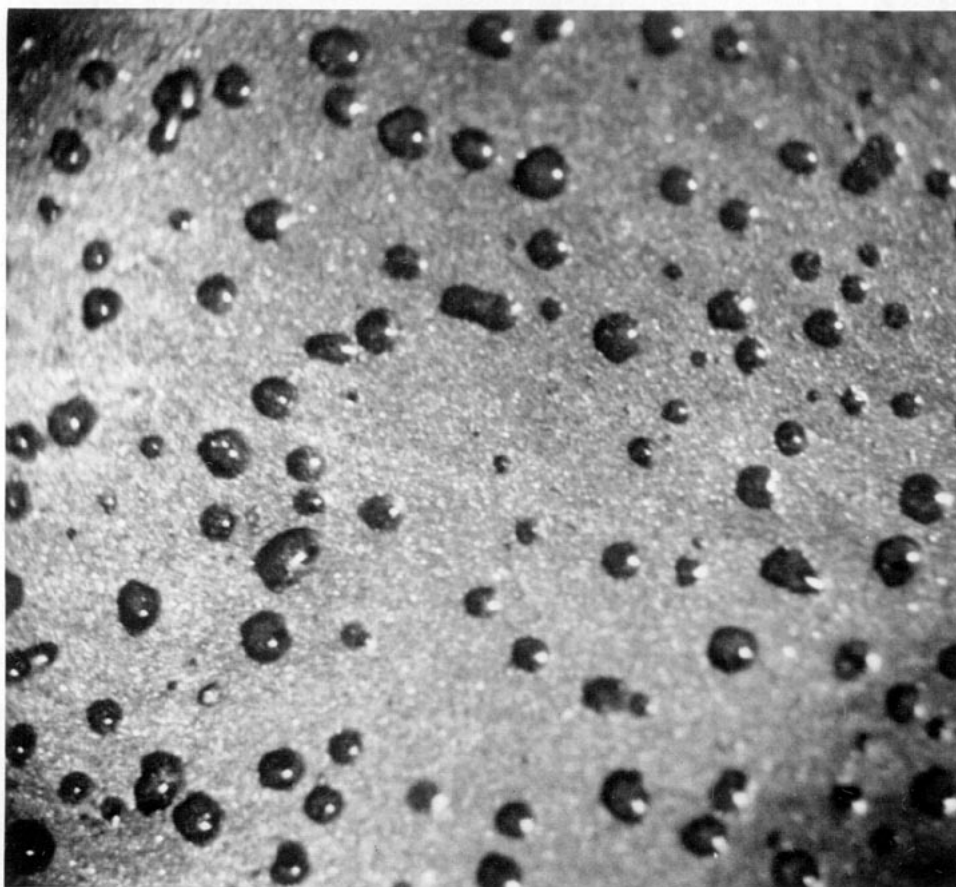


FIGURE 1. Photograph obtained through a dissecting microscope of hillocks on tantalum-coated canine tracheal epithelium produced by electrical stimulation of a superior laryngeal nerve. The tantalum prevented the normal dispersion of the secretions from the mucus glands. The hillocks, caused by accumulated secretions, averaged 0.2 mm in diameter.

mucosal gland secretion is increased by electrical stimulation of the superior laryngeal and recurrent laryngeal nerves (9). We are able to prevent the secretomotor effect of stimulating the motor branches of the vagus nerve by atropine sulfate given intravenously or locally, indicating that the secretomotor effect involves postganglionic cholinergic pathways.

The measurement of the number of hillocks per square centimeter of tracheal epithelium does not provide evidence about secretion from individual submucosal glands; therefore we modified our photographic measuring system (16). Now we visualize the hillocks with a television camera attached to a dissecting microscope, record their images with a videotape recorder and view the field during the experiment on a television monitor. A record of time is simultaneously displayed on the monitor and recorded on videotape. Thus, we make a continuous record of the secretion from individual submucosal glands; the rate of secretion can be estimated from the increase in the size of a hillock with time. We divide the observed field into six sections and measure the diameter of one round hillock in each section if that is possible.

We measure the diameters of the hillocks using a split-video image measuring device (Instrumentation for Physiology and Medicine, San Diego). The images of the hillocks are played back from a videotape recorder onto the screen of a modified television monitor. The image is held on the screen by stopping the videotape recorder at any desired time. To measure the diameter of a hillock, we select the line in the video image which runs along the diameter of the hillock, offset electrically the upper part of the image

of the hillock, and displace it from the lower part of the image of the hillock by the length of one diameter (Fig. 2). The distance moved, which equals the diameter of the hillock, is shown on a digital scale, which we convert to mm with a calibration factor. This measuring device allows us to measure the diameters of hillocks at frequent short time intervals (Fig. 3). To estimate the rate of secretion of individual glands we assume that each hillock is hemispherical, and calculate the volumes of the hemispheres from the measured diameters. Although this provides only an estimate of the volume of secretion, it emphasizes the large changes in volume that occur with small changes in the diameter of a hillock (volume of a hemisphere =  $\pi d^3/12$ ).

To examine the secretory response of the submucosal glands to nervous or pharmacological stimuli, we measure the increase of number of hillocks per square centimeter during baseline conditions and after a stimulus. This method allows us to measure the pattern of the secretory response of individual glands to different stimuli (Fig. 3). Our studies with the method show that an  $\alpha$ -adrenergic agonist, phenylephrine, causes secretion from canine tracheal mucus glands. The effect is smaller than the stimulatory effect of electrical stimulation of cholinergic efferent fibers in the superior laryngeal nerve (17). The difference in the response to the two stimuli may be due to the fact that phenylephrine has a constrictive effect on the local blood supply to the trachea (Fig. 4).

Another technique for studying submucosal gland secretion, which has been developed in our laboratory (18), allows the secretion from a single submucosal gland to be collected into a micropipet. Cat

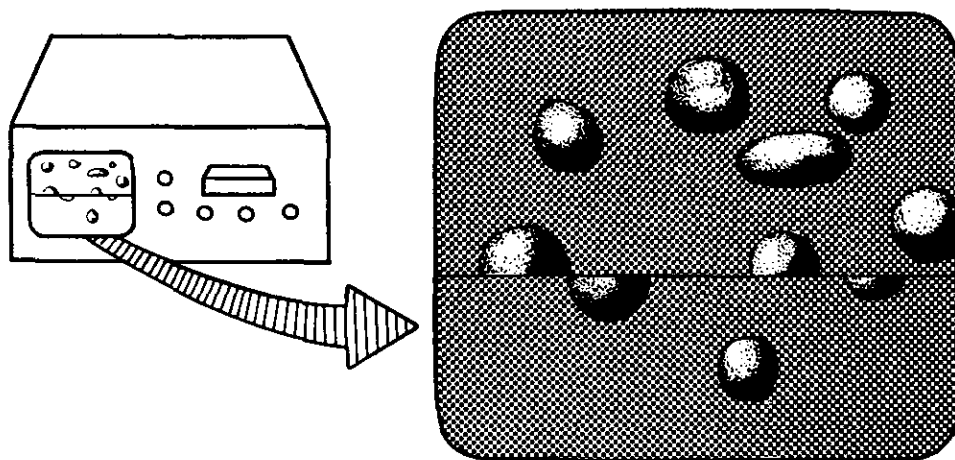


FIGURE 2. Drawing of the screen of a video-image measuring monitor showing a picture of hillocks projected from a tape recorder. The image of one hillock has been split along its diameter by offsetting electrically the upper part of the picture from the lower part. The distance moved by the offset image was measured electrically.

tracheal glands are studied because the secretions from these glands are watery and can be collected easily into a capillary tube. In anesthetized cats ventilated via the lower trachea, the tracheal mucosa is exposed and covered with HEPES oil equilibrated with water. To visualize the gland duct openings we stain them *in vivo* with neutral red dye (0.1% solution) applied to the luminal surface of the epithelium and observe them through a dissecting microscope. With experience we are able to recognize the gland duct openings by their elliptical shape and therefore we no longer use neutral red. To collect the secretion we use oil-filled constant-bore pipets bent at their ends to an angle of 30°-45°. The tips of the pipets are fire-polished to prevent damage to the epithelial surface. The tip of the pipet is placed over a gland duct opening, so that the opening is surrounded and can only discharge its secretion into the capillary lumen. We start the flow by applying slight negative pressure with a syringe to the end of the micropipet. Secretion is collected for 1 min, and then oil is aspirated into the capillary lumen to isolate the sample; two or three samples are collected into one micropipet and the volume of each sample is calculated by measuring the length of the fluid column using a vernier micrometer and a stereomicroscope. The volumes of sequential 1-min samples collected from the same gland duct vary very little over periods up to 4 hr. We used this method to show that the rate of secretion from the glands was increased by electrical stimulation of the vagus nerves and by intravenous injection of phenylephrine (15, 18).

## In Vitro Techniques

The effect of physiological and pharmacological agents on secretion of mucus cannot at present be adequately studied using only tissues *in vivo*. Therefore, we developed an *in vitro* preparation of cat trachea for the study of mucus secretion. We mount pieces of the anterior part of the trachea (exposed area, 1.3 cm<sup>2</sup>) as flat sheets between the two halves of a modified Ussing chamber (Fig. 5). The pieces of tissue, composed of pseudostratified epithelium, submucosal glands, connective tissue, and cartilage, are approximately 1 mm thick. We placed a thin Parafilm ring between the epithelial surface of the tissue and one-half of the Ussing chamber to help form a seal between the tissue and the chamber, and to help minimize edge damage.

One-half of the Ussing chamber has six sharpened pins equally spaced around the medial edge which we push through a section of the trachea, pinning it to a block of paraffin wax. The pins hold the tracheal section flat across the chamber. After cutting the section free from the remaining trachea, we insert

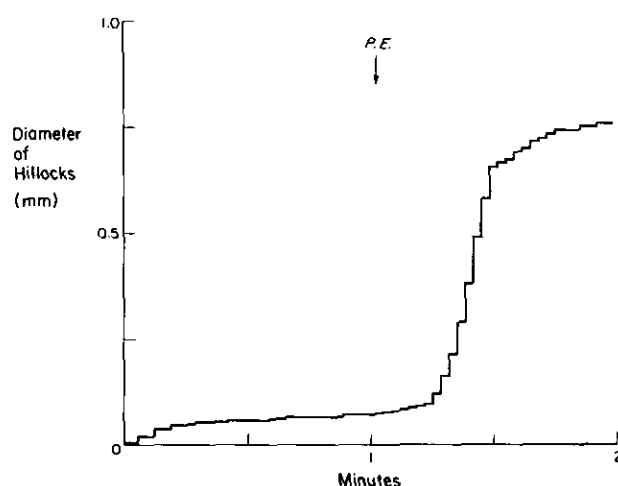


FIGURE 3. Graph of measurements of diameter of hillocks made each 2.5 sec during a control period of 60 sec and during a 60 sec period after injection of 40  $\mu$ g of phenylephrine (PE), into a cranial thyroid artery of a dog.

the pins into corresponding holes in the other half of the chamber to close it. We connect each half of the Ussing chamber to a glass perfusion chamber (MRA, Clearwater, Florida) and perfuse both sides of the chamber with 10 ml of oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) Krebs-Henseleit solution, warmed to 37° C to maintain viability (Fig. 5).

We measure electrical potential difference across the epithelium via two agar-KCl (1 M) bridges positioned in the electrolyte solution on either side of the trachea 2 mm from the surface and connected through calomel half cells to a high impedance millivoltmeter. We measure the short-circuit current (expressed as  $\mu$ A/cm<sup>2</sup> of tissue) via two agar-NaCl (0.15 M) bridges which carry direct current to the electrolyte solution from an automatic voltage clamp connected to the agar-salt bridges by silver/silver chloride junctions. To calculate the resistance of the tissues, we divide the potential difference by the short-circuit current. The relationship between the clamped voltage and the current needed to clamp it is linear over the range +40 mV to -60 mV. Potential difference and short-circuit current, measured every 15 min during the experiment, are used to monitor the viability of the tissue.

To study the effects of a drug on sulfated mucin secretion and Cl<sup>-</sup> and Na<sup>+</sup> fluxes, we measure sulfated mucin secretion, bidirectional fluxes of Cl<sup>-</sup> and bidirectional fluxes of Na<sup>+</sup> in separate pieces of tissue before and after the addition of the test drug.

<sup>35</sup>S-Sulfate is taken up by the mucus-secreting cells of the cat trachea, and is released into the airway lumen bound to glycoproteins (19). For this reason, we use the secretion of bound <sup>35</sup>S into the luminal side of the chamber as a measure of

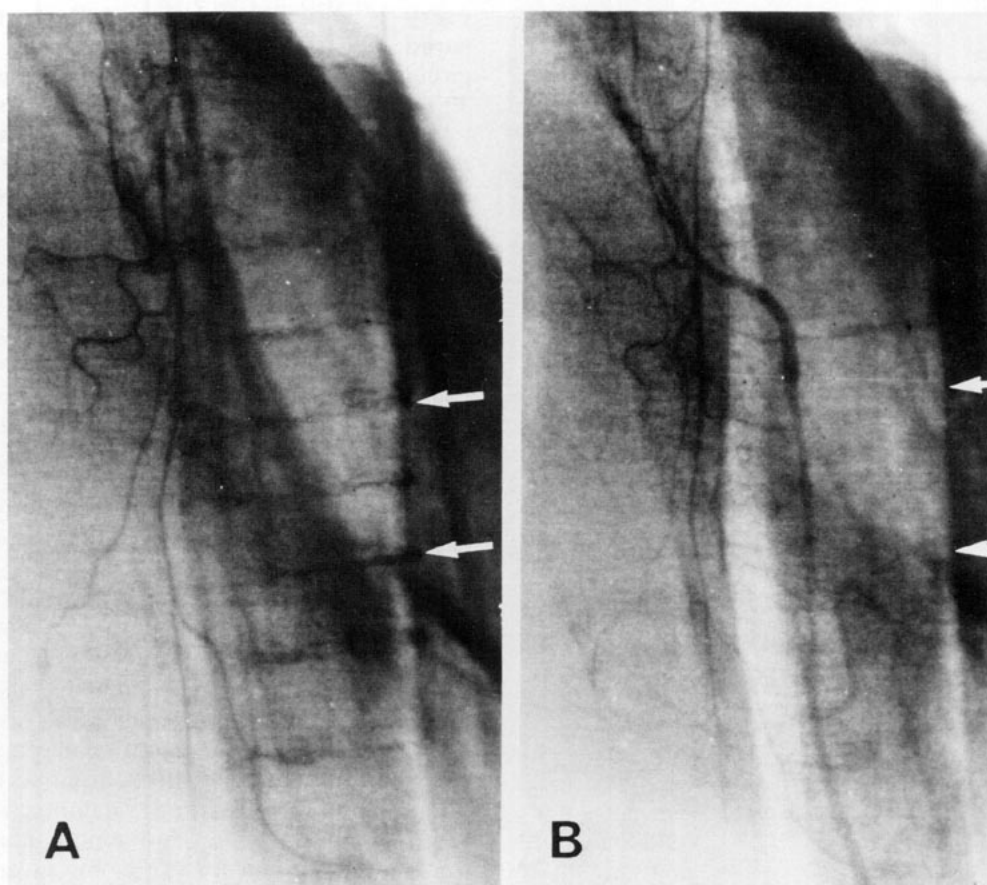


FIGURE 4. Angiograms of canine tracheal vessels produced by injecting dye through a cannula in the right cranial thyroid artery (A) 1 min after injection of 2 ml of saline and (B) 1 min after injection of phenylephrine 40  $\mu$ g in 2 ml of saline. Phenylephrine caused constriction of the tracheal vessels (arrows).

sulfated-glycoprotein secretion. We add 1.0 mCi sodium  $^{35}\text{S}$ -sulfate to the submucosal side of the tissue; every 30 min we collect the solution bathing the luminal side of the tissue and replace it with fresh Krebs-Henseleit solution. Samples are collected for a control period of 3-4 hr. The test drug is added to the submucosal side of the tissue for one 30-min sampling period and subsequently washed out. Samples are collected for 1-2 hr after the drug is washed out. The samples obtained are placed in dialysis tubing bags (average pore radius permeability 24 Å, VWR Scientific) and dialyzed against distilled water to remove unbound  $^{35}\text{S}$ -sulfate. Up to 30 of these samples are placed in 4 liters of distilled water; this water is exchanged six times during a 48 hr period. To each of the first four volumes of dialysis water we add nonradioactive sodium sulfate (0.5 g) to displace noncovalently bound  $^{35}\text{S}$ -sulfate, and we add sodium azide (0.5 g) to prevent fungal and bacterial growth. The last two dialyses are against 20mM phosphate

buffer, which helps to disperse the mucins. On the completion of dialysis, a 0.6 ml aliquot of each sample is taken, mixed with 4.0 ml of scintillation fluid (PCS; Amersham) and the bound  $^{35}\text{S}$  is counted in a  $\beta$ -scintillation counter (Liquid Scintillation System MK III, Searle Analytic). The output is expressed as counts per minute per square centimeter of tissue per hour (cpm/cm<sup>2</sup>-hr).

To measure the fluxes of  $\text{Cl}^-$  and  $\text{Na}^+$  across the tissue, we add Krebs-Henseleit solution which contains either 5-10  $\mu\text{Ci}$   $^{36}\text{Cl}$  or 2-5  $\mu\text{Ci}$   $^{22}\text{Na}$  to one side of the chamber, and measure the rate of appearance of radioactivity on the other side. We obtain samples by collecting the solution from the appropriate side of the chamber and replacing it with fresh Krebs-Henseleit solution every 30 min. We collect samples for a control period of 2 hr, add the test drug to the submucosal side of the tissue for one 30 min sampling period, and subsequently remove the drug by washing. Samples are collected for 1 hr after the drug is

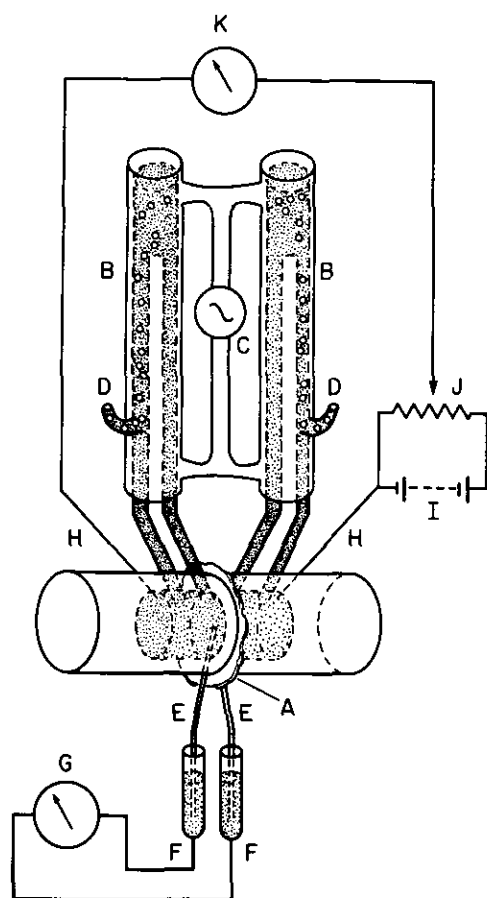


FIGURE 5. Ussing chamber, glass perfusion chamber and electrical circuits. A piece ( $1.2 \text{ cm}^2$ ) of trachea (A) bathed on each surface by Krebs-Henseleit solution warmed to  $37^\circ\text{C}$  in a heating jacket (B) which is connected to a water pump and heater (C). The solution is circulated and oxygenated by bubbling it with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  (D). Electrical potential difference is measured with KCl-agar bridges (E) connected via calomel half cells (F) to an electrometer (G). The trachea is short-circuited by passing current to the electrolyte solution via saline-agar bridges (H) from an external source (I) regulated by an automatic voltage clamp (J) to keep the spontaneous electrical potential difference at zero. The short-circuit current is measured by a milliammeter (K).

removed. A 0.6-ml aliquot of each of these samples is taken, mixed with 4.0 ml scintillation fluid, and the radioactivity counted in a  $\beta$ -scintillation counter. From the specific activities of the  $^{36}\text{Cl}$  and  $^{22}\text{Na}$  source solutions we are able to calculate the ion fluxes in microequivalents per square centimeter of tissue per hour ( $\mu\text{Eq}/\text{cm}^2\text{-hr}$ ). When we have measured the flux of an ion in one direction, we wash out both sides of the chamber and measure the flux of the same ion in the opposite direction, before and after the test drug, in the same piece of tissue.

All fluxes are measured under open-circuit conditions, except for a few seconds (less than 5 sec)

every 15 min when short-circuit current is measured. Electrical potential difference and short-circuit current are measured every 5 min during the 30 min period that the test drug is in the chamber.

This new method for studying respiratory tract secretion has the following advantages over other *in vitro* methods: we can measure mucus output and ion transport in similar pieces of trachea from the cat, under well controlled conditions for periods up to 10 hr. We can assess the viability of the tissue by electrical measurements during the experiments and by electron microscopy after the experiments. Using this method we found that an  $\alpha$ -adrenergic agonist (phenylephrine) and a  $\beta$ -adrenergic agonist (terbutaline) both stimulated the secretion of sulfated mucins and net ion movement into the lumen (10, 20). The effects of  $\alpha$ -adrenergic and  $\beta$ -adrenergic stimuli were different. Phenylephrine caused large increases in the output of sulfated mucin, and in the net fluxes of  $\text{Cl}^-$  and  $\text{Na}^+$  toward the lumen; terbutaline caused a moderate increase in the output of sulfated mucin, and a small increase in net  $\text{Cl}^-$  flux toward the lumen; it did not affect either unidirectional flux of  $\text{Na}^+$ . From these findings we predict that phenylephrine will cause more ion-mediated water secretion than terbutaline. Thus, phenylephrine may act to produce more water in the respiratory tract secretions.

Originally we used trachea mounted in an Ussing chamber to study ion movement across canine tracheal epithelium (21) (Fig. 6). In epithelia, ion movement induces transepithelial movement of water by creating local osmotic gradients in the lateral intercellular spaces (22, 23). Therefore, we reasoned that active ion transport was likely to regulate the rate of water secretion into the airway. Changes in ion mediated secretion of water may alter the consistency of respiratory tract fluid and change the depth of the periciliary fluid layer. The periciliary fluid layer is important for the regulation of mucociliary clearance. With too much periciliary fluid, the propulsive effect of the cilia will not reach the mucus gel layer; with too little periciliary fluid, the ciliary motion will be blocked by the weight and resistance of the gel layer. Thus, the regulation of water secretion into the periciliary fluid could be an important determinant of mucociliary clearance rates.

To study ion movement we used Ussing's short-circuit current method and the posterior membranous part of the trachea of dogs. We found net movement of  $\text{Cl}^-$  toward the lumen ( $2.7 \pm 0.6 \mu\text{Eq}/\text{cm}^2\text{-hr}$ ) and net movement of  $\text{Na}^+$  toward the submucosa ( $0.8 \pm 0.2 \mu\text{Eq}/\text{cm}^2\text{-hr}$ ). These net ion fluxes measured under short-circuit conditions were associated with a spontaneous transepithelial electrical potential ( $30.7 \pm 2.7 \text{ mV}$ ) and a short-circuit current ( $108 \pm 8$



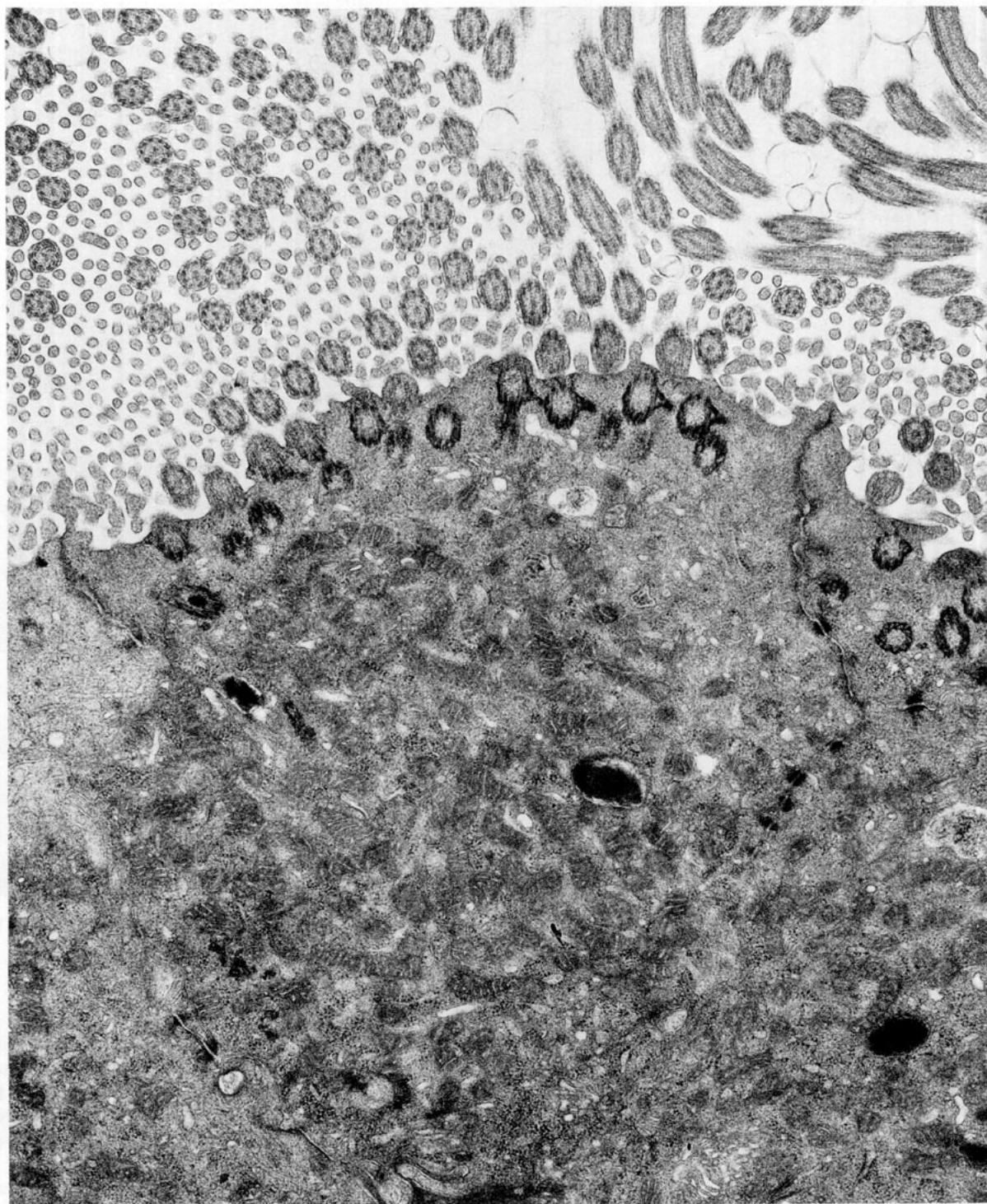


FIGURE 6. Electron micrograph of a ciliated cell prepared from canine tracheal epithelium which had been mounted in an Ussing chamber for 8 hr. The ultrastructure of the cell and junctions appear normal.

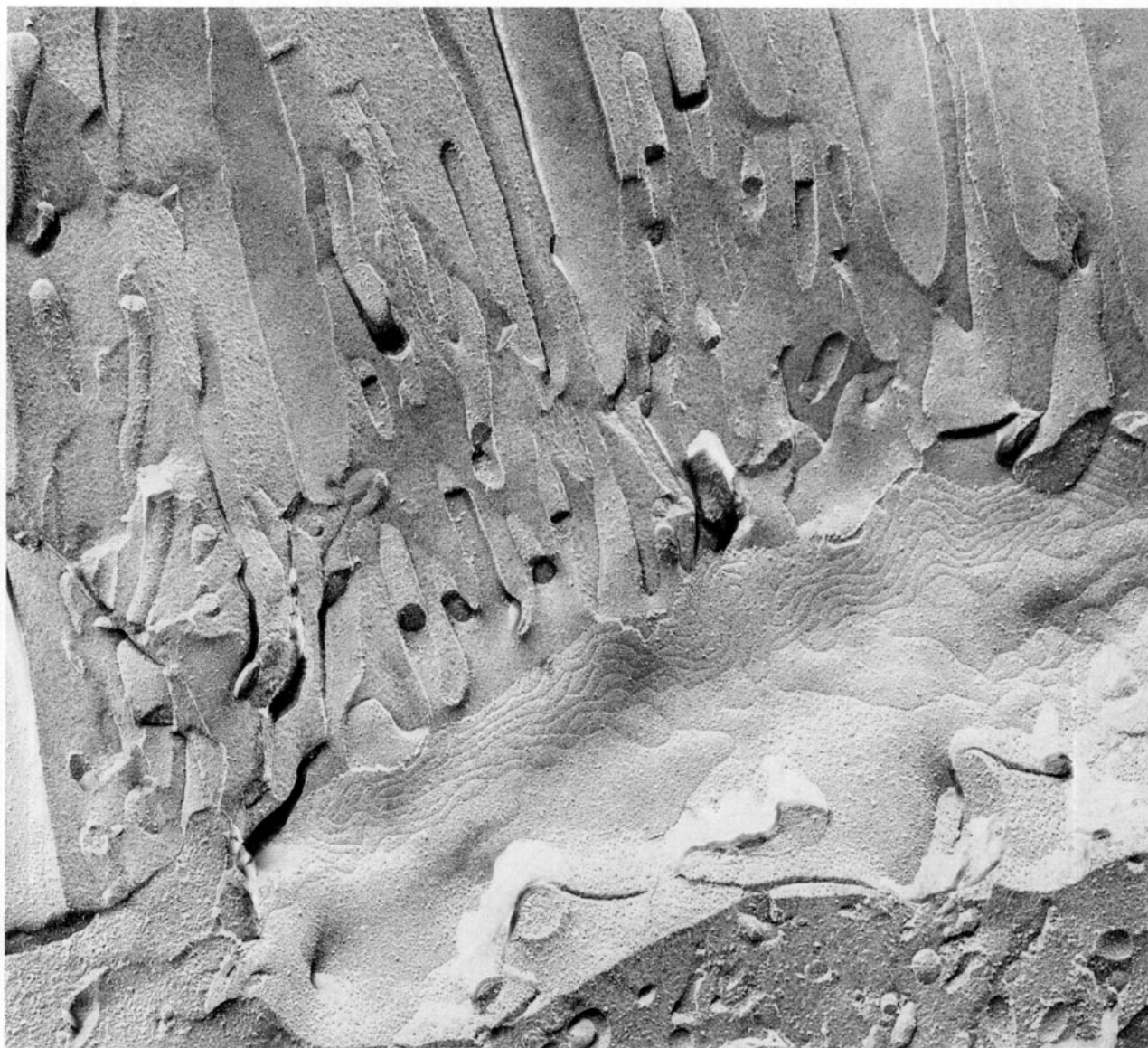


FIGURE 7. Freeze fracture through the tight junction of a ciliated cell in cat trachea which was removed from an Ussing chamber after 6 hr. The junction is formed by parallel strands with a few interconnections.

$\mu\text{A}/\text{cm}^2$ ) (21). Thus,  $\text{Cl}^-$  and  $\text{Na}^+$  must be actively transported across canine tracheal epithelium. By forming local osmotic gradients ions may regulate movement of water into the tracheobronchial sections (14, 24).

Mediators and drugs which mimic the actions of the autonomic nervous system affect ion movement across the trachea. Histamine increased net movement of  $\text{Cl}^-$  and  $\text{Na}^+$  toward the tracheal lumen; the response was prevented by an  $\text{H}_1$ -antagonist but not by an  $\text{H}_2$ -antagonist (25). Acetylcholine increased net movement of  $\text{Cl}^-$  and  $\text{Na}^+$  toward the lumen; the

response was prevented by small concentrations of atropine (26). Terbutaline, a specific  $\beta_2$ -adrenergic agonist, increased net movement toward the lumen of  $\text{Cl}^-$ , but not of  $\text{Na}^+$ ; the effect was prevented by propranolol (27, 28). We speculate that ion-mediated water secretion into the airway can be altered by mediators and neurotransmitters.

Active transport in tracheal epithelium was affected by drugs which inhibit ion transport in other epithelia. Furosemide reduced net  $\text{Cl}^-$  movement toward the lumen when added to the submucosal side, but not when added to the luminal side of the



trachea (29). Ouabain was bound to more sites on the submucosal membranes than the luminal membranes of the tracheal surface epithelial cells (30); the drug inhibits  $\text{Na}^+$  transport when added to the submucosal side of the trachea but has little effect when added to the luminal surface of the epithelium. The transepithelial movement of  $\text{Cl}^-$  depends on co-existing active  $\text{Na}^+$  transport, since net  $\text{Cl}^-$  movement is greatly reduced by replacement of  $\text{Na}^+$  in the bathing solution (31-33) and by adding ouabain, an inhibitor of sodium pumps, to the submucosal bathing solution (31, 33). Further studies will be necessary to determine the cells responsible for the active ion transport and the exact systems involved.

We use the *in vitro* preparation of cat trachea in an Ussing chamber to study epithelial permeability and electrical properties and the pattern of tight junction strands in the same piece of epithelium (11). In each tissue we measure electrical potential difference, short-circuit current and electrical resistance for periods up to 6 hr. Simultaneously, we measure the flux of  $^{14}\text{C}$ -sucrose (an extracellular tracer) from submucosa to lumen during 30-min periods. At a predetermined time during the experiment, we remove the tissue, fix it, and prepare it for freeze fracture. Small pieces of the trachea are fractured and replicated in a freeze-etch device (Balzers, Liechtenstein) with a double replica stage. We use electron micrographs of freeze-fractured tracheal tight junctions (Fig. 7) to quantitate the changes in tight junctions associated with changes in electrical resistance and permeability of the epithelium. We used this method to study the effect of removing  $\text{Ca}^{2+}$  from the bathing solutions on the properties of the epithelium. The  $\text{Ca}^{2+}$  free solution caused a decrease in electrical resistance, an increase in permeability to  $^{14}\text{C}$ -sucrose and produced areas of disarray and reorientation of the tight junction strands (11).

## Summary

The control systems for the production and removal of respiratory tract secretions may be altered in disease. For instance, patients with cystic fibrosis do not adequately clear the sticky secretions which form in their airways, patients with severe asthma plug their small airways with altered mucus, and patients with chronic bronchitis produce excessive amounts of secretion that must be removed from the airway. Direct methods for studying the regulation of the system in man are not available because the mucus glands are inaccessible and the secretions obtained at the mouth are contaminated. Previous methods for studying the respiratory tract secretions in animals did not identify the sources of the secretions. We have developed new techniques using

animal tracheas *in vitro* and *in vivo* which allow us to study the submucosal glands. We have shown that the glands have a secretory response to cholinergic agonists and stimulation of the vagus nerve and to  $\alpha$ -adrenergic and  $\beta$ -adrenergic agonists.

We have shown that tracheal epithelium actively transports  $\text{Cl}^-$  and  $\text{Na}^+$  and that the net movement of these ions towards the lumen is increased by autonomic agonists. Net movement of these ions may be an important control of ion-mediated water flux into the lumen. Furthermore, changes in the permeability of the tissue may modify ion-mediated water movement. Tight junctions appeared to be altered reversibly by changes in their external milieu. We speculate that epithelial permeability may also be modified by the autonomic nervous system. In the future, we expect that a better understanding of the controls of airway epithelial functions will lead to advances in the treatment and prevention of airway disease.

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