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# ISOLATION OF TRANSPORTING PLASMA MEMBRANE VESICLES FROM BOVINE TRACHEAL EPITHELIUM *

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**A method is described for isolating plasma membrane vesicles from bovine tracheal epithelium. The procedure yields highly purified apical membranes which are enriched 19-fold in the marker enzyme, alkaline phosphatase. Contamination of this fraction by other organelles is minimal. Basolateral membranes isolated from the same preparation have a 4-fold enrichment of ($Na^+ + K^+$)-ATPase and a 2-fold reduction in alkaline phosphatase specific activity compared to the starting material. Assays of $Na^+$ uptake by the apical membrane vesicles demonstrate their suitability for transport studies. Transport of $Na^+$ into an intravesicular space was demonstrated by (1) a linear inverse correlation between $Na^+$ uptake and medium osmolarity; (2) complete release of accumulated $Na^+$ by treatment with detergent; and (3) a marked temperature-dependence of $Na^+$ uptake rate. Other features of $Na^+$ transport were (1) inhibition by amiloride; (2) insensitivity to furosemide; and (3) anion-dependence of uptake rate with the following selectivity: $SCN^- > Cl^- >$ gluconate$^-$.**

## Introduction

Tracheobronchial secretions of mucus, fluid and electrolyte are produced by the combined activities of the submucosal glands and the surface epithelium [1]. These secretions are a critical component of the mucociliary apparatus which serves to clear the lungs of inhaled foreign particles and maintain the patency of the smaller airways. The tracheal epithelium has been identified as a $Cl^-$-secreting tissue displaying a serosa-positive potential difference and a short-circuit current which are largely determined by the rate of active $Cl^-$ secretion from serosa to mucosa [2,3]. This finding has stimulated investigations of tracheal epithelial ion transport properties aimed at defining the humoral, pharmacological and pathological factors that govern respiratory tract secretion. Results from a variety of studies indicate that the basic characteristics of tracheal secretion are common to those of other $Cl^-$-secreting epithelia [4]. These characteristics have been summarized by Frizzell et al. [5] in the form of a cellular model for epithelial $Cl^-$ secretion. According to the proposed model, a NaCl transport mechanism drives movement of $Cl^-$ across the basolateral membrane whereas $Cl^-$ exit across the apical membrane occurs by diffusion down its electrochemical gradient. The rate of

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid.

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secretion is determined primarily by apical membrane $Cl^-$ permeability, which may be altered by a variety of secretagogues acting through the intracellular second messengers cAMP and/or $Ca^{2+}$. While this model is consistent with available data from tracheal epithelial studies, it remains largely untested. Recent electrophysiological studies [6–8] have demonstrated that stimulation of active $Cl^-$ secretion is associated with an increase in the $Cl^-$ conductance of the apical membrane. However, kinetic evidence for coupled NaCl uptake across the basolateral membrane is lacking, and the intracellular regulatory roles of the second messengers cAMP and $Ca^{2+}$ have yet to be defined.

In addition to $Cl^-$ secretion, a smaller rate of active $Na^+$ absorption occurs across tracheal epithelium which is independent of $Cl^-$ [2] and is inhibited by mucosal amiloride [4]. The existence of two active transport processes in opposing directions may allow for fine control of the overall rate of fluid and electrolyte secretion across the respiratory epithelium.

Previous studies of tracheal epithelial ion transport properties have chiefly employed transepithelial flux measurements and conventional microelectrode techniques. The trachea, with its sheet-like arrangement of cells, relatively simple cellular organization and superficial location of the bulk of the secretory cells, is a particularly suitable tissue for studies of this kind. However, the existence of the paracellular shunt pathway makes it impossible to simply determine apical or basolateral membrane ion permeabilities with microelectrodes. In addition, the thickness of the connective tissue 'dead-space' (500–1000 $\mu$m) underlying the tracheal mucosa makes it impractical to study ion fluxes across the basolateral plasma membrane. Isolation of tracheal epithelial cells [9] circumvents these problems but results in loss of the normal cell polarity. An alternative approach is to physically isolate apical and basolateral plasma membranes and to use the purified membrane vesicles to characterize the transport properties of the respective cell surfaces. This technique is particularly advantageous in the study of epithelial cells which have a clear dichotomy of function with respect to apical vs. basolateral membranes. There is ample precedent for the successful study of transport reactions in epithelia using plasma membrane vesicle preparations. The advantages and limitations of this experimental approach have recently been thoroughly reviewed by Murer and Kinne [10].

The aim of the present study was to develop a technique for isolating purified plasma membrane vesicles from tracheal epithelium, and thus to open new avenues for the investigation of ion transport mechanisms in this tissue. A method is described for obtaining highly purified and efficiently transporting apical membrane vesicles from bovine tracheal epithelium. Preliminary flux studies with these vesicles demonstrate amiloride-sensitive $Na^+$ transport in the tracheal apical membrane; uptake of $Na^+$ is unaffected by furosemide and shows an anion-dependence consistent with an electrogenic transport mechanism. The membrane isolation procedure also yields a fraction enriched in basolateral membranes, which, with further purification, will considerably extend the transport studies possible from a single preparation. By providing the means to assay ion transport properties independently at each surface of the tracheal epithelial cell, this preparation will permit a critical evaluation of the proposed cellular model for epithelial $Cl^-$ secretion and definition of the mechanism and intracellular regulation of tracheal fluid and electrolyte secretion.

## Materials and Methods

### *Dissection and harvesting of tracheal mucosa*

Cattle tracheae were obtained from a local slaughterhouse. The trachea was removed 10–15 min after each animal had been killed and immediately placed on ice for transport to the laboratory. Tissue from five animals provided sufficient material for a conveniently scaled preparation. Each trachea was divided into two or three segments. The segments were opened anteriorly and washed in ice-cold oxygenated Ringer's solution containing 1 mM dithiothreitol to remove debris and mucus from the lumen. The cartilage was removed by rapid dissection. The resulting strips of mucosa and underlying connective tissue (1–2 inch wide) were incubated for 15 min at 0°C in a 250 mM sucrose, 5 mM Hepes-Tris solution (pH 7.8) containing 2 mM EGTA and 1 mM dithiothreitol and bubbled vigorously with air. Tracheal mucosa was harvested by firmly scraping

the luminal surface with glass microscope slides. The epithelial scrapings (5 g) were collected in 60 ml of an ice-cold 50 mM mannitol, 5 mM Hepes-Tris solution (pH 7.4) containing 0.25 mM $MgCl_2$ and 1 mM dithiothreitol (homogenization medium). All subsequent procedures were performed at 0–4°C.

*Homogenization and membrane isolation*

Mucosal scrapings were homogenized with 12 strokes in a Thomas (Philadelphia, PA) glass homogenizer with a motor-driven teflon pestle (Size C). The homogenate was centrifuged at $300 \times g$ for 8 min and the supernatant ($S_0$) was saved. The pellet ($P_0$) contained numerous intact cells as revealed under light microscopy by trypan blue exclusion. To maximize the yield of the preparation, $P_0$ was resuspended in 20 ml homogenization medium, re-homogenized with 6 strokes in the glass and teflon pestle aparatus and mixed with the reserved $S_0$. The combined $S_0$ and $P_0$ mixture was centrifuged at $2000 \times g$ for 8 min to remove nuclei and large debris ($P_1$). Centrifugation of the supernatant ($S_1$) at $9500 \times g$ for 10 min sedimented a dense muddy brown pellet ($P_2$) with a loose lighter halo. The light portion was sloughed from the pellet by gentle swirling and collected with the supernatant ($S_2$). Centrifugation of $S_2$ at $35000 \times g$ for 40 min yielded a mixed plasma membrane fraction ($F_1$) enriched in markers for both apical and basolateral membranes.

Resolution of $F_1$ into purified apical and basolateral membrane fractions was achieved by the following steps. $F_1$ was resuspended in 20 ml isolation medium (100 mM mannitol, 5 mM Hepes-Tris, pH 7.4) and $MgCl_2$ was added as a 1 mM solution to a final concentration of 10 mM. The suspension was incubated on ice with occasional stirring for 1 h, during which time an increase in turbidity was observed. Subsequent low speed centrifugation ($1500 \times g$ for 12 min) sedimented a fraction enriched in basolateral membranes ($F_3$), leaving a supernatant which contained apical membranes ($F_2$). The supernatant was diluted with isolation medium containing 1 mM EDTA to remove excess $Mg^{2+}$, and centrifuged at $100000 \times g$ for 15 min. The resulting pellet ($F_4$), containing the apical membranes, was resuspended in appropriate buffer at a protein concentration of 5–7 mg/ml and was not purified further. The basolateral membrane fraction was similarly extracted by resuspension of the pellet ($F_3$) in isolation medium containing 1 mM EDTA and centrifugation at $100000 \times g$ for 15 min. Apical membrane contamination in the resulting fraction ($F_5$) was significantly reduced by a further purification step. The membranes were incubated in isolation medium containing 1 M NaBr and 1 mM EDTA for 15 min at 0°C. Basolateral membranes were then collected by centrifugation at $100000 \times g$ for 15 min. The pellet ($F_6$) was resuspended in appropriate buffer at a protein concentration of 5–7 mg/ml.

Both the apical and basolateral membrane preparations, if not used immediately, were stored at −80°C for subsequent biochemical or transport studies.

*Assay procedures*

The membrane isolation procedure was performed analytically by following standard markers for nuclei, mitochondria, endoplasmic reticulum and plasma membranes. $(Na^+ + K^+)$-ATPase was used as a specific marker for basolateral membrane [11]. $(Na^+ + K^+)$-ATPase activity ($\mu$mol inorganic phosphate produced per hour per mg protein) was measured at 37°C by a regenerating assay using the medium of Kagawa [12] modified by the inclusion of 120 mM NaCl (or 120 mM choline chloride in $Na^+$-free medium) and 20 mM KCl. Protein samples were subjected to snap freeze-thaw using liquid $N_2$ immediately prior to assaying. Inorganic phosphate produced by ATP hydrolysis was determined by the method of Taussky and Shorr [13] with the modifiation of Radominska-Pyrek et al. [14]. $(Na^+ + K^+)$-stimulated ATPase activity was estimated as that fraction of total ATPase activity inhibited by 1.0 mM ouabain and by the absence of $Na^+$. Alkaline phosphatase was chosen as the apical membrane marker. This is the marker most commonly used to identify luminal membranes isolated from other epithelial tissues, and therefore allowed for a comparative assessment of the purity of the tracheal apical membrane preparation. Alkaline phosphatase activity ($\mu$mol inorganic phosphate produced per hour per mg protein) was measured at 20°C in 2.5 ml of a medium which contained: 5 mM $MgCl_2$; 100 mM Tris-HCl buffer (pH 9.0);

0.1% Triton X-100, and 1 mM *p*-nitrophenyl phosphate. The reaction was initiated by addition of protein and monitored by recording the change in absorbance at 405 nm. Using a value of 22.2 $l/cm^2$ per mmol for the extinction coefficient of *p*-nitrophenyl phosphate at 405 nm, enzyme activity was then calculated from the rate of change of absorbance.

DNA served as a marker for nuclei and was measured according to the method of Burton [15]. Succinate dehydrogenase activity, a marker for mitochondria, and NADH dehydrogenase activity, an endoplasmic reticulum marker, were assayed as described by Hochstadt et al. [16]. Protein was estimated according to Lowry et al. [17] using bovine serum albumin as standard.

*Transport measurements*

Radiolabelled $Na^+$ influx was assayed using Dowex 50W-X8 (50–100 mesh, $Tris^+$ form) columns according to the procedure of Gasko et al. [18]. Disposable Pasteur pipettes (5.75 inch) were packed with 1.5 ml cation-exchange resin supported by a dacron wool plug. The columns were washed with 100 mM mannitol, 2 mM Tris-sulfate (pH 7.4). Apical membrane vesicles were equilibrated with 100 mM mannitol, 2 mM Tris-sulfate (pH 7.4) by homogenizing the membranes in this solution and centrifuging the the suspension at 100 000 × *g* for 15 min. The protein concentration of the final vesicle suspension was adjusted to 1.75 mg/ml. The incubation medium contained 2 mM Tris-sulfate (pH 7.4), 50 M KSCN and 1 mM $^{22}NaSCN$. In parallel experiments, $SCN^-$ was replaced by $Cl^-$ or $gluconate^-$. Reactions were initiated by addition of 22 μl vesicles to 198 μl incubation medium at 20°C. Any addition to the reaction mixture or to the vesicle suspension was made prior to the start of the reaction. At predetermined times, 200 μl of the reaction mixture was placed directly on to a column of Dowex and immediately washed into the column with 0.25 ml of the ice-cold buffered 100 mM mannitol solution, then eluted with 1.75 ml of the same solution. Transit time through the column was less than 15 s. The eluent was dissolved in 8 ml scintillation fluid and counted for radioisotope content. The specific activity of $^{22}Na$ was determined from a standard sample of the total reaction mixture and fluxes were calculated in terms of nmoles of $Na^+$ transported per mg protein. Zero-time points were obtained in matched incubation media at 0°C. All experiments were done in duplicate.

Uptake of [$^{14}$C]urea was assayed by a modification of a method originally described by Penefsky [19] for separating free and protein-bound ligand. Disposable 1 ml tuberculin syringes were plugged with dacron wool and filled to the 0.9 ml mark with Sephadex G-50 fine, previously swollen in distilled water. The column was equilibrated with 2 mM Tris-sulfate/100 mM mannitol (pH 7.4), then placed in a glass 13 × 100 mm test tube and centrifuged at 300 × *g* for 2 min, which caused significant shrinkage of the column. The transport reaction was initiated by addition of 20 μl vesicles (3–4 mg/ml) to 80 μl incubation medium containing 5 mM [$^{14}$C]urea (7 mCi/mmol). The incubation medium was 2 mM Tris-sulfate/50 mM KZ (pH 7.4), where Z = $SCN^-$, $Cl^-$ or $gluconate^-$, as specified in the text. After a predetermined interval, 90 μl total reaction mixture was placed on to the column via an automatic pipeptte tip resting on top of the syringe; the tip retained the sample until a second centrifugation at 300 × *g* for 2 min was performed, using a clean test tube to collect the effluent. Recovery of protein under these conditions was 100%. The effluent was diluted with 1 ml $H_2O$, transferred to a scintillation vial and dissolved in 8 ml scintillation fluid. The specific activity of [$^{14}$C]urea was calculated from a standard sample of radiolabelled urea in 1 ml $H_2O$. The assay allowed calculation of intravesicular space volume in units of μl/mg protein. All assays were done in duplicate,

*Materials*

$^{22}Na$ (carrier-free) and [$^{14}$C]urea were obtained from New England Nuclear (Boston, MA). Furosemide was obtained from Hoechst Pharmaceuticals Inc. (Cincinnati, OH) and Amiloride-HCl from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ). Hepes, Tris, and Dowex were obtained from Sigma Chemical Co. (St. Louis, MO), and dithiothreitol from Boehringer-Mannheim Biochemicals (Indianapolis, IA). All other chemicals were of the highest quality available.

## Results

*Isolation of apical and basolateral membranes*

The recovery of activity of alkaline phosphatase (apical membrane marker) and $(Na^+ + K^+)$-ATPase (basolateral membrane marker) during the isolation procedure is shown in table I. Early centrifugation steps at $2000 \times g$ and $9500 \times g$ removed > 95% of the total DNA, > 75% of the succinate dehydrogenase actvity and > 65% of the NADH dehydrogenase activity (see below, and Table II). After centrifugation at $35000 \times g$, a crude plasma membrane fraction ($F_1$) was obtained, which comprised approx. 13% of the total protein of the tracheal mucosal homogenate. This mixed membrane fraction retained 63% of the $(Na^+ + K^+)$-ATPase activity and 39% of the alkaline phosphatase activity. Incubation with $MgCl_2$ (10 mM) followed by low-speed centrifugation separated $F_1$ into two membrane populations. Membranes remaining in the supernatant were diluted with isolation buffer containing 1 mM EDTA and collected by high speed centrifugation. This fraction ($F_4$) retained 15% of the total alkaline phosphatase activity and less than 4% of the total $(Na^+ + K^+)$-ATPase. The membrane fraction sedimented after $Mg^{2+}$ treatment ($F_5$) contained 34% of the total $(Na^+ + K^+)$-ATPase and 17% of the alkaline phosphatase activity. The $(Na^+ + K^+)$-ATPase in this fraction could be further resolved from alkaline phosphatase by incubation with 1 M NaBr and 1 mM EDTA. Virtually all of the $(Na^+ + K^+)$-ATPase activity was retained after this step (34–32% recovery), but more than 75% of the alkaline phosphatase was removed.

Table I also indicates the protein yields of the isolation procedure. 0.9% of the total crude homogenate protein was isolated in the purified apical membrane fraction ($4.8 \pm 0.8$ mg). 9.4% was isolated in the $Mg^{2+}$-precipitated fraction enriched in basolateral membranes ($F_5$); after the final NaBr purification step ($F_6$), this was reduced to 8.4% ($44.8 \pm 4.5$ mg).

The purity of the membrane preparations was assessed biochemically from the specific activities of the markers used to analyse the isolation procedure. The activities of the two plasma membrane marker enzymes are shown Table I. The apical membranes ($F_4$) showed a 19-fold purification of alkaline phosphatase activity compared to the homogenate and a 40-fold enrichment compared to the NaBr-treated basolateral membranes ($F_6$). $(Na^+ + K^+)$-ATPase specific activity was enriched 4-fold in the basolateral membrane fractions ($F_5$, $F_6$). Ouabain-inhibitable $(Na^+ + K^+)$-ATPase comprised 30–40% of the total ATPase activity in the basolateral membranes.

TABLE I

RECOVERY AND SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE AND $(Na^+ + K^+)$-ATPase IN STARTING MATERIAL AND IN PURIFIED MEMBRANES

Enzyme activity is expressed in μmol inorganic phosphate produced per hour per mg protein. Results are means ± S.E. for four preparations. Tracheae from five animals were used for each preparation. Since the yield of mucosal scrapings varied from preparation to preparation, the protein and total enzyme activities have been normalized to the mean homogenate values. $F_1$ = mixed plasma membrane fraction; $F_4$ = apical membrane fraction; $F_5$ = basolateral membrane fraction; $F_6$ = basolateral membrane fraction following treatment with 1 M NaBr; $F_6$ (Sup) = supernatant remaining after centrifugation of NaBr-treated basolateral membrane fraction. Yields presented as percentages of the respective homogenate values.

| Fraction | Protein (mg) | Alkaline phosphatase | | | $(Na^+ + K^+)$-ATPase | | |
|---|---|---|---|---|---|---|---|
| | | Total | Yield | Spec. act. | Total | Yield | Spec. act. |
| Crude homogenate | 523.1 ± 48.3 | 1266.7 ± 123.0 | 100 | 2.6 ± 0.12 | 1476.8 ± 98.8 | 100 | 2.96 ± 0.39 |
| $F_1$ | 68.8 ± 3.1 | 498.0 ± 43.7 | 39 | 7.71 ± 0.40 | 936.7 ± 138.5 | 63 | 9.70 ± 1.30 |
| $F_4$ | 4.8 ± 0.8 | 193.2 ± 16.8 | 15 | 49.04 ± 5.57 | 56.6 ± 10.0 | 4 | 11.77 ± 2.23 |
| $F_5$ | 49.3 ± 9.1 | 211.1 ± 45.1 | 17 | 4.57 ± 0.28 | 499.5 ± 142.3 | 34 | 10.75 ± 0.23 |
| $F_6$ | 40.0 ± 4.5 | 50.8 ± 10.5 | 4 | 1.23 ± 0.27 | 432.3 ± 54.2 | 30 | 12.17 ± 1.92 |
| $F_6$ (Sup) | 10.8 ± 2.3 | 56.9 ± 10.9 | 4 | 6.41 ± 1.52 | 42.2 ± 8.7 | 3 | 4.21 ± 0.71 |

TABLE II

RECOVERY OF BIOCHEMICAL MARKERS DURING A REPRESENTATIVE MEMBRANE ISOLATION PROCEDURE

Tracheae from six animals were used for the preparation. The total recovery and percentage yield of protein and all subcellular markers are given for fractions recovered at each stage of the isolation procedure. Alkaline phosphatase and $(Na^+ + K^+)$-ATpase activities are expressed in μmol inorganic phosphate produced per hour; succinate dehydrogenase and NADH dehydrogenase activities are expressed in μmol substrate oxidized per hour. Yields presented as percentages of the respective homogenate (H) values.

| Fraction | Protein | | DNA | | Alkaline phosphatase | | $(Na^+ + K^+)$-ATPase | | Succinate dehydrogenase | | NADH dehydrogenase | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg | Yield | mg | Yield | Total | Yield | Total | Yield | Total | Yield | Total | Yield |
| H | 780.2 | 100 | 32.7 | 100 | 3016.4 | 100 | 2117.7 | 100 | 4360.2 | 100 | 49.2 | 100 |
| $S_1$ | 607.4 | 78 | 1.2 | 4 | 2282.1 | 76 | 1731.8 | 82 | 2973.9 | 68 | 38.5 | 78 |
| $P_1$ | 183.2 | 23 | 30.9 | 95 | 798.4 | 26 | 338.9 | 16 | 1593.9 | 37 | 13.8 | 28 |
| $S_2$ | 517.6 | 66 | – | – | 1996.1 | 66 | 1549.5 | 73 | 489.1 | 11 | 17.4 | 35 |
| $P_2$ | 77.2 | 10 | 1.2 | 4 | 384.0 | 12 | 245.4 | 12 | 2534.2 | 58 | 21.2 | 43 |
| $S_3$ | 411.2 | 53 | – | – | 510.0 | 17 | 310.8 | 15 | 275.8 | 6 | 7.5 | 15 |
| $F_1$ | 83.1 | 11 | – | – | 1453.1 | 48 | 950.4 | 45 | 304.1 | 7 | 11.2 | 23 |
| $F_2$ | 13.5 | 2 | – | – | 712.3 | 24 | 146.0 | 7 | 4.7 | 0.1 | 0.17 | 0.3 |
| $F_3$ | 68.9 | 9 | – | – | 763.2 | 25 | 849.5 | 40 | 302.8 | 7 | 11.0 | 22 |
| $F_4$ | 5.4 | 1 | – | – | 507.2 | 17 | 114.8 | 5.5 | 3.6 | < 0.1 | 0.08 | 0.2 |
| $F_4$ (Sup) | 5.4 | 1 | – | – | 44.1 | 2 | 21.4 | 1 | – | – | 0.06 | 0.1 |
| $F_5$ | 59.1 | 8 | – | – | 559.7 | 19 | 824.9 | 39 | 279.5 | 6.4 | 11.1 | 22 |
| $F_5$ (Sup | 4.0 | 0.5 | – | – | 30.6 | 1 | 37.1 | 2 | 8.3 | 0.2 | 0.12 | 0.2 |
| $F_6$ | 45.5 | 6 | – | – | 183.9 | 6 | 727.3 | 34 | 234.8 | 5.4 | 8.8 | 18 |
| $F_6$ (Sup) | 13.6 | 2 | – | – | 138.5 | 5 | 73.5 | 3.5 | 13.0 | 0.3 | 1.7 | 3.5 |

Following treatment of the basolateral membrane fraction ($F_5$) with 1 M NaBr, the ($Na^+ + K^+$)-ATPase : alkaline phosphatase ratio was increased from 2.4 : 1 to 10 : 1 (Table I). 95% of the ($Na^+ + K^+$)-ATPase activity was recovered following this procedure, but only 51% of the alkaline phosphatase activity. While this suggests that NaBr treatment is resulting in some inactivation of alkaline phosphatase activity, it is also producing further resolution of the membrane populations: more than 90% of the recovered ($Na^+ + K^+$)-ATPase activity is retained in the membrane pellet ($F_6$) after this step, whereas 52% of the recovered alkaline phosphatase activity is eliminated in the supernatant ($F_6$ Sup).

The recoveries of biochemical markers used to follow the tissue fractionation and membrane isolation procedure are given in Table II. The final membrane preparations had no nuclear contamination. The apical membrane fraction ($F_4$) was also essentially free of mitochondria and endoplasmic reticulum. However, 5–6% of the total succinate dehydrogenase activity and approx. 20% of the total NADH dehydrogenase activity was retained by the basolateral membrane fractions. This mitochondrial and endoplasmic reticulum contamination might account at least in part for the relatively low enrichment of ($Na^+ + K^+$)-ATPase in these fractions. Centrifugation of the basolateral membrane fraction ($F_5$) in Ficoll or sucrose density gradients failed to improve the purification (data not shown). While NaBr treatment reduced the alkaline phosphatase contamination of $F_5$, the enrichment of the basolateral membrane marker enzyme was not significantly enhanced.

In some preparations, the isolation procedure was modified by homogenizing the mucosal scrapings in a Waring blender (2 min at top speed) and omitting the first $300 \times g$ centrifugation step. This method was suitable only for the collection of apical membranes, since it resulted in nuclear disruption and heavy contamination of $F_5$ with DNA. The apical membrane fraction, however, remained free from nuclear and mitochondrial contamination. Purification of alkaline phosphatase varied from 19- to 35-fold following this method of homogenization and in some cases the ratio of alkaline phosphatase:($Na^+ + K^+$)-ATPase was also improved. Further experiments are required to determine whether or not there is a significant increase in the purity of the apical membranes obtained by this method. In the $Na^+$ transport experiments described below, apical membranes from both preparative procedures were used, and exhibited similar characteristics and rates of transport.

*$Na^+$ transport studies with apical membrane vesicles*

Tracheal apical membrane vesicles exhibited time-dependent uptake of $Na^+$ as demonstrated in Fig. 1. In order to distinguish net $Na^+$ uptake into the intravesicular space from non-specific binding to membrane proteins, the osmolarity of the incubation medium was varied from 100 to 1100 mosM. As shown in Fig. 2, $Na^+$ uptake was inversely proportional to medium osmolarity ($r = 0.99$), indicating transport into an osmotically active space. Extrapolation to infinite osmolarity showed a binding component of only 0.74 nmol/mg protein, a small fraction of the total $Na^+$ uptake. Additional evidence for accumulation of $Na^+$ within an intravesicular space is provided by the effect of the nonionic detergent Tween 20 (0.1%), also shown in Fig. 2. Tween caused complete release of accumulated $Na^+$. Indeed the value of vesicle $Na^+$ obtained after treatment with Tween (0.41 nmol/mg protein) is lower than the calculated nonspecific binding of $Na^+$ (0.74 nmol/mg protein), suggesting that the latter may be an overestimate. Finally, as further independent evidence for transport, lowering the assay temperature from 20°C to 0°C reduced the initial rate of $Na^+$ uptake from 1.44 nmol/min per mg protein to 0.52 nmol/min per mg protein.

The data presented in Fig. 1 also show the inhibition of $Na^+$ uptake by amiloride, a specific inhibitor of $Na^+$ transport in several epithelial [20]. Maximal inhibition of the initial rate of uptake was > 90% by 1 mM amiloride. At 0.25 mM, amiloride reduced the initial rate of $Na^+$ uptake by only 25% (data not shown). At 60 min, $Na^+$ uptake is at, or near, equilibrium both in presence and absence of amiloride. The value for $Na^+$ uptake at equilibrium is considerably lower in presence of amiloride. In separate experiments, the equilibrium uptake of urea was determined in presence and absence of 1 mM amiloride, using a

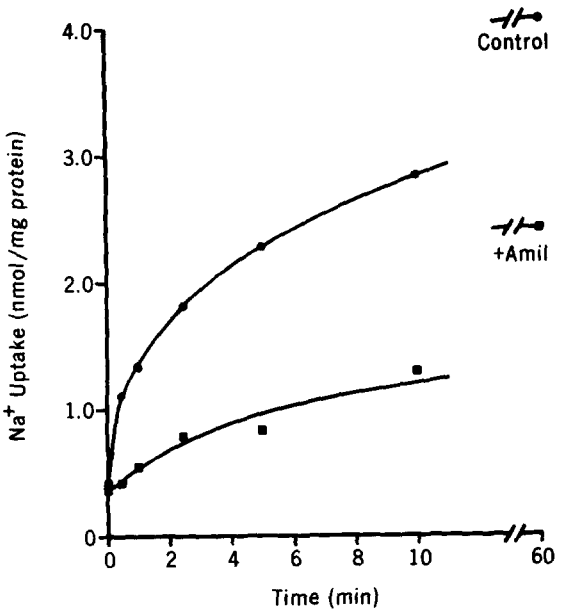


Fig. 1. Uptake of $Na^+$ by tracheal apical membrane vesicles. Vesicles were equilibrated with 2 MM Tris-sulfate, 100 mM mannitol (pH 7.4). The incubation medium was 1 mM $^{22}NaSCN$ in 2 mM Tris-sulfate, 50 mM KSCN (pH 7.4). Each point is the mean of duplicate assays using vesicles from $n$ preparations. ●, control vesicles ($n = 4$); ■, vesicles pre-incubated with 1 mM amiloride for 20 min and assayed in presence of 1 mM amiloride ($n = 2$).

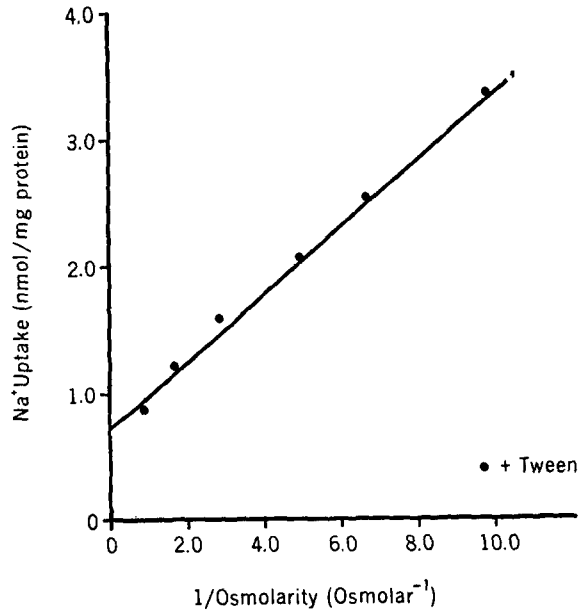


Fig. 2. Effect of medium osmolarity on $Na^+$ uptake. Vesicles were equilibrated with 2mM Tris-sulfate, 100 mM mannitol (pH 7.4). the incubation medium was 1 mM $^{22}NaSCN$ in 2 mM Tris-sulfate, 50 mM KSCN (pH 7.4) with mannitol added to increase the osmolarity. The incubation period was 60 min. The line, drawn by linear regression analysis, has a correlation coefficient $r = 0.99$. Also shown is the effect of addition of Tween 20 (0.1%).

KSCN incubation medium matching that used for $Na^+$ assays. Osmotically-active uptake of urea was confirmed by a marked reduction in uptake at equilibrium measured in presence of 1 M sucrose (data not shown). Intravesicular space volumes calculated for control and amiloride-treated vesicles were $2.14 \pm 0.13$ and $2.11 \pm 0.19$ $\mu$l/mg protein ($n = 6$), respectively, indicating that amiloride did not cause a change in vesicle volume to which the disparity in $Na^+$ equilibrium influxes might be attributed. In addition, the lack of an effect of amiloride on urea uptake attests to the specificity of its effect on $Na^+$ transport.

In the amiloride experiments described above, vesicles were preincubated with the diuretic for 20 min at the same concentration and temperature employed in the assay. Full expression of the inhibitory effect of amiloride required this preincubation. Uptake of $Na^+$ at 1.0 min (zero-time subtracted) in the presence of 1 mM amiloride was $0.24 \pm 0.04$ nmol/mg protein if the vesicles were preincubated with the drug, a decrease of 73% (control $0.88 \pm 0.07$ nmol/mg protein). The uptake without preincubation was $0.50 \pm 0.01$ nmol/mg; a decrease of only 43%. In contrast to the effect of amiloride, 0.5 mM furosemide did not alter $Na^+$ uptake ($0.88 \pm 0.05$ nmol/mg protein at 1.0 min), despite a similar preincubation with this drug.

An anion-dependence of $Na^+$ uptake by the tracheal apical membrane vesicles is demonstrated in Fig. 3. The rate of $Na^+$ transport exhibited the following anion selectivity: $SCN^- > Cl^- >$ gluconate$^-$. At 60 min, $Na^+$ uptake is approaching a maximum in presence of all three anions, but the equilibrium values are not the same. One possible explanation for this is that the vesicles may be undergoing volume changes due to the different anionic composition of the incubation medium. To test this hypothesis, the uptake of urea at equilibrium (60 min) was measured in matched SCN, Cl and gluconate media. The intravesicular space

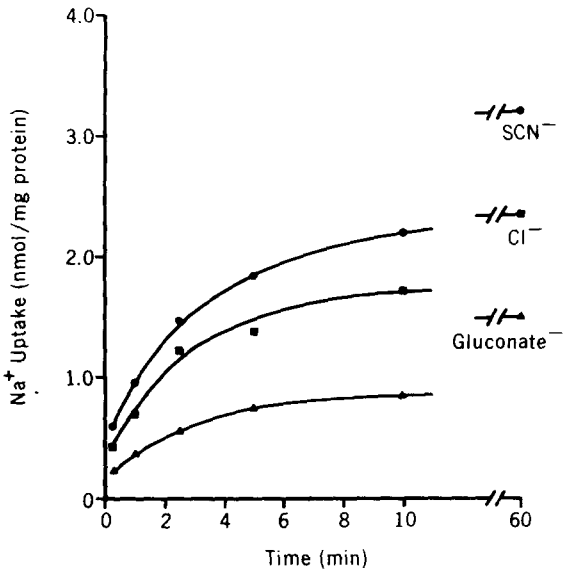


Fig. 3. Anion-dependence of $Na^+$ uptake by tracheal apical membrane vesicles. Vesicles were equilibrated with 2mM Tris-sulfate, 100 mM mannitol (pH 7.4). Incubation media contained 1 mM $^{22}NaX$ in 2 mM Tris-sulfate, 50 mM KX (pH 7.4) where $X = SCN^-$ (●), $Cl^-$ (■) or $gluconate^-$ (▲). Each point is the mean of duplicate assays using vesicles from two experiments.

volumes calculated from these equilibrium measurements were 2.14 ± 0.13 (SCN), 2.14 ± 0.24 (Cl) and 2.11 ± 0.19 (gluconate) μl/mg protein, $n = 6$. Thus, volume changes cannot account for the difference in $Na^+$ equilibrium uptake values. An alternative explanation may lie in an effect of the different anions on the Donnan equilibrium across the membrane. This aspect of the transport awaits further analysis.

## Discussion

*Comments on the isolation procedure*

This paper presents the first report of plasma membrane vesicle isolation from tracheal epithelium. Bovine trachea was selected as a starting material because of the availability of an abundant and inexpensive supply of tissue from healthy animals. Canine tracheal epithelium has been investigated more extensively than that of other species. In a recent study by Durand et al. [21], bovine tracheal mucosa obtained from slaughterhouse material was found to possess electrical properties qualitatively similar to those of dog tracheal epithelium. These eletrical properties remained stable for 12-16 hrs attesting to the viability of the tissue.

The purity of the membranes isolated by the present procedure and the efficiency of the separation process may be assessed by comparison to established procedures for isolating membranes from renal and intestinal epithelia. The specific activity of alkaline phosphatase (49.0 μmol/h per mg protein) in the purified tracheal apical membranes lies within the range of those reported for intestinal and renal brush borders (8.3–165.0 μmol/h per mg protein) from a variety of species [22–26]. Enhancement of alkaline phosphatase specific activity (19-fold) exceeds the 10–15-fold commonly reported for renal and intestinal luminal membranes [23,26–30]. The purity of the preparation can also be evaluated by considering the amount of cross-contamination between apical and basolateral marker enzymes. Purified apical membranes contained 11 times the specific activity of alkaline phosphatase compared to the first basolateral membrane fraction ($F_5$) and 40 times the acivity in the NaBr-treated basolateral membranes ($F_6$). This separation of alkaline phosphatase meets or exceeds other preparations in qualtity [22,24]. The activity of $(Na^+ + K^+)$-ATPase in the apical membranes is four times that of the homogenate. This is comparable to the 2–5-fold enrichment of cross-contaminating basolateral marker enzyme reported for renal brush borders [22,26,28]; this level of contamination was considered acceptable in the renal preparations despite a considerably lower enrichment of alkaline phosphatase (10–15-fold) than is obtained in the tracheal preparation. Contamination of the tracheal apical membranes by nuclei and mitochondria was at negligible levels. Thus, by a variety of criteria, the present preparation yields highly purified apical membranes which compare favorably to luminal preparations from other epithelia.

The specific activity of $(Na^+ + K^+)$-ATPase in the tracheal basolateral membranes (10.8–12.2 μmol/h per mg protein) and the 4-fold enrichment of the marker enzyme are at present lower than those of renal and intestinal basolateral membrane preparations [24,28,31]. While NaBr treatment lowered the specific activity of alkaline phos-

phatase in these membranes, the enrichment of $(Na^+ + K^+)$-ATPase was not significantly improved. Contamination with mitochondria and endoplasmic reticulum may be at least in part responsible for the relatively low marker enrichment, since 5–6% of the total succinate dehydrogenase activity and approx. 20% of the NADH dehydrogenase activity were retained in the basolateral membrane fraction. Transport assays in the present study were confined to the highly purified apical membrane vesicles pending further efforts, currently underway, to improve the purification of the basolateral membranes. These attempts are encouraged by the fact that a large proportion (34%) of the total $(Na^+ + K^+)$-ATPase is retained by this fraction. In addition, the measured ouabain-inhibitable $(Na^+ + K^+$-ATPase activity represents 30–40% of the total ATPase activity; a similar proportion has been reported for purfied renal basolateral membranes [32,33].

*Transport studies with tracheal apical membranes*

The suitability of the tracheal apical membrane vesicles for transport studies is evident from the assays of $Na^+$ uptake. Transport of $Na^+$ into an intravesicular space is unequivocally demonstrated by the following standard criteria: (1) there was a linear inverse correlation between $Na^+$ uptake and medium osmolarity; (2) $Na^+$ uptake exhibited marked temperature-dependence; and (3) accumulated $Na^+$ was completely released when vesicles were disrupted with the detergent Tween 20 (0.1%). The calculated binding component for $Na^+$ (0.74 nmol/mg protein) represented only a small fraction of the total $Na^+$ uptake. Amiloride inhibited the uptake of $Na^+$. Amiloride-sensitive $Na^+$ absorption has been demonstrated in intact tracheal mucosa [4], suggesting that membrane transport functions remain intact through the isolation procedure. The lack of effect of furosemide on $Na^+$ uptake by the apical membrane vesicles is also in accord with the results of intact tissue studies [34]. The rate of $Na^+$ uptake varied with the anion in solution, with the order $SCN^- > Cl^- >$ gluconate$^-$. This finding is consistent with an electrogenic $Na^+$ transport mechanism. An apical membrane $Na^+$ conductance has recently been proposed on the basis of electrical measurements in intact tracheal mucosa [35]. Further studies with the vesicles will be aimed at directly assessing the relationship between $Na^+$ transport and membrane potential.

In summary, the membrane isolation procedure described in this paper yields highly purified, efficiently transporting apical membrane vesicles from bovine tracheal epithelium. The same preparative procedure yields a fraction enriched in basolateral membranes. These purified membrane vesicle preparations provide a new and simplified approach by which to characterize the mechanisms and intracellular regulation of tracheal epithelial secretion.

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