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# X-RAY MICROANALYSIS OF HAMSTER TRACHEAL EPITHELIUM

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## Abstract

Studies of ion transport across respiratory epithelia are of great interest if we are to understand the pathophysiology of diseases such as cystic fibrosis in which ion transport is abnormal. Concentrations of elements were determined in various subcellular regions of normal or isoproterenol-treated hamster tracheal epithelium, using X-ray microanalysis of freeze-dried cryosections. Samples of trachea were taken from animals under anesthesia and either frozen in situ or dissected and plunge frozen. Concentrations of Mg, P, S, Cl, K and Ca were higher in cytoplasm and nuclei of control epithelial cells in dissected samples than in cryoneedle samples. Following treatment with isoproterenol, a large decrease in the concentration of Cl was observed. The results confirm that cyclic AMP-regulated chloride secretion is unaffected by anesthesia.

Key Words: X-ray microanalysis, ion transport, epithelium, trachea, isoproterenol, cryobioptic needle, dissection.

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## Introduction

Transport of ions and water across airway epithelia is of vital importance to the normal function of the airway, particularly in the provision of the correct environment for effective clearance of inhaled particles and mucus by cilia (Welsh 1986). Many studies have shown that the normal tracheal epithelium secretes $Cl^-$ and absorbs $Na^+$ (Al-Bazzaz and Al-Awqati 1979, Welsh 1986). However, in the disease cystic fibrosis (CF), a great deal of evidence suggests that there is a malfunction of $Cl^-$ transport in respiratory epithelia, leading to obstruction of airways with viscous secretions (Knowles et al. 1986). The molecular defect in CF is not known. Further studies of the basic mechanism of normal ion transport across tracheal epithelium are therefore important to form the basis for improved management of the disease. The technique of X-ray microanalysis has great potential as a tool for the study of CF, which has been demonstrated clearly in studies in animal models of the disease (Roomans 1989). We have therefore used the technique of X-ray microanalysis to study elemental distribution in hamster trachea, following two slightly different methods of sample preparation.

## Materials and Methods

Samples of the mid region of the trachea were obtained from male golden hamsters (110-130 g body weight) under sodium pentobarbital anesthesia. Samples were taken either using a cryobioptic technique, which simultaneously excises and freezes the tissue (von Zglinicki et al. 1986), or by rapid dissection of a 1-2 mm length of trachea followed by plunge-freezing in liquid propane. Tissue was taken from untreated animals and from hamsters injected 5 min previously with isoproterenol (20 mg/kg body weight, intraperitoneally, prior to administration of the anesthetic).

Dissected samples were fractured under liquid nitrogen in such a way that sections could be taken from areas at least 20 micrometers away from the dissected edge and the pieces mounted in a flat-jaw chuck or onto stubs using heptane (Steinbrecht and Zierold 1984). Cryobioptic needle samples were mounted in the flat-jaw chuck and sectioned directly at the edge. Cryosections (200 nm nominal thickness) were cut on an LKB SuperNova/CryoNova ultramicrotome, using dry glass knives at a

temperature of -130°C and transferred onto hinged Cu grids (75 mesh) previously coated with Formvar and carbon. Grids were transferred into the electron microscope (a Philips 400T fitted with a field emission gun and a LINK QX200/AN10000 analyzer system) with a Gatan 626 cryotransfer device. Sections were then freeze-dried in the electron microscope column by raising the specimen temperature initially to -80°C and then to -40°C over a period of approximately 1 h.

Spectra (200-400s livetime) were obtained in the TEM mode at -171°C. Quantitation was performed using the LINK Systems QUANTEM/FLS program, which is based upon the continuum method of Hall (Hall and Gupta 1982), with reference to gelatin standards (Roomans and Seveus 1977), using a continuum range of 4.2-4.6 keV. Spectra were corrected for extraneous background from the holder, grid, and film (Roomans 1988). Since small, but significant peaks for S and Cl were detected in film spectra, these were also subtracted from specimen spectra before quantitation.

## Results

Cells in freeze-dried cryosections of trachea taken from "dissected" samples were structurally well-preserved, allowing identification of subcellular components of the epithelial and submucosal regions (Figs. 1 and 2). "Cryoneedle" samples were difficult to obtain due to the presence of the cartilage rings, through which the needle must pass before reaching the epithelial layer. Although the epithelial layer appeared less well preserved than dissected/plunge frozen samples, areas for analysis were still recognizable, especially in submucosal regions (Fig. 3). No morphological differences were seen between control and isoproterenol-stimulated samples.

Comparisons of the concentrations of Na, P, S, Cl, K and Ca in trachea epithelial cells, goblet cell mucus and submucosal gland mucus, from control samples obtained by the two cryofixation techniques, are shown in Table 1. Concentrations of Mg, P, S, Cl, K and Ca were, with only one exception ([S] in basal cytoplasm), higher in the cytoplasm and the nucleus of dissected samples, and in most cases significantly higher. In contrast, concentrations of Na in dissected samples were lower than in cryoneedle samples. Concentrations of elements were generally higher in apical and basal cytoplasm than in core cytoplasm and no concentration gradients from apical to basal cytoplasm were thus observed (at least not on a dry weight basis). Data from goblet cell mucus and submucosal gland mucus were pooled from both dissected and cryoneedle samples, since no significant differences were seen between samples prepared by either technique. Goblet cell mucus showed higher [Ca], but lower [S] than submucosal gland mucus granules.

Concentrations of elements in samples of trachea from isoproterenol-stimulated animals are shown in Table 2. Spectra from samples obtained with the cryobioptic needle were obtained only from apical and core cytoplasm. In contrast to the differences seen between concentrations of elements in dissected and cryoneedle samples from control animals, the only significant differences between samples obtained by the two preparation techniques after isoproterenol stimulation were in [Na] in apical cytoplasm and [S] in core cytoplasm.

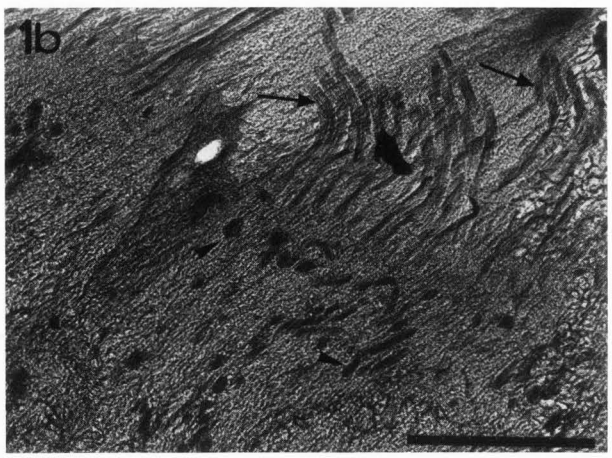
When compared with controls, [Na] was decreased in the cytoplasm of tracheal epithelium from isoproterenol-stimulated hamsters and significantly so in cryoneedle samples. [Cl] was significantly decreased in both cytoplasm and nucleus of dissected samples and in apical cytoplasm of cryoneedle samples. Concentrations of the other elements were decreased (except [S] which increased), in apical and basal cytoplasm of dissected, isoproterenol-treated samples. [Na] and [Cl] were also decreased in submucosal gland mucus and [S] increased in goblet cell mucus, following isoproterenol treatment.

## Discussion

Although data derived from isoproterenol-stimulated animals are limited, the results obtained agree well with previous studies. Addition of isoproterenol to cultured normal tracheal epithelial cells leads to an increase in intracellular cyclic AMP (cAMP) levels and increased short-circuit current across cell sheets, consistent with increased $Cl^-$ secretion (Widdicombe 1986). X-ray microanalysis of cultured normal human respiratory epithelial cells also showed that a decrease in intracellular chloride concentration occurred following stimulation with isoproterenol (Roomans et al. 1988).

It has been shown previously that preparation of tissue samples using the cryobioptic needle technique gives an unbiased estimation of intracellular ion distribution (von Zglinicki et al. 1986). Dissection required to yield a piece of tissue small enough for optimum plunge freezing may alter intracellular ion concentrations from what they were in vivo. It therefore appears most logical to assume that the difference in ionic concentrations between cryoneedle and dissected specimens reflects damage to the tissue prepared by the latter method. Tissue damage would cause influx of $Na^+$ and $Cl^-$ ions and water. Cells normally show an increase in [Na] and in the [Na]/[K] ratio following cell damage. However, [Na]/[K] ratios were lower in control dissected samples than in cryoneedle samples. It should be remembered though, that there are a number of different ion transport mechanisms operative, even in the resting state, that influence the intracellular Na and K concentrations: the $Na^+/K^+/Cl^-$cotransporter and the $Na^+/K^+$ pump in the basolateral membrane and an amiloride-sensitive $Na^+$ uptake mechanism in the apical membrane. Changes in the activities of these transport mechanisms could result in a decrease of [Na], counteracting the increase due to cell damage. On the other hand, comparison of elemental concentrations in dissected and cryoneedle samples shows that there are few significant differences after beta-adrenergic stimulation. Following stimulation with isoproterenol, $Cl^-$ ions are rapidly excreted via the apical chloride channel which could counteract the increase in [Cl] due to cell damage.

It is unclear whether higher element concentrations in apical and basal cytoplasm than in core (perinuclear) cytoplasm indicate a higher degree of hydration in the former regions. Measurement of the intracellular water distribution could be determined using X-ray microanalysis of thicker, frozen-hydrated cryosections (Hall and Gupta 1982) or by optical

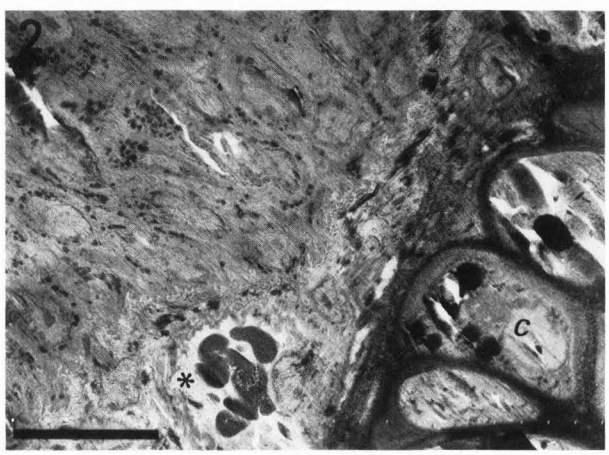





**Fig. 1**: Freeze-dried cryosection of dissected hamster tracheal epithelium. (a) Nuclei (n), basal lamina (asterisk), goblet cell mucus (arrows) and cilia (arrowheads) are clearly visible. Bar = 10 micrometers. (b) Higher magnification of upper left portion of Fig 1(a); cilia (arrows), mitochondria (arrowheads). Bar = 5 micrometers.

**Fig. 2**: Freeze-dried cryosection of dissected hamster trachea, sectioned obliquely through core and basal region of the epithelial layer, a blood vessel (asterisk) and cartilage (c). Bar = 10 micrometers.

**Fig. 3**: Submucosal gland cell from hamster trachea obtained using the cryobioptic needle. Dense mucus granules (asterisk), nucleus (n), connective tissue (c). Bar = 5 micrometers.

transmission of electron micrographs (von Zglinicki et al. 1987). Recent studies showed that gradients of ions and water exist across intestinal epithelium prepared by the cryobioptic needle technique (von Zglinicki and Roomans 1989). No clear element gradients from the apical to the basal part of the cell were seen in this study. Further data is needed for cryoneedle fixed samples from isoproterenol-stimulated animals to assess whether any such gradients exist following stimulation of secretion. However, it should be taken into account, that apart from similarities, the respiratory and intestinal epithelia also show marked structural and functional differences, that could explain the difference in ion distribution between the two types of epithelia.

Goblet cell mucus granules were characteristically high in calcium, whereas submucosal gland mucus was high in S but low in Ca. The concentrations of elements in goblet cell mucus in this study are in good agreement with values obtained for human nasal epithelium goblet cells (Herlong et al. 1988). On the other hand, Roomans et al. (1986) showed that in human bronchial epithelium the goblet cell mucus contained less Ca than the submucosal gland mucus. The increase in [S] in goblet cell mucus after isoproterenol stimulation is in line with changes seen in chronically isoproterenol-treated rat submandibular glands (Muller and Roomans 1984). However, the reason for the decrease in [Na] and [Cl] in submucosal gland mucus is unclear.

It is interesting to note that the anesthesia does not appear to interfere with chloride secretion

Table 1. X-ray microanalysis of control hamster epithelium

| Compartment | | | Concentration (mmol/kg dry weight) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | (n) | Na | Mg | P | S | Cl | K | Ca |
| Apical cytoplasm | Dissected | 31 | 86 ± 8 | 22 ± 2 | 518 ± 28 | 171 ± 10 | 358 ± 15 | 630 ± 34 | 24 ± 3 |
| | Cryoneedle | 18 | 106 ± 5 | 12 ± 2 | 426 ± 16 | 118 ± 9 | 216 ± 5 | 597 ± 14 | 8 ± 1 |
| | p | | | <0.01 | <0.05 | <0.001 | <0.001 | | <0.001 |
| Core cytoplasm | Dissected | 8 | 73 ± 10 | 21 ± 5 | 429 ± 22 | 117 ± 10 | 341 ± 17 | 543 ± 52 | 11 ± 2 |
| | Cryoneedle | 13 | 79 ± 5 | 10 ± 1 | 332 ± 12 | 126 ± 9 | 194 ± 15 | 463 ± 15 | 7 ± 1 |
| | p | | | <0.01 | <0.001 | <0.01 | <0.001 | | <0.05 |
| Basal cytoplasm | Dissected | 17 | 74 ± 10 | 33 ± 3 | 723 ± 43 | 97 ± 6 | 369 ± 22 | 880 ± 37 | 12 ± 4 |
| | Cryoneedle | 22 | 156 ± 19 | 16 ± 5 | 555 ± 48 | 136 ± 16 | 235 ± 14 | 570 ± 47 | 9 ± 3 |
| | p | | <0.01 | <0.05 | <0.05 | <0.05 | <0.001 | <0.01 | |
| Nucleus | Dissected | 13 | 71 ± 10 | 33 ± 7 | 882 ± 60 | 141 ± 12 | 297 ± 11 | 981 ± 42 | 26 ± 5 |
| | Cryoneedle | 5 | 156 ± 15 | 25 ± 7 | 833 ± 62 | 130 ± 15 | 202 ± 14 | 653 ± 62 | 16 ± 6 |
| | p | | <0.01 | | <0.001 | <0.01 | <0.001 | | <0.05 |
| Goblet cell mucus | | 8 | 90 ± 26 | 38 ± 5 | 200 ± 34 | 118 ± 8 | 267 ± 46 | 233 ± 63 | 84 ± 9 |
| Submucosal gland mucus | | 31 | 128 ± 10 | 8 ± 4 | 165 ± 14 | 1279 ± 72 | 274 ± 20 | 237 ± 16 | 15 ± 3 |

Concentrations of elements in various subcellular regions of control hamster tracheal epithelium. Results are expressed as mean ± s.e.m. for analyses from 3 animals in the "dissected" group and 2 animals in the "cryoneedle" group; n = number of analyses, p = probability as determined by Student's t-test; dissected versus cryoneedle samples.

Table 2. X-ray microanalysis of isoproterenol-stimulated hamster trachea

| Compartment | | | Concentration (mmol/kg dry weight) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | (n) | Na | Mg | P | S | Cl | K | Ca |
| Apical cytoplasm | Dissected | 9 | 58 ± 11 | 10 ± 2 | 496 ± 32 | 199 ± 13 | 142 ± 17 | 583 ± 66 | 8 ± 1 |
| | p | | <0.05 | | | <0.001 | <0.01 | | |
| | Cryoneedle | 7 | 24 ± 6* | 12 ± 4 | 477 ± 36 | 222 ± 21 | 152 ± 29 | 574 ± 65 | 9 ± 1 |
| | p | | <0.001 | | | <0.001 | <0.01 | | |
| Core cytoplasm | Dissected | 7 | 47 ± 12 | 9 ± 2 | 444 ± 27 | 162 ± 18 | 144 ± 7 | 458 ± 65 | 9 ± 1 |
| | p | | | | | | <0.01 | | |
| | Cryoneedle | 3 | 30 ± 10 | 15 ± 5 | 492 ± 22 | 240 ± 25* | 178 ± 50 | 643 ± 88 | 10 ± 1 |
| | p | | <0.001 | | <0.001 | <0.001 | | <0.01 | |
| Basal cytoplasm | Dissected | 12 | 56 ± 5 | 7 ± 1 | 579 ± 12 | 135 ± 19 | 112 ± 14 | 524 ± 25 | 7 ± 1 |
| | p | | | <0.01 | <0.05 | <0.05 | <0.001 | <0.001 | |
| Nucleus | Dissected | 8 | 95 ± 13 | 23 ± 7 | 864 ± 33 | 155 ± 15 | 179 ± 29 | 824 ± 30 | 16 ± 3 |
| | p | | | | | | <0.01 | <0.05 | |
| Goblet cell mucus | | 4 | 94 ± 13 | 46 ± 9 | 171 ± 12 | 242 ± 17 | 116 ± 33 | 189 ± 33 | 64 ± 9 |
| | p | | | | | <0.001 | | | |
| Submucosal gland mucus | | 10 | 62 ± 21 | 9 ± 5 | 155 ± 20 | 1419 ± 37 | 130 ± 38 | 255 ± 13 | 19 ± 5 |
| | p | | <0.01 | | | | <0.01 | | |

Concentrations of elements in subcellular regions of tracheal epithelium from isoproterenol-treated hamsters. Mean ± standard error for analyses from 1 animal in each group; n = number of analyses, p = probablity as determined by Student's t-test: control versus isoproterenol-stimulated. * indicates significant difference between concentrations in samples obtained by the two different methods.

in hamster tracheal epithelium to such an extent that the secretory response (decrease in [Cl]) cannot be recognized. If the same is true in humans, it may be practical to carry out similar in situ studies on normal and CF respiratory epithelium in humans.

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## Discussion with Reviewers

B.L. Gupta: There are many internally inconsistent results in the elemental concentrations between the two methods of cryopreparation used. Your explanation of an influx of water after excision and plunge freezing does not seem satisfactory because an influx of water would normally be expected to follow influx of both Na and Cl. Any comments?

G.A.J. Kuijpers: The difference between the dissected and cryoneedle sample is a confusing issue. Altogether, it seems just as, or more likely, that the cryoneedle samples have suffered damage leading to $Na^+$ influx and $K^+$ efflux in those cells. $Cl^-$ may leave these cells if it were actively accumulated in the healthy state. In fact, the paper states that the structure of the cryoneedle samples was less well preserved, especially the epithelial layer, which would agree with this explanation. Please comment.

Authors: Although we agree that some of the results are puzzling, we think that the explanation advanced by us is, at present, the most probable. The higher [Ca] in the dissected samples is an additional argument that cell damage may have occurred. The apical chloride channel is cAMP-dependent and therefore energy-dependent; that it would be activated under anoxic and therefore low-energy conditions seems less likely. We would like to point out, however, that the most important finding is that efflux of chloride upon beta-adrenergic stimulation can be demonstrated even under conditions where slight tissue damage during preparation cannot be avoided.

T. von Zglinicki: I wonder why S concentrations should be so significantly affected by the preparation schedule and by isoproterenol treatment. I would rather expect that to be radiation damage even at low temperature. What beam current density was used?

G.A.J. Kuijpers: Can you explain the changes in the concentration of Mg, P, S and Ca after stimulation?

Authors: It is possible that stimulation changes the intracellular distribution of elements of the Golgi system, endoplasmic reticulum and other cellular macromolecules (containing P and S) to which Mg and Ca may be bound. We could not measure the beam current directly, but given the low count rate (about 700 counts per second) and the low temperature we have difficulty in accepting beam damage as the most likely explanation for our findings.

G.A.J. Kuijpers: What cation accompanies $Cl^-$ upon $Cl^-$ secretion? Or, how is electroneutrality maintained?

R. Rick: In agreement with previous data by the same group the results show a significant drop in the Cl concentration during isoproterenol stimulation. In addition, the study reports significant reductions in the Na concentrations in several cellular regions. How do you explain the Na decrease in the light of the prevalent model of epithelial chloride secretion?

Authors: It is thought that beta-adrenergic stimulation not only activates the chloride channel but also stops $Na^+$ influx across the apical membrane. Since the $Na^+/K^+$ pump at the basolateral membrane is presumably still operative, this could explain the decrease in [Na].

B.L. Gupta: The mucosal surface mucus has been shown to have an important role in the ionic homeostasis of the transporting epithelia (see e.g., Gupta BL 1989 Soc Exptl Biol Symp 43). Did you measure surface mucus and if not why not? After all an important consequence of cystic fibrosis is a change in the viscoelastic properties of mucus probably due to a change in ionic composition. Measurements of surface mucus would also have allowed you to make a better assessment of the changes in the mucous granules.
T. von Zglinicki: Your Fig 1 suggests the possibility of measuring elemental concentrations in the luminal mucus using frozen-dried sections. Is that right?
Authors: Unfortunately too few sections such as the one shown in Fig. 1 were obtained to make quantitative analysis of luminal mucus feasible. We agree that such an analysis would be very interesting and it will be the subject of further studies.

B.L. Gupta: In the mucosal gland mucous granules there is inordinately high sulfur (1279 mM/kg dry weight). This sulphur is most likely to represent sulphate residues in sulphated proteoglycans and hence "fixed" negative charges. What provides electronneutrality since there is a great shortage of cations?
Authors: The intrinsic limitations of X-ray microanalysis do not permit us to be certain about the state of sulfur in the mucus granules. If the sulfur were present in the form of -S-S- bridges, no cations would be needed. In addition, the technique cannot measure organic cations (amines) that could provide electroneutrality.

B.L. Gupta: The use of Cu grids is likely to introduce inconsistencies in the Na estimates due to Cu L X-rays. Have you considered using multislot Ni grids, possible with a heavy electroplating of Ni on your specimen stage for a better management of extraneous correction?

R. Rick: Of course, it is extremely important to consider film and instrumental contributions to X-ray spectra. Nevertheless, none of the correction schemes applied can fully account for extraneous radiations since the conditions applied during recording of the film or instrumental spectrum do not exactly match the conditions during acquisition of the cellular spectrum. In this context, I would like to know how large the corrections are for the grid spectrum, in particular in the region of the Na K-alpha/Cu-L overlap and whether or not correcting for the grid does significantly affect your Na numbers?
Authors: The software used may be assumed to be capable of deconvoluting partially overlapping peaks such as the Na K and Cu L lines. We find Ni grids more difficult to handle than Cu grids because of their magnetic properties, and therefore prefer to use Cu. We are indeed aware that the correction for extraneous radiation applied is not ideal, because the actual physical situation in the electron microscope column is more complicated than assumed in the rather simple theoretical model that underlies the applied correction. However, it is the best we can do at present. We always applied the correction, and did not assess the effect of omitting it.

B.L. Gupta: Is it possible that there might be differences between heptane mounted samples and vice-held samples which could explain some of the inconsistencies in the data?
Authors: No systematic differences were observed between vice-held and heptane mounted samples within one group of samples (cryoneedle or dissected). The heptan only is in contact with the back of the sample, which is not sectioned.

B.L. Gupta: Since you use a gelatin standard, why did you not use this as a mass standard for the local estimation of dry mass and hence water contents as is done by the Munich group with apparent success?
Authors: This would require the gelatin standard sections to have the same thickness as the specimen sections. Since we use thin sections (200 nm, about one-tenth of the thickness that the Munich group uses) constancy of section thickness cannot be guaranteed.