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X-RAY MICROANALYSIS OF MAMMALIAN SALIVARY GLANDS

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Abstract

X-ray microanalysis was used to study the elemental composition of major salivary glands in rat and man. The elemental composition of the acinar cells is influenced by a variety of factors. Stimulation of fluid secretion by cholinergic, α-adrenergic or peptidergic agonists leads to a sustained increase of the Na/K ratio. The elemental composition and ultrastructure of the acinar cells of rat submandibular gland, as well as flow rate and composition of pilocarpinestimulated submandibular saliva are significantly affected by thyroxine, but less by the synthetic glucocorticoid dexamethasone. Hypercalcemia (induced by chronic vitamin D treatment) as well as hypocalcemia (induced by calcitonin) cause accumulation of intracellular mucus in the submandibular acinar cells, with concurrent increase in the cellular calcium concentration, and a significantly decreased flow rate after pilocarpine stimulation.

Several animal models have been proposed for the generalized exocrinopathy cystic fibrosis (CF), which may be caused by a defective regulation of chloride transport in epithelial cells. A potential new model is proposed: the chronically furosemide-treated rat, which shows several abnormalities in salivary gland structure and function that parallel the abnormalities found in the human disease. Another experimental model of potential interest in the study of CF is short-term treatment of experimental animals with the adenylate-cyclase inhibitor alloxan.

KEY WORDS: salivary glands, submandibular gland, parotid gland, oral biology, thyroxin, glucocorticoids, hypercalcemia, alloxan, furosemide, cystic fibrosis.

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Introduction

The salivary glands have several important functions in mammals: they provide lubrication to aid the swallowing of food, they keep the oral cavity moist (which is e.g., necessary for speech) and clean (which can be important for oral health) and they play a role in digestion (amylase in parotid saliva). Saliva has antimicrobial properties. The calcium and phosphate content of the saliva is, at least in man, an important factor in the prevention of enamel etching (Blair-West et al. 1967). Also salivary proteins play a role in the formation of the enamel pellicle. In some species the salivary glands produce hormones, e.g., nerve growth factor and epidermal growth factor, controlling epidermal keratinization and tooth eruption. In fur-bearing animals, such as the rat, that wet their fur with saliva in response to heat stress, the salivary glands function in thermoregulation.

The morphology of the salivary glands is extremely diverse (Young and Van Lennep 1978). The cell types of the secretory endpiece are called serous, mucous, or seromucous according to the nature of the material synthesized and packed in intracellular granules. However, due to different methods of investigation, there is no general agreement on the classification of the acinar cells. The submandibular gland in rat is often classified as (sero-)mucous, the parotid gland as serous, and the sublingual gland as mucous (with serous demilunes). The human submandibular and parotid gland are often classified as seromucous, the sublingual gland as mucous. The ductal system of the salivary glands comprises a varied network of ducts characterized by progessively larger members (Fig 1). In the rat submandibular gland, the duct system can be divided into (1) intercalated ducts, (2) granular ducts, (3) striated ducts, and (4) excretory or terminal ducts. The rat parotid gland lacks granular ducts (2). In the rat submandibular gland in addition a special

type of granular cell has been described at the junction between the acinus and the intercalated duct (Qwarnstrom and Hand 1983). Granular duct cells (2) occur in the submandibular glands of many rodents, but are not found in man.

The current models of salivary secretion are based on the "two-stage hypothesis" (Thaysen et al. 1954): (1) endpieces produce an isotonic primary secretion, rich in Na^+, and are the site of fluid secretion by the gland; (2) the primary fluid, during its passage from the endpiece to the mouth, is rendered hypotonic by a process of Na+ reabsorption in excess of water reabsorption across an epithelium with relatively low water conductivity (Fig 1).

Fluid secretion in the secretory endpiece is assumed to be mediated by two separate transport mechanisms, located in the baso-lateral membrane of submandibular and parotid acinar cells: a $Na^+/K^+/2Cl^-$ symport (or cotransport) and a pair of antiports for Na^+/H^+ and $Cl^-/HCO3^-$ exchange (Fig 2) (Case et al. 1984, Novak and Young 1986, Pirani et al. 1987). In the non-stimulated steady state condition, active uptake of K^+ (under simultaneous extrusion of Na^+) by the Na-K-ATP-ase is balanced by a passive efflux through K^+-channels in the basolateral membrane (Petersen 1980). The electrochemical gradient for Na^+ established by the Na-K-ATPase is utilized by the cotransport system in the baso-lateral membrane to energize an uphill influx of Cl^- (Poulsen and Kristensen 1982, Martinez and Cassity 1985). Consequently, the Na-K-ATPase and the cotransport system act together as a "chloride pump" which results in an intracellular chloride concentration well above electrochemical equilibrium. The passive efflux of Cl^- across the luminal cell membrane seems to occur by electrodiffusion through Cl^- channels. The transepithelial potential difference thus created is assumed to provide the driving force for a paracellular passive flux of Na^+. Water supposedly follows by osmosis. Fluid secretion is mainly driven by the cotransport mechanism, and to a lesser extent by the pair of antiport mechanisms (Novak and Young 1986).

In the salivary glands, stimulation of fluid secretion is mediated by muscarinic cholinergic, α-adrenergic and peptidergic receptors, and Ca^{2+} acts as a second messenger (Young and van Lennep 1979). Upon stimulation, an increase in the intracellular free calcium concentration will open Ca^{2+}-activated K^+-channels in the basolateral membrane of the cell. K^+ released into the narrow intercellular clefts will be taken up again, primarily through the $Na^+/K^+/2Cl^-$ cotransport system (Case et al. 1984). It is still unclear what activates the cotransport system.

During unstimulated or reflex stimulated salivation, the primary fluid is modified while passing through the duct. When ducts of unstimulated glands are perfused, the concentrations of Na^+ and Cl^- fall in the excretory duct fluid, whereas those of K^+ and HCO_3^- rise to a small extent, so that the osmotic pressure falls. This suggests that the ducts reabsorb Na^+ (actively) and Cl^-, that they are impermeable to water, and that they secrete K^+ and HCO_3^- actively, so that the concentrations of these latter ions exceed those in plasma. Knauf et al. (1976) propose the presence of two distinct transport mechanisms on the luminal cell membrane: one exchanges intracellular K^+ for intraluminal H^+ and the other couples the Na^+-flux from the lumen to the flux of K^+ or H^+ in the opposite direction.

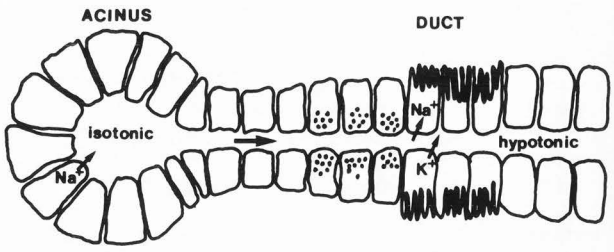

Fig 1. Schematic drawing of a typical secretory portion (rat submandibular gland) composed of acinus and duct elements. The arrow indicates the direction of the salivary flow.

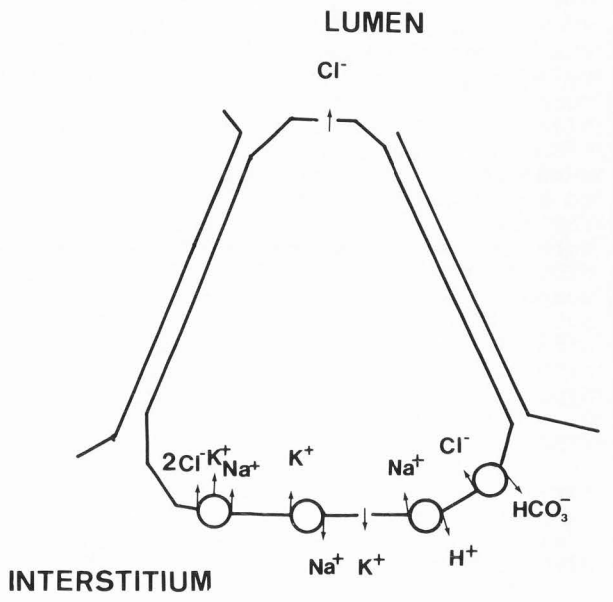

Fig 2: Ion transport mechanisms in the membrane of the acinar cells of the salivary gland.

Furthermore, a basally located Na-K-ATPase appears to be responsible for active Na^+ and K^+ transport.

Secretion of protein-rich saliva is mediated by ß-adrenergic receptors, and cyclic AMP (cAMP) acts as intracellular second messenger. However, it has become clear that also calcium is involved in this process. For the parotid gland, it has been shown that adenylate cyclase is sensitive to variations in intracellular Ca^{2+} concentrations and may be regulated by calmodulin (Argent and Arkle 1985).

Because of the importance of ion fluxes in salivary gland function, energy-dispersive X-ray microanalysis appears a potentially very interesting technique to apply to these tissues. Insect salivary glands have been studied by the group of Gupta and Hall (e.g., Gupta and Hall 1983). However, microprobe studies on mammalian salivary glands in health and disease are quite rare.

Among the diseases affecting the salivary glands, the generalized exocrinopathy cystic fibrosis (CF) should be mentioned. Although in CF patients the changes in salivary gland function are relatively minor (Quinton 1984) and do not significantly contribute to the clinical problems, the salivary glands provide an interesting system for research on the basic defect and have especially been used in studies using animal models for CF (Martinez 1985). X-ray microanalysis of the labial glands of CF patients was carried out by Izutsu et al. (1985), who showed that the acinar cells of these minor salivary glands in CF patients had a higher Na concentration than in healthy controls.

In this review we report studies on the elemental composition of resting and stimulated salivary glands, and on the effect of hormones (thyroxin, dexamethasone), factors affecting the serum calcium level (calcitonin, vitamin D), and alloxan on salivary gland structure and function. In addition, studies on a possible new animal model for CF, the chronically furosemide-treated rat, are reported. Other animal models for CF have been the subject of a recent review (Müller and Roomans 1985). X-ray microanalytical studies were complemented with ultrastructural studies and chemical analysis of saliva obtained after stimulation of the gland.

General methods

Human tissue

Human parotid and submandibular gland tissue was obtained during tumor operations: small pieces of clinically normal tissue were frozen as rapidly as possible in liquid nitrogen (Wróblewski et al. 1987).

Animal tissue

Male Sprague-Dawley rats (initial weight 150-175 g) were used in all studies (except where indicated). Details about the experimental protocol used are given with the respective study. The animals were deprived of food the night before sacrifice, but had access to water ad libitum. Tissue for microanalysis and electron microscopy was taken from animals heavily anesthesized with sodium pentobarbital.

Tissue preparation

For X-ray microanalysis at the cellular level, 1-2 mm pieces of salivary gland were rapidly excised and immediately frozen in liquid nitrogen. Thick (16 µm) sections were cut on a conventional cryostat at -20°C, mounted on carbon specimen holders, freeze-dried in the cryostat, and coated with a conductive carbon layer (Wróblewski et al. 1978). In some studies, 4-6 µm thick sections, cut at -30°C, were used. These sections were mounted on carbon specimen holders with a central hole (2 mm diameter) covered with a Formvar film (Wroblewski et al. 1983).

For analysis at high resolution, thin sections of freeze-dried embedded material were prepared. The tissue was frozen in Freon subcooled by liquid nitrogen, and freeze-dried in a modified conventional freeze-dryer (Wroblewski and Wróblewski 1986). Freeze-drying was started at about -90°C; the temperature was then kept at -85°C for the next 10-12 h, and then slowly raised to -65°C over a period of two days. The vacuum in the freeze-dryer was below 10^{-3} Torr. The condenser temperature was -120°C. After freeze-drying, the samples, still at -65°C in vacuum, were infiltrated with Lowicryl HM23, which was polymerized by indirect UV light (360 nm) without changing the temperature or breaking the vacuum. The vacuum during infiltration was about 10^{-1} Torr. After polymerization, the specimens were slowly brought to room temperature. Thin sections (100-200 nm) were cut on a dry glass knife (Wroblewski and Wróblewski 1986).

X-ray microanalysis

The 16 µm thick sections were viewed in the secondary electron mode and analyzed at 20 kV (Wróblewski et al. 1978). This allows analysis with a spatial resolution of about 10 µm. Quantitative analysis of the acinar cells was carried out as described previously, using standards consisting of mineral salts dissolved in a gelatin/glycerol matrix, that had been prepared and analyzed in the same way as the specimens (Roomans 1981, Müller and Roomans 1985). The 4-6 µm sections were viewed in the scanning transmission mode and analyzed at 80 kV; quantitative analysis was carried out as described by Wroblewski et al. (1983). The thin sec-

tions of freeze-dried, embedded tissue were analyzed qualitatively, in the scanning transmission mode at 80 kV.

Morphological and morphometrical studies

Tissue for light microscopy and transmission electron microscopy (TEM) was fixed in cacodylate-buffered glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in a graded ethanol series and embedded in Polarbed 812 (Polaron, Watford, U.K.). Ultrathin sections for TEM were stained with uranyl acetate and lead citrate, sections for light microscopy with toluidine blue. Morphometrical determinations of acinar size and relative mucus volume were carried out on prints of light or transmission electron microscopic images (final magnification 3000 x and 6000 x, respectively) with the point lattice method (Rohr et al. 1978).

Cannulation

Cannulation of the submandibular duct was carried out to collect stimulated saliva. The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), and tracheotomized to provide clear airways during cannulation. The animals were placed on a heating pad to maintain body temperature at 37°C. A fine polyethylene cannula (Clay-Adams PE10) drawn to a tip diameter of about 100 μm was inserted in the main excretory ducts under a dissecting microscope. Salivation was induced by pilocarpine (8 mg/kg body weight). The saliva produced in the initial 4 min was discarded, the saliva produced in the subsequent 10 min was collected, and the amount of saliva determined gravimetrically. After the end of the collection period, the submandibular gland was removed, separated from the sublingual gland, and weighed. The saliva samples were analyzed for sodium, potassium, and calcium by means of atomic absorption spectrometry and for protein by the method of Lowry et al. (1951).

Resting glands

The data for the elemental composition of rat and human salivary gland acinar cells are given in Tables 1 and 2. In addition, data for the granular duct cells in the rat submandibular gland are shown. The morphology of the acinar cells of submandibular and parotid gland is shown in Fig 3. In general, the mucous cells (submandibular and sublingual) had a higher calcium concentration than the serous cells (submandibular granular duct, parotid). Our data for the parotid gland are in general comparable to those of other groups (Sasaki et al. 1983, Izutsu et al. 1987). Analysis of thin sections (with a spatial resolution of about 100 nm) of the rat submandibular gland (Fig 4) showed that this calcium was mainly localized in the mucous granules, whereas endoplasmic reticulum, nucleus and mitochondria contained relatively little calcium (Fig 5). A striking feature is the high Cl content of the mucus granulus. The rat granular duct cells contain a variable number of secretory granules with a relatively high sulfur content. These granules are probably responsible for the relatively high sulfur levels found in analysis of thick cryosections.

The data for the elemental composition of the human salivary gland showed higher Na and Cl concentrations than those for the rat parotid and submandibular glands. This is presumably an artefact due to the fact that sampling of the human tissue during an operation is only of secondary importance, whereas in animal experiments the speed of sampling can be optimized. Post-mortem changes in the elemental composition of salivary gland acinar cells mainly affect Na, Cl (increase) and K (decrease) (Roomans and Wroblewski 1985).

A recent analysis of submandibular gland tissue of a CF patient (autopsy material, kindly provided by Drs R.L. Dormer and M.A. McPherson, Cardiff) showed higher levels of calcium in the acinar cells of the CF gland (57 ± 9 mmol/kg dry weight; mean and standard deviation of 12 cells), and lower chloride levels (152 ± 36 mmol/kg dry weight) than in the acinar cells of the control material. The lower chloride levels are remarkable, since the chloride concentration would be expected to be high in autopsy material.

Stimulated glands

In vitro studies were carried out as follows: small pieces of the submandibular gland were incubated in fortified Krebs-Ringers buffer at 37°C; the medium was continually gassed with 95% O_2 / 5% CO_2 (Bogart and Picarelli 1978). After an equilibration period of 20-30 min with several changes of buffer, agonist was added and the pieces of gland were removed from the incubation medium and frozen in liquid nitrogen after 5 to 30 min (as specified in Table 3 and Fig 6) of incubation.

The effect of cholinergic stimulation (20 μm carbachol) on the elemental content of rat submandibular gland acinar cells is shown in Fig 6. The potassium concentration was slightly decreased (most at 5 min incubation), whereas the Na concentration was significantly increased. No significant changes were observed in the Cl concentration. The sustained increase of the cellular Na/K ratio appears typical for stimulation of cholinergic, α-adrenergic and peptidergic receptors that

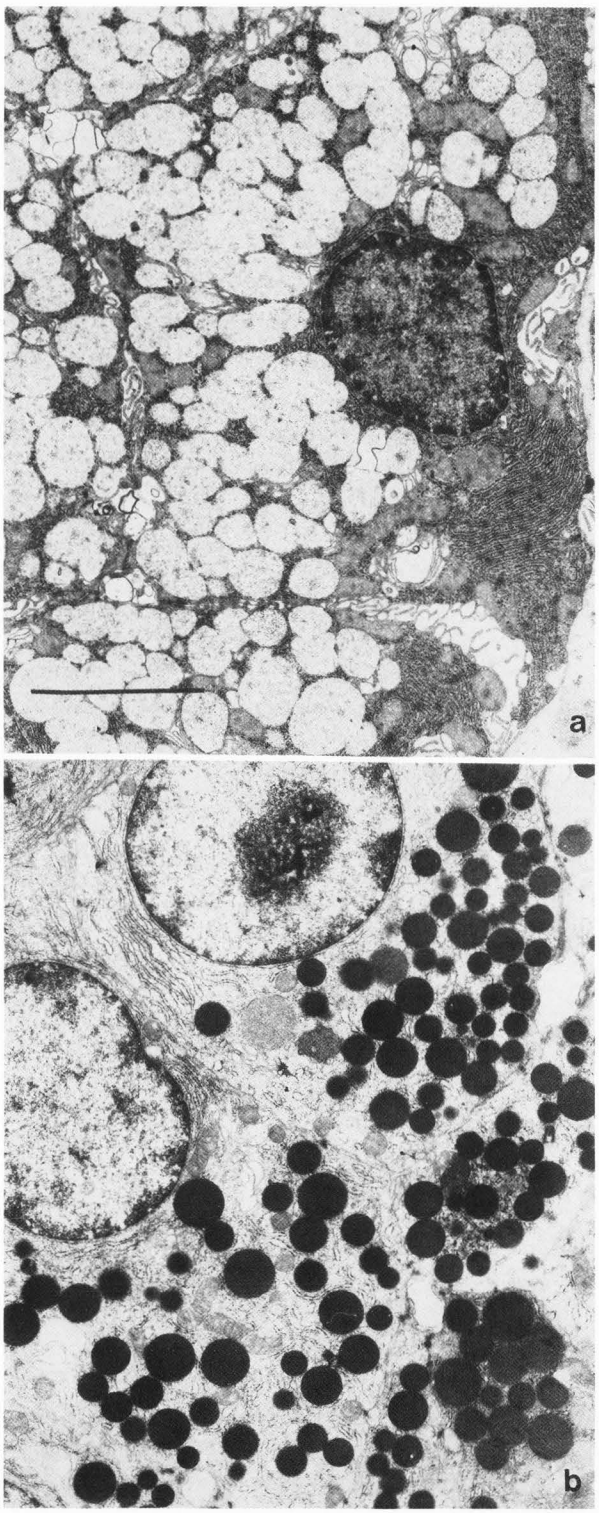

Fig 3: Transmission electron micrographs of conventionally fixed, embedded, rat salivary glands: (a) submandibular acinar cell, (b) parotid acinar cell (fasted animal) with electron dense zymogen granules. Bar = 5 μm.

Fig 4: Transmission electron micrograph of rat submandibular gland: frozen, freeze-dried, embedded in Lowicryl and sectioned on a dry knife. Bar = 5 μm.

Table 1
X-ray microanalysis of human salivary glands

	submandibular	parotid
Na	179 ± 9	108 ± 10
Mg	40 ± 6	23 ± 2
P	593 ± 30	566 ± 19
S	251 ± 13	216 ± 9
Cl	246 ± 17	306 ± 42
K	536 ± 31	427 ± 19
Ca	34 ± 5	28 ± 4

Values represent mean and standard error of 2 (submandibular gland) and 7 (parotid gland) donors and are given in mmol/kg dry weight. In each specimen 12-20 cells were analyzed. Thick (16 μm) cryosections were used for the analysis.

results in fluid secretion by the gland; ß-adrenergic stimulation resulted in loss of Ca from the acinar cells, but did not affect the Na/K ratio (Table 3) (von Euler et al. 1983, 1985).

In vivo studies on stimulation of rat salivary glands were carried out as follows: cholinergic stimulation was elicited by i.p. injection of pilocarpine, ß-adrenergic stimulation by i.p. injection of isoproterenol. Gland tissue was removed 1 h after the injection and frozen immedia-

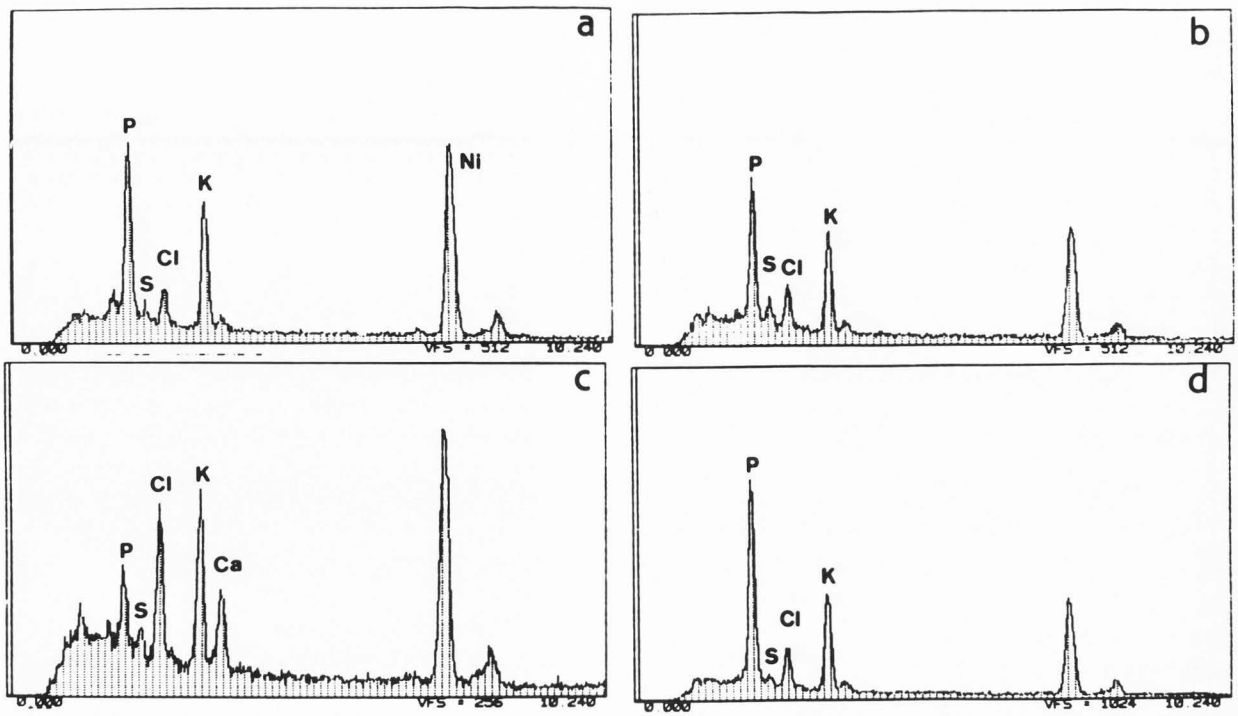

Fig 5: X-ray spectra from thin sections of freeze-dried, low-temperature embedded rat submandibular gland. All spectra are from acinar cells: (a) nucleus, (b) endoplasmic reticulum, (c) mucus granule, (d) mitochondrion. The main peaks are identified by their elemental symbols. The Ni peak is due to the grid.

Table 2
X-ray microanalysis of rat salivary glands

	submandibular gland acinar cells	submandibular gland granular duct cells
Na	85 ± 8	99 ± 14
Mg	60 ± 3	47 ± 5
P	716 ± 40	356 ± 10
S	234 ± 15	357 ± 20
Cl	212 ± 8	186 ± 5
K	527 ± 29	489 ± 6
Ca	39 ± 3	25 ± 12

	parotid gland	sublingual gland
Na	71 ± 6	74 ± 7
Mg	32 ± 2	72 ± 4
P	691 ± 41	485 ± 28
S	208 ± 30	233 ± 18
Cl	232 ± 19	229 ± 24
K	449 ± 55	457 ± 30
Ca	26 ± 6	42 ± 3

The data represent mean and standard error of 7-8 glands; in each gland, 8-12 cells were analyzed (4-6 or 16 μm thick cryosections). The values are given in mmol/kg dry weight.

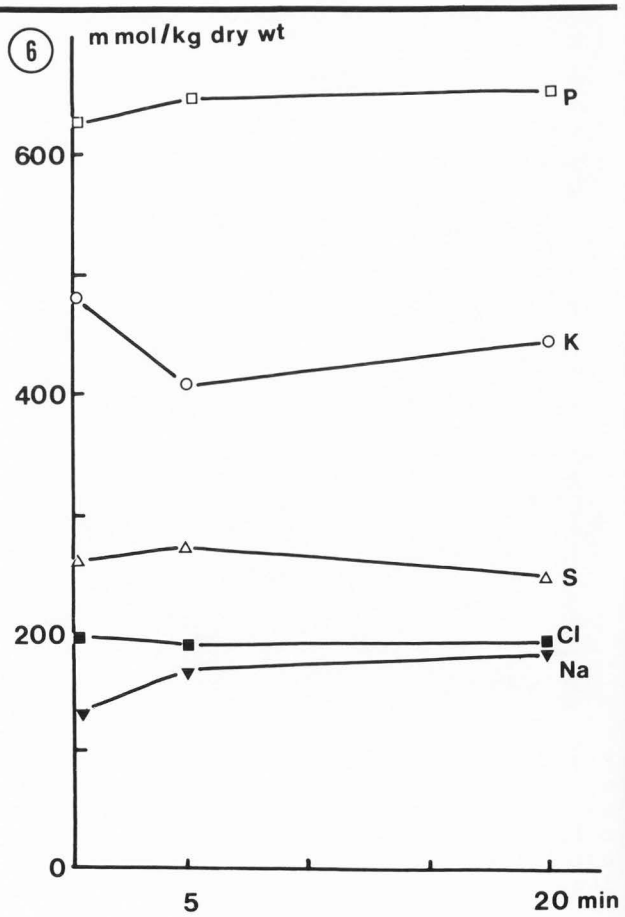

Fig 6: Effect of cholinergic stimulation on the elemental content of rat submandibular gland acinar cells. Data points represent means of four experiments (Wroblewski et al. 1983).

Table 3
Elemental changes in stimulated rat submandibular gland

	Na/K ratio
in vitro studies	
control	0.53 ± 0.06
phenylephrine	0.56 ± 0.08
carbachol	0.62 ± 0.05
substance P	0.68 ± 0.05
isoproterenol	0.43 ± 0.03
in vivo studies	
control	0.20 ± 0.02
pilocarpine	0.39 ± 0.08
isoproterenol	0.22 ± 0.04

In the in vitro studies, slices of submandibular gland (female rats) were stimulated for 10 min with phenylephrine (20 μM), carbachol (20 μM), substance P (0.1 μM), or isoproterenol (20 μM). Values are mean and standard error of 3-6 experiments; in each slice 10-15 cells were analyzed (von Euler et al. 1983, 1985). In the in vivo studies, the animals were stimulated with pilocarpine (20 mg/kg body weight), or isoproterenol (2 mg/kg body weight). The submandibular glands were removed 1 h after stimulation. Values are mean and standard error of 4 experiments; in each gland 8-12 cells were analyzed (Sagulin et al. 1985b).

tely. Also after in vivo cholinergic stimulation, a sustained increase in cellular Na/K ratio was noted (Table 3), whereas β-adrenergic stimulation resulted in a loss of Ca from the cells but did not affect the Na/K ratio. The changes in cellular calcium and magnesium levels following β-adrenergic stimulation have been reviewed by Müller et al. (1985) and Müller and Roomans (1985).

The Na/K ratio in unstimulated cells in situ is significantly lower than that in unstimulated cells in slices in vitro, indicating that some damage to the cells occurs during disection and incubation of the tissue, and that, under the experimental circumstances, the damage was not (completely) reversible.

The increase in the Na/K ratio in submandibular gland acinar cells after pilocarpine stimulation agrees with microprobe studies by Izutsu and Johnson (1986) on rat parotid gland, where an increase in Na in zymogen granules, nucleus and cytoplasm, and a decrease in K in nucleus and cytoplasm was noted.

Hormonal control of salivary glands

The salivary glands are concerned with protein synthesis and secretion and

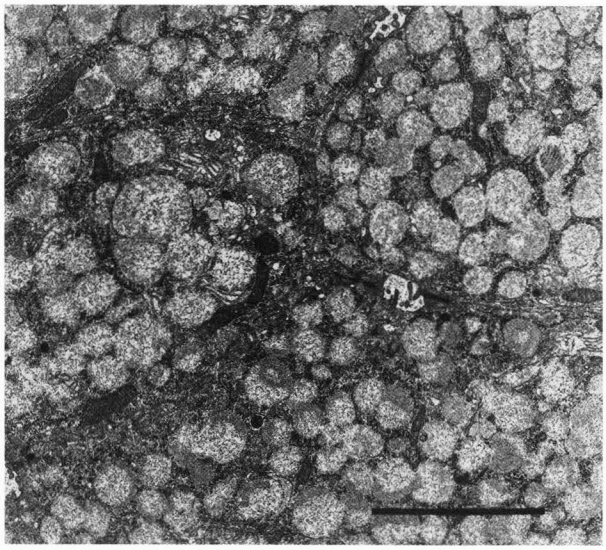

Fig 7: Transmission electron micrograph of rat submandibular gland acinar cells after chronic treatment with thyroxin. The cells are enlarged and have accumulated mucus. Bar = 5 μm.

in common with other tissues having these functions they are affected by somatotrophin and thyroid hormones, but they appear less dependent on glucocorticoids than other such tissues (Blair-West et al. 1967). In the present study, male rats were given 100 μg/kg body weight/day of the synthetic glucocorticoid, dexamethasone, or 200 μg/kg body weight/day of thyroxine (Johnson et al. 1987). After eight days of treatment portions of the submandibular and parotid gland were removed for microanalytical and morphological studies. In addition, pilocarpine-stimulated submandibular saliva was collected by cannulation of the submandibular duct.

Thyroxine induced an increase in size in the submandibular acinar cells, and an intracellular accumulation of mucus (Fig 7). X-ray microanalysis at the cellular level showed a significant increase in the calcium content of the acinar cells (Table 4). The flow rate of the submandibular saliva after pilocarpine stimulation was significantly reduced, and Ca and K levels were slightly increased (Table 5). The decrease in flow rate of submandibular saliva does not agree with reports of increased flow rate of parotid saliva after thyroxine treatment (Johnson et al. 1987). However, in that study salivation was elicited by combined stimulation with pilocarpine and isoproterenol, whereas in our study only chloninergic stimulation was used. The parotid gland acinar cells mainly contained the electron-lucent type of zymogen granule. The elemental composition of parotid acinar cells was not markedly affected by thyroxine treatment.

Table 4
Calcium concentration in rat submandibular gland acinar cells

Treatment	Ca
control (8)	39 ± 3
thyroxin (8)	58 ± 4*
dexamethasone (8)	36 ± 5
vitamin D (7)	49 ± 4*
calcitonin (6)	72 ± 5*

The number of animals used in each experiment is given in parentheses. Data are expressed in mmol/kg dry weight and represent mean and standard error.
* denotes a significant difference from the control value ($p<0.05$).

Dexamethasone did not induce any marked morphological changes in the acinar cells of submandibular and parotid gland, and did not significantly affect the elemental composition of these cells. However, a slight decrease of the pilocarpine-stimulated flow rate of the submandibular saliva, and a slight increase of Ca and K levels was noted (Table 5). Na levels were not significantly affected, in contrast to what has been reported to occur in human saliva; the increase in K agrees, however, with data on human saliva (Blair-West et al. 1967). A more significant decrease in flow rate after dexamethasone treatment was reported for the rat parotid gland (Johnson et al. 1987) after combined stimulation with pilocarpine and isoproterenol.

Effects of vitamin D-induced hypercalcemia

The importance of the extracellular calcium concentration for the secretory process in exocrine glands in vitro has been extensively documented. In general, experiments have demonstrated calcium dependency of secretion by lowering the calcium concentration in the bath fluid or complexing the calcium by a chelating agent (Petersen and Ueda 1976). The effect of high calcium concentrations on secretion by exocrine glands has been much less extensively investigated. However, this question may be of clinical interest since elevated calcium levels in saliva caused by or associated with hypercalcemia have been reported (Freeman and Welt 1965, Weinberger et al. 1974), and a positive correlation between salivary calcium and phosphate levels and prevalence of periodontal disease has been suggested (Maijer and Klassen 1972).

In the present study rats were injected for a period of 5 days with 1α-vitamin D3 (Leo Pharmaceutical Products, Copenhagen, Denmark), which resulted in an increase of the blood Ca^{2+} levels to 1.8 ± 0.03 mM (from 1.3 ± 0.04 mM in the control animals). In the submandibular acinar cells, intracellular accumulation of mucus was noted, and concurrent with this, a significant increase in the cellular calcium concentration (Table 4). In the parotid gland, a relative increase in the number of electron-translucent zymogen granules was noted (with a concurrent decrease of the number of electron-dense zymogen granules). No significant changes in the elemental content of the parotid acinar cells were found. The flow rate of the submandibular saliva after pilocarpine stimulation was significantly reduced in vitamin-D treated rats, and a tendency to increased Na and decreased K levels (increased Na/K ratio) could be observed (Table 5). No significant changes in Ca and protein levels could be observed. Also in studies on human salivary glands during experimentally induced hypercalcemia no significant changes in Ca and protein levels in parotid and mixed saliva could be found (Sagulin et al. 1985a, 1986).

Strikingly, similar changes in acinar cell ultrastructure and elemental composition, and a reduction in the flow rate of submandibular saliva were noted after treatment with calcitonin, which lowers blood Ca^{2+} (Sagulin et al. 1985b) (Tables 4 and 5).

Effects of alloxan

Alloxan is known for its specific cytotoxic effect on pancreatic B cells, and is used to experimentally induce diabetes in a variety of laboratory animals (reviewed by Cooperstein and Watkins, 1981). It has also been shown that alloxan in vitro interferes with the exocrine function of the pancreas and that it inhibits pancreatic fluid secretion (Scratcherd, 1974). There are a few studies of the effect of alloxan on salivary glands but these appear to be limited to long-term effects (Anderson and Shapiro 1979, Anderson and Johnson 1981, Anderson 1983).

There is no agreement on the immediate mechanism by which alloxan exerts its cytotoxic effects. Some authors have suggested an inhibition of the Na-K-pump (Idahl et al 1977). Others have suggested that alloxan inhibits adenylate cyclase (Cohen and Bitensky 1969, Scratcherd 1974, Kempen 1976, Tomita and Scarpelli 1977).

In many experimental conditions, a marked accumulation of mucus in the acinar cells of the submandibular gland can be observed (see above, e.g., thyroxine, vitamin D, and calcitonin). Also in various animal models for cystic fibrosis such as the chronically reserpinized rat (Martinez et al. 1975a,b), this phenomenon

Table 5

Flow rate and composition of rat submandibular saliva

Treatment	Flow rate	protein	Ca	Na	K
control (23)	79 ± 4	4.1 ± 0.3	0.9 ± 0.04	2.1 ± 0.4	70 ± 4
thyroxin (10)	43 ± 6*	4.6 ± 0.4	1.2 ± 0.1*	2.3 ± 0.4	86 ± 5*
dexamethasone (16)	66 ± 7	4.7 ± 0.4	1.4 ± 0.1*	2.6 ± 0.4	83 ± 3*
vitamin D (8)	48 ± 5*	4.0 ± 0.4	1.1 ± 0.1	3.4 ± 0.6	59 ± 3*
calcitonin (21)	60 ± 4*	4.7 ± 0.3	1.0 ± 0.05	3.2 ± 0.5	89 ± 5*
alloxan (8)	81 ± 15	4.0 ± 0.4	1.2 ± 0.2	2.7 ± 0.5	79 ± 6
furosemide (17)	38 ± 5*	4.4 ± 0.3	1.1 ± 0.1*	3.9 ± 0.7*	58 ± 2*

The number of glands for each experiment is given in parentheses. Flow rate is expressed in mg saliva/g gland/min, protein in g/l, Ca, Na and K in mM. * denotes a significant difference from control ($p<0.05$ or better). Data from Scarlett et al. (1988) and unpublished results.

was found (Müller and Roomans 1985, 1987). Similar findings were made in studies of early effects of reserpine (Müller et al. 1985). Also, mucus release from the acinar cells on ß-adrenergic stimulation was reduced (Müller and Roomans, 1985), despite the fact that chronic reserpine treatment induces an increase in the number of ß-adrenergic receptors (Bylund et al. 1981). It was therefore concluded that the reserpine-induced accumulation of intracellular mucus was due to a post-receptor inhibition of ß-adrenergic stimulation, and a comparison with the acute effects of alloxan, a putative adenylate cyclase inhibitor, appeared of interest.

First, in a study on a plasma membrane fraction from rat submandibular gland we confirmed that alloxan in vitro (10 mM) was a potent inhibitor of adenylate cyclase activity, even after activation of the enzyme by forskolin (Sagström et al. 1987).

In acinar cells of submandibular gland removed 3 h and 6 h after i.p. alloxan injection (175 mg/kg body weight) (Anderson and Johnson 1981), the amount of endoplasmic reticulum was markedly decreased and the relative amount of mucus in the cells was increased (Fig 8). Morphometry on light microscopic images confirmed the impression from the electron micrographs and showed that the acini were enlarged and that the cells contained significantly more mucus after alloxan injection (Fig 9). The effect of alloxan subsided and the cells appeared close to normal after 24 h. X-ray microanalysis of acinar cells showed an increase of calcium and (to a lesser extent) of magnesium after alloxan injection, whereas the Na/K ratio was not significantly affected.

The flow rate of the submandibular saliva after pilocarpine stimulation was not significantly affected by alloxan, and

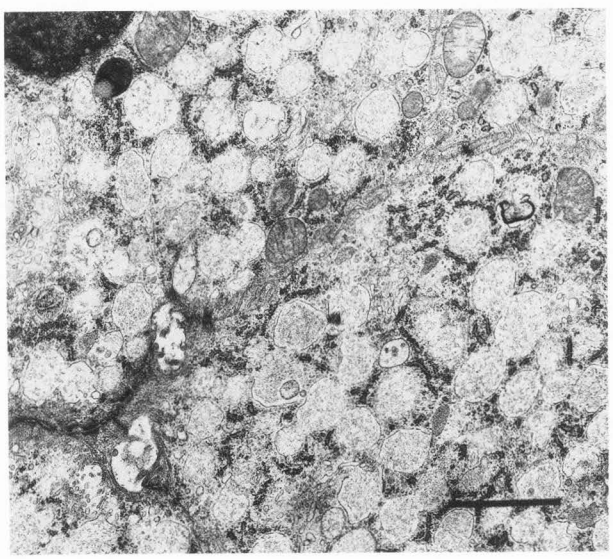

Fig 8: Transmission electron micrograph of rat submandibular gland acinar cells 3 h after injection with alloxan. Intracellular accumulation of mucus is evident. Bar = 2 µm.

the concentrations of Na, K, Ca and protein in saliva from alloxan injected animals were not significantly different from those in submandibular saliva of control animals (Table 5).

The chronically furosemide-treated rat

There is increasing evidence that a defective regulation of epithelial ion and water transport plays a central role in the pathogenesis of CF. Because of observed abnormalities in the calcium con-

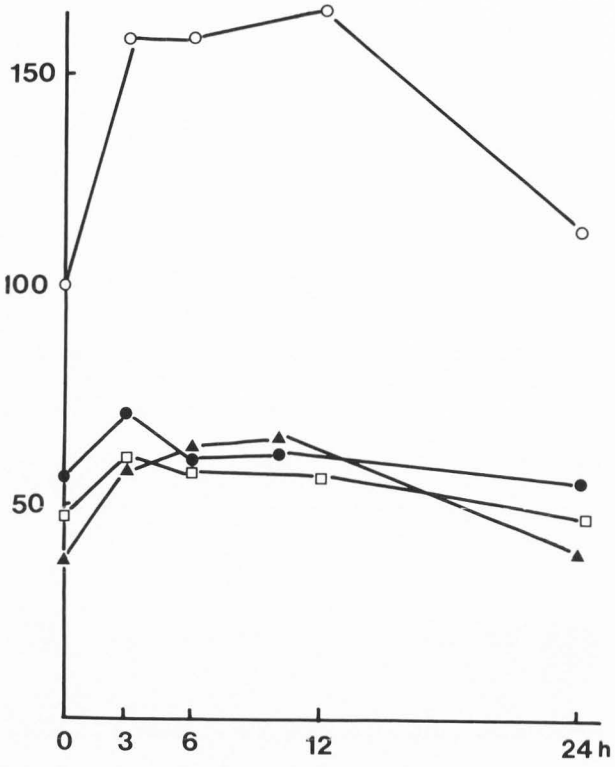

Fig 9: Effects of alloxan on: (○) relative acinar size (% of control), (●) Mg concentration in acinar cells (mmol/kg dry weight), (▲) Ca concentration in acinar cells (mmol/kg dry weight, (□) relative volume of intracellular mucus (in %). Data from Sagström et al. (1987).

tent of some exocrine secretions in CF and because of the central role of calcium ions in the secretory process a defective regulation of intracellular calcium levels has been proposed as the primary cause for CF (reviewed by Katz et al. 1984 and Roomans 1986). There is strong evidence that chloride transport is abnormal in at least two tissues in CF patients: sweat glands (Quinton 1983, Bijman and Quinton 1984) and respiratory epithelium (Knowles et al. 1983a,b). Defective reabsorption of chloride (and sodium) in the duct of the sweat gland would explain the abnormally high NaCl concentration in the sweat of CF patients (Bijman and Quinton 1984). Defective secretion of chloride by the respiratory epithelium would result in reduced water transport and ultimately in the formation of a water deficient secretion blocking the smaller airways.

Because of the inherent difficulties in investigating the tissue of interest in CF patients, it has been attempted to develop animal models for this disease. The most frequently used animal model is the chronically reserpinized rat (Martinez et al. 1975a,b, Martinez 1985), which

Table 6
Elemental composition of rat submandibular and parotid gland after chronic furosemide treatment

	submandibular gland	parotid gland
Na	87 ± 11	70 ± 5
Mg	70 ± 3*	33 ± 3
Cl	186 ± 9*	183 ± 9*
K	518 ± 24	383 ± 25
Ca	56 ± 3*	31 ± 3

Data are given as mean and standard error of 8 animals; in each gland 8-12 cells were measured. All data in mmol/kg dry weight. * denotes a significant difference with control values (see Table 2). Data from Scarlett et al. (1988).

displays several abnormalities in exocrine gland structure and function resembling the situation in CF patients, and also appears to have a defective chloride transport across the respiratory epithelium (Martinez and Quinton 1985). Despite the usefulness of this animal model, several problems remain: the model does not display all clinical symptoms associated with CF, and the cellular mechanism underlying the changes actually observed in the exocrine glands of the reserpinized rat is not always clear.

If a defective chloride transport is a factor of importance in the pathogenesis of CF, chronic treatment of experimental animals with inhibitors of chloride transport would be expected to produce CF-like symptoms. Therefore, in continuation of a series of studies on the reserpinized rat and other established animal models of CF (Müller and Roomans 1984a,b, 1985), we investigated the effect of chronic treatment with the chloride transport inhibitor furosemide on structure and function of rat salivary glands.

In the present study, the rats (initial weight 150-175 g) were fed with food to which furosemide had been added. The animals were kept for 4-5 weeks on this diet and the average daily intake of furosemide per animal during this period was about 40 mg (Warshaw et al. 1980). Water was provided ad libitum. Weight increase during the period was not significantly different from normal. Rats receiving standard laboratory chow were used as control.

After furosemide treatment, an increase in the relative volume of intracellular mucus in the submandibular acinar cells was noted. X-ray microanalysis of the acinar cells of the submandibular gland showed a significant increase in the cellular Ca and Mg concentration; in addition, a small decrease in Cl levels was noted (Table 6).

The volume of saliva secreted in the first 10 min-period by the submandibular gland after pilocarpine stimulation was markedly reduced in furosemide-treated rats; the concentration of Na and Ca was significantly higher, the concentration of K significantly lower after furosemide treatment; the protein concentration was not significantly affected (Table 5).

In the parotid gland, virtually all acinar cells contained relatively large, electron translucent zymogen granules after furosemide treatment. Also in the parotid gland, a significant decrease of the cellular Cl concentration was noted (Table 6).

Discussion

A number of apparently completely different experimental treatments induces similar changes in the submandibular gland acinar cells: intracellular accumulation of mucus and concurrent increase of the cellular calcium concentration. In most cases the increase in cellular calcium concentration can be quantitatively explained by the increase in the relative intracellular mucus content (Roomans 1986): this appears to be the case for chronic metabolic acidosis (Roomans and Bardon 1984), metabolic alkalosis (Roomans 1986), the effects of calcitonin (Sagulin et al. 1985b), and the effects of thyroxin, alloxan, vitamin D-induced hypercalcemia, and chronic furosemide treatment reported in this paper. In these cases, the local calcium content of the mucus granules remains constant, and no marked changes are expected in the calcium/protein ratio of the submandibular saliva, even if the salivary concentration of calcium or that of protein changes. In other cases, notably in animal models for cystic fibrosis such as the reserpinized rat, the chronically isoproterenol-treated rat, and the chronically pilocarpine-treated rat, the increase in cellular calcium levels exceeds that expected on the basis of the increase in the relative volume of intracellular mucus, due to an increase in the local calcium concentration of the mucous granules (Müller and Roomans 1985, Roomans 1986). This increase may be associated with changes in the relative proportions of different (glyco)-proteins in the mucus (Wells and Humphreys-Beher 1985, Klein and Sarras 1986), which could lead to increased affinity of the mucus for calcium.

The mechanism responsible for the intracellular accumulation of mucus is not completely clear. In rats treated chronically with isoproterenol, a desensitization of β-adrenergic receptors may cause decreased secretion of mucus. In the reserpinized rat, however, the number of β-receptors is actually increased (Bylund et al. 1981, Cutler et al. 1981) and also thyroxin has been reported to increase the number of β-receptors in rat submandibular gland (Medina et al. 1984). On the other hand, in the submandibular gland of the reserpinized rat, the response of the acinar cells to low concentrations of isoproterenol is reduced (Cutler et al. 1981; Müller and Roomans 1985); this is also the case for rats treated with calcitonin (Sagulin et al. 1985b) and for the chronically furosemide-treated rats (Scarlett et al. 1988). In these cases one would suggest a post-receptor inhibition of β-adrenergic stimulation. In agreement with this notion is the fact that the adenylate cyclase inhibitor alloxan also induces a transient intracellular accumulation of mucus. The post-receptor inhibition can, however, take place at several levels and need not be similar for all experimental conditions discussed above. Apart from interference with stimulus-secretion coupling at the level of the second messenger (cAMP), or changes in the energy metabolism of the cells, in some cases the exocytosis process as such may be inhibited due to changes in intracellular free Ca^{2+} concentrations or intracellular pH. More recently, a regulatory role for calcium also in cAMP-mediated secretory processes has been proposed. Argent and Arkle (1985) found that in the parotid gland adenylate cyclase activity was maximal at a Ca^{2+} concentration of 10^{-7} M. Any factor altering the intracellular free calcium concentration or affecting the mobilization of calcium from intracellular stores might therefore affect the secretory process, and it is conceivable that factors that would affect intracellular calcium levels in different ways could have the same effect on secretion.

In most cases where intracellular accumulation of mucus in the submandibular gland acinar cells is noted, the presence of electron-lucent (possibly immature) rather than condensed, electron dense ("mature") granules in the parotid acinar cells is found. Often, more granules than normal are present (Müller and Roomans 1987). Cellular calcium levels may be unaffected or even decreased (Müller and Roomans 1984b). These changes point to the possibility that in addition to a disturbance of the condensation process of the zymogen granules, changes in the proteins forming the granules have occurred. These changes are well-documented for chronic isoproterenol treatment (Robinovitch et al. 1977) but have not been studied for other experimental conditions discussed here.

Some of the changes induced by chronic furosemide treatment in rats resemble abnormalities found in CF patients, even though the agreement is not complete. Hypertrophy of mucous cells (Tandler 1987), decreased responsiveness

to ß-adrenergic stimulation (Sato and Sato 1984) and disturbed fluid and electrolyte transport appear to be features common to animal model and human disease. On the other hand, the extensive changes of the pancreas in CF patients are not found in the furosemide-treated rat (Scarlett et al. 1988). Generally, furosemide is assumed to inhibit the $Na^+/K^+/2Cl^-$ cotransport mechanism (Martinez and Cassity 1983), although it recently has been suggested that it might inhibit a Ca-regulated chloride channel (Evans et al. 1986). The chloride channel involved in the defective ion and water transport in CF epithelia is assumed to be localized in the apical membrane and may therefore be different from that primarily affected by chronic furosemide treatment. However, presumably inhibition of chloride transport across the basal membrane would indirectly result in inhibition of chloride and water transport over the apical membrane of epithelial cells. The final result would therefore be expected to resemble the situation in CF patients.

The chronically furosemide-treated rat also displays, at least with regard to salivary gland structure and function, many parallels with other animal models of CF, in particular the chronically reserpinized rat. This could imply that inhibition of chloride transport in the chronically reserpinized rat is a factor underlying some of the pathological changes observed.

Since the action of furosemide on salivary glands appears to be better defined than that of reserpine, which has a multitude of cellular and systemic side-effects, the chronically furosemide-treated rat may be an interesting animal model to study the effect of chronic changes in fluid transport in the salivary gland. Also alloxan may be of interest in this respect. Since the basic defect in CF is assumed to be located in cAMP controlled ion and water processes across epithelial cell membranes (Frizell et al. 1986, Welsh and Liedtke 1986), it could be speculated that alloxan might provide a tool to mimic at least some of the disturbances found in ion-transporting epithelia in CF.

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Discussion with Reviewers

K. Izutsu: Could you give more details on the tests that were performed to show statistically significant results? In particular, did the degrees of freedom reflect the number of spectra in the two groups or the number of animals?
Authors: The degrees of freedom reflect the number of animals in each group and not the number of spectra. We feel that cells within one gland cannot be regarded as independently variable. If a simple test, such as Student's t-test is used, the degrees of freedom should therefore not reflect the number of spectra. Possibly one should consider more detailed statistical analysis of the data by analysis of variance.

S.H. Ashrafi: What is the advantage of using freeze-dried and embedded specimen sections over freeze-dried cryosections?
Authors: In a tissue type such as the submandibular gland there is little advantage in using freeze-dried embedded tissue sections compared to freeze-dried cryosections. The use of freeze-dried embedded tissue has particular advantages in tissues where orientation is very important (Wróblewski et al. 1987), since the plastic blocks can easily be reorientated. To check the reliability of the freeze-dried embedded tissue technique it is, however, advantageous to try this technique on tissues that also can be cryosectioned with relative ease.

M.B. Engel: In some instances, liquid nitrogen alone was used for freezing. However, fewer ice crystals are generated if an intermediate immersion medium is used. Please comment.
Authors: In routine work with thick sections, the tissue is stored at -80°C in a freezer and cut at -20 to -30°C. Under such conditions recrystallization is unavoidable. Since the ice crystals are small compared to the analyzed volume, in low resolution analysis, acceptable results can be obtained. Since we have to accept ice crystals, there is little point in using more complicated and expensive freezing techniques than liquid nitrogen, since any advantages of such techniques are nullified by our subsequent handling of the tissue. We have, however, compared tissue frozen in liquid nitrogen cooled freon with tissue frozen in liquid nitrogen only and could at the cell level not find a significant difference between the two methods.

S.H. Ashrafi: Is the quantitative data of X-ray microanalysis of 16 μm thick sections at 20kV comparable to the data obtained from 4-6 μm thick sections at 80kV?
Authors: Analysis of 4-6 μm cryosections may allow the separate analysis of nucleus and the extranuclear part of the cell. Unless, however, a specific attempt is made to analyze these parts of the cell separately, the results of 4-6 μm cryosections are quantitatively comparable to those obtained on 16 μm cryosections.

A.R. Hand: The dose of isoproterenol used (Table 3) is quite low compared to other published studies. What is the effect of this dose on granule exocytosis and flow rate from the submandibular gland?
Authors: Isoproterenol doses in the order of magnitude of 10^{-5} M (10 μM) are commonly used in in vitro studies on rat salivary glands (see e.g., Argent and Arkle 1985). In our hands, 20 μM isoproterenol gives virtually maximal stimulation of amylase secretion from the parotid gland. We do not have any data on in vitro mucin secretion by the submandibular gland.

K. Izutsu: In Fig 5, an X-ray spectrum was shown as originating from a mitochondrion. How did you establish that the structure was in fact a mitochondrion and not some other intracellular organelle?
Authors: The organelles identified as mitochondria are rodlike structures with a higher electron density than the surrounding cytoplasm and secretory structures. In the submandibular gland acinar cells, there is little possibility that such a structure is not a mitochondrion.

G.A.J. Kuijpers: Do you have any quantitative data on morphometry of acinar cell size and relative mucus volume as a result of the various treatments?
Authors: Furosemide causes an increase in the relative volume of intracellular mucus from 43% in the control animals to 64% (Scarlett et al. 1988). The data for other treatments are given in Roomans (1986).

A.R. Hand: You suggest that the methods for preparation and incubation of the slices need improvement, perhaps in this as well as in other studies. What significance does this have for the interpretation of the results of in vitro stimulation experiments?
Authors: The preparation method for slices used in this study was a standard method, and we find that slices of pancreas or parotid gland prepared by this method show a 'normal' (i.e., in accordance with the literature) secretory response to cholinergic or β-adrenergic stimulation, respec-

tively. The problem whether isolated cells have the same ionic composition as their in situ counterparts is indeed very fundamental. A recent study by Warley (1987) indicates that even under favourable conditions (mild isolation, single thymocytes) this may not be taken for granted. We have during the past years had the occasion to analyze various types of isolated cells or groups of cells used in biochemical studies and invariably observed a higher Na/K ratio than in the in situ cells. Usually, such preparations are tested for a particular biochemical/ physiological response and no particular attention is given to whether or not the ionic composition of the cell has changed. This point will, however, definitely require more attention, so that preparation methods can be improved.

S.H. Ashrafi: What do you mean by saying that the local calcium content of the mucus granules remains constant, although you noticed changes in cellular calcium levels under experimental conditions?
Authors: If the relative amount of mucus in the cell increases, the cellular calcium concentration will increase, since mucus contains more calcium than other cellular structures. From the known (local) calcium content of mucus in normal cells and the relative amount of mucus in the cell under different experimental conditions one can calculate the expected calcium concentrations in the cell, assuming that the local calcium concentration of the mucus remains the same (Roomans 1986). If in a particular experiment this calculation holds, one may assume that the local calcium concentration of the mucus has not been affected by the experiment.

M.B. Engel: To explain their results, the authors adhere to the conventional view which involves ion channels, ion pumps, and energy-generating mechanisms. Could an alternative view be considered? Receptors on the cell surface or in the cell undergo perturbations and conformational changes in response to neurotransmitters, drugs or hormones. These changes in cellular macromolecules could affect the state of associated water and electrolytes in the cell and its secretion (cf Belleau, J. Medicinal Chem, 7:776, 1964, Freitag and Engel, Anat Record, 167:87, 1970).
Authors: The data obtained in the experiments described in the paper can be explained within the framework of the ion pump/ion channel models, and do not, in themselves, warrant consideration of alternative models. As you are aware, the ion pump/ion channel model is the most common theory used. On the other hand, our data do not disprove any alternative models, and could also be explained by theories such as the one suggested by you. To distinguish between two models for translocation of ions over the cell membrane, very specific experiments have to be set up, and discrimination criteria have to be established. Our study was not set up for this purpose. A general comment pertaining to this question is, that much of the present discussion on the mechanism of ion and water transport in cells is due to the lack of easily verifiable discrimination criteria. In the meantime, one should probably regard all models as tools to plan experiments and generate progress in research rather than as an absolute description of the truth.

G.A.J. Kuijpers: Does the change in Na/K concentration in the submandibular gland coincide, precede or follow the actual secretion from the (intact) gland?
Authors: The time-scale of 5 to 20 minutes used in the experiment shown in this paper is not adequate to answer your question. We have now carried out preliminary experiments with rapid freezing in situ, so that tissue could be frozen within a minute of stimulation. Under those conditions the increase in Na/K ratio is not significant. This might indicate that the increase in Na/K ratio is a slower process than fluid secretion.

G.A.J. Kuijpers: Do you have an explanation for the increased Na/K ratio upon stimulation of the salivary gland? Why is it not reversible?
Authors: An initial depolarization is known to occur after pilocarpine stimulation and agrees with an increased Na/K ratio. One would expect the Na/K ratio to eventually return to the resting value. The dose of pilocarpine used in the in vivo experiment supports, however, fluid secretion for more than 30 min, which exceeds the time at which the final sample for X-ray microanalysis was taken. On the other hand, Leslie and Putney (1983) have demonstrated vacuole formation in submandibular acinar cells after cholinergic stimulation. Although the origin of the vacuoles could not be determined with certainty, it is not implausible that the composition of the vacuolar fluid resembles that of the extracellular fluid (high Na, low K). Since any vacuoles of this type would be included in our measurements, they could well affect the elemental composition of the cells observed by X-ray microanalysis of thick cryosections.

A.R. Hand: Could you offer an explanation as to why vitamin D and calcitonin have the same effect on the submandibular gland ?
Authors: We cannot give a definite answer to your question at this point. It is possible that any deviation from the 'ideal' intracellular calcium concentration (either decrease or increase) inhibits a step in stimulus-secretion or exocytosis. As pointed out by Argent and Arkle (1985) parotid gland adenylate cyclase might be calmodulin-regulated, and displays a

calcium-optimum at 0.1 μM. Shifts in the intracellular (free) calcium concentration during stimulation (either increase or decrease) could conceivably inhibit secretion. With regard to calcitonin, our experiments so far indicate a slight but significant inhibition of adenylate cyclase (Sagulin et al., unpublished results). Other techniques apart from X-ray microanalysis will have to be used to provide a definite answer.

S.H. Ashrafi: Do you think that an animal model used to study cystic fibrosis (CF) is suitable to investigate CF in humans?
Authors: This question has been discussed extensively by us (Müller and Roomans 1985) and others (Martinez 1985). There is no completely satisfactory animal model for cystic fibrosis but there are several models that display one or more features found in the human disease. The chronically furosemide-treated rat may come closer to the human disease because in this animal model the transepithelial chloride flux is reduced. Differences between the regulation of chloride - water transport in rat vs human respiratory epithelium may restrict the value of this animal model. The chronically reserpinized rat also appears to have a reduced transepithelial chloride flux (Martinez and Cassity 1985) although the reason for that is not immediately evident. The effect on chloride transport, rather than the known effects of reserpine on innervation, may make this animal model interesting. The alloxan-model may give information of cAMP-regulated processes, but the systemic effects of alloxan are so severe that the usefulness of the model is limited to very specific questions that can be investigated in short term experiments.

Nonetheless, the use of animal models is justified because we still do not understand how a defective transepithelial chloride transport causes all the clinical symptoms seen in CF.

Additional References

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