Scanning Electron Microscopy

Volume 1986
Number 1 *Part I*

Article 17

3-24-1986

Calcium and Cystic Fibrosis

Godfried M. Roomans
University of Stockholm

SCANNING ELECTRON MICROSCOPY/1986/I (Pages 165-178)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507 USA

0586-5581/86$1.00+05

CALCIUM AND CYSTIC FIBROSIS

Godfried M. Roomans

Department of Ultrastructure Research, Wenner-Gren Institute,
University of Stockholm, S-10691 Stockholm, Sweden

(Received for publication February 21, 1986, and in revised form March 24, 1986)

Abstract

Cystic fibrosis (CF) is a generally lethal, congenital, genetic disease of unknown etiology. It is likely that a defective regulation of ion and water transport in exocrine glands and possibly also in other epithelial cells has a central role in the pathogenesis of this disease. Calcium has been implicated in the basic defect underlying CF because of findings of abnormally high calcium levels in some secreted fluids and some cells of CF patients.

Using X-ray microanalysis, we have demonstrated elevated calcium concentrations in cultured fibroblasts and in goblet cells of the bronchial epithelium of CF patients. A factor produced by CF fibroblasts in culture can increase the calcium concentration in healthy cells, although this may be an indirect effect. In animal models for CF, such as the chronically reserpinized rat and the chronically isoproterenol-treated rat, abnormally high calcium levels in the acinar cells of the submandibular gland could be demonstrated, similar to the situation in CF patients. In the acinar cells of the parotid gland in these animal models, the calcium levels are, however, abnormally low. This suggests that the changes in cell calcium content are secondary to other changes, possibly changes in the secretory proteins. A study of the effect of the serum calcium level and of the calciotropic hormone calcitonin suggested that neither of these factors could be directly linked with CF.

It is concluded that several lines of evidence point to a secondary rather than a primary role for calcium in the pathogenesis of CF.

KEY WORDS: Cystic fibrosis, calcium, exocrine glands, fibroblasts, goblet cells, animal models, reserpine, acidosis, calcitonin, X-ray microanalysis.

* Address for correspondence:
Godfried M. Roomans, Department of Ultrastructure Research, Biology Building E4, University of Stockholm, S-10691 Stockholm, Sweden.
Phone No: (+46)8-164093.

Introduction

Cystic fibrosis (CF) is a generally lethal, congenital, genetic disease occurring predominantly in the white population, in some countries with a frequency higher than 1 in 2000 newborns. It has recently been shown that the CF gene is localized in the middle part of the long arm of chromosome 7 (Knowlton et al. 1985; Wainwright et al. 1985; White et al. 1985). However, the CF gene itself has not been identified and the molecular basis of the disease is still unknown. Nevertheless, there is a consensus that a defective regulation of ion and water transport in exocrine glands and possibly also in other epithelial cells has a central role in the pathogenesis of CF.

Two theories have dominated the field in recent years: the 'calcium hypothesis' (Katz et al. 1985) and the 'chloride hypothesis' (Knowles et al. 1983a,b; Quinton 1983). The 'calcium hypothesis' of CF is based on the finding that in several exocrine glands the secreted fluid has an abnormally high calcium content. Katz et al. (1985) have reviewed the often conflicting data on this subject. Moderate to strong evidence indicates that elevated calcium concentrations can be found in submandibular and parotid saliva, tears, and sweat of CF patients and possibly also in their tracheobronchial secretions.

Also a number of cell types in CF patients have abnormally high levels of total or free calcium: cultured fibroblasts (Baur et al. 1976; Feigal and Shapiro 1979; Roomans et al. 1983, Shapiro and Lam 1982), neutrophils (Cabrini and De Togni 1985), lymphocytes (Schöni et al. 1985) and isolated parotid cells (Mangos and Donnelly 1981). On the other hand, other groups have failed to find significantly elevated levels in lymphocytes (Grinstein et al. 1984) and acinar cells of the labial gland (Izutsu et al. 1985). Waller et al. (1984) noted normal free calcium levels, but elevated total calcium levels in CF lymphocytes.

A possibly defective regulation of the

This paper was presented at the Symposium on 'Cell Structure and Cell Biology' in honor of Björn Afzelius, December 19 and 20, 1985 in Stockholm, Sweden.

intracellular calcium concentration could exert its influence on two levels: (1) abnormally high concentration of free calcium could affect many important cellular processes, including stimulus-secretion coupling, (2) abnormally high concentrations of bound calcium - in exocrine cells mainly calcium bound to secretory proteins - would result in elevated calcium levels in the fluid secreted by the acinar cells.

This in its turn could influence (a) the ductal modification of the primary secretion, (b) the rheological properties of the secreted fluid, which could result in the production of viscous mucus and obstruction of the ducts, and (c) the composition of the bacterial flora in the airways; in particular it could promote the growth of certain pathogenic mucoid forms of Pseudomonas typical for CF.

Among the molecular mechanisms that could be responsible for the defective regulation of intracellular calcium, the following have been suggested: (1) abnormal Ca-ATPase (reviewed by Katz et al. 1985), (2) compositional alterations in membrane lipids, affecting membrane transport (Motta et al. 1985; Rogiers et al. 1980), (3) disturbed energy metabolism, in particular a disturbance in pyruvate metabolism leading to lactic acidosis, which could affect calcium homeostasis (Kollberg et al. 1983), (4) a circulating factor in the blood of CF patients that increases the calcium permeability of the epithelial cells (Bogart et al. 1982), (5) abnormal levels of calciotropic hormones, in particular of calcitonin (Kilbourn 1984), and (6) alterations in the composition of intracellular mucus (Boat et al. 1974) resulting in increased binding of calcium. Possible abnormalities in calmodulin activity or in phosphatidylinositol metabolism that could affect the regulation in intracellular calcium levels are also being investigated (Doughney et al. 1985; McPherson et al. 1985). A schematic survey of the 'calcium hypothesis' of CF is given in Fig 1.

The 'chloride hypothesis' has not yet been developed in equal detail. However, there is strong evidence that chloride transport in sweat glands and respiratory epithelium is abnormal in CF. In the sweat glands, the abnormality appears to reside in the duct (Bijman and Quinton 1984). Defective ductal reabsorption of chloride from the isotonic primary sweat produced in the coil would result in elevated NaCl levels in the final sweat. The abnormally high NaCl levels in sweat are the primary diagnostic sign of cystic fibrosis. Also in the respiratory epithelium an abnormally low permeability for chloride has been demonstrated (Knowles et al. 1983a,b). Chloride transport over the apical membrane of the cells of the respiratory epithelium is one of the factors regulating water transport. The result of the defective chloride transport could be the production of a water-deficient airway secretion, with a high concentration of protein and other ions (Boucher et al. 1984) However, insufficient data are as yet available to explain exactly how the defect

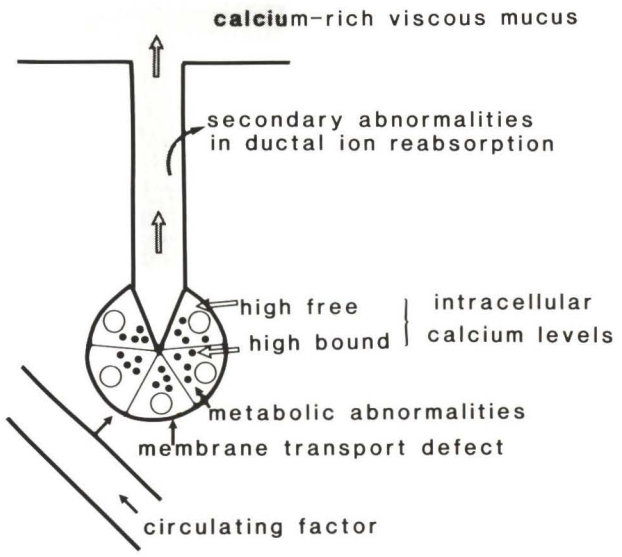

Fig 1. The 'calcium hypothesis' of CF.

in chloride permeability could cause all the clinical symptoms associated with CF.

It is also not yet established whether the defect in chloride permeability is due to a defective transport mechanism (chloride channels) or to a defective regulation of the chloride channel. Boucher et al. (1984) appear to favour the second possibility. In this respect it is of interest to note that intracellular calcium is one of the factors regulating chloride permeability.

In this paper, our work on abnormal calcium handling in CF and in animal models for CF will be reviewed. The main part of this work was carried out with electron probe X-ray microanalysis, which allows quantitative localization of calcium (and other elements) at the cellular or subcellular level.

Methods

Specimen preparation

Since virtually all calcium is lost from the cells during aldehyde fixation, throughout this study cryomethods for specimen preparation were used. For analysis at low spatial resolution, freezing in liquid nitrogen generally was sufficient. For analysis at high spatial resolution, the tissue was frozen in Freon 13 subcooled by liquid nitrogen, to a temperature of around -185 to 190°C. Although the use of ethane, and possibly of propane, may result in higher freezing velocities, the use of these liquids was absolutely precluded when the freezing had to be carried out in or near the operating theatre.

The further processing of the tissue depended on the type of specimen and the resolution of analysis. Cell cultures were, after freezing in liquid nitrogen, freeze-dried at -80°C and coated with a conductive layer. Low resolution

analysis was carried out on 16 µm cryosections, cut at -20 to -30°C on a conventional cryostat and mounted on a carbon specimen holder (Wroblewski et al. 1978). High resolution analysis was carried out on thin (less than 200 nm) cryosections, cut at -100 to -120°C on an LKB Ultrotome III with LKB CryoKit (Roomans et al. 1982a). The sections were placed on a Formvar-covered copper or nickel grid, freeze-dried in the cryochamber, brought to room temperature and covered with a second Formvar film. Bronchial biopsies were prepared for analysis by freeze-substitution. The frozen tissue was kept for 3 weeks at -78°C in dry ethyl ether over molecular sieve, brought slowly to room temperature, infiltrated with and finally embedded in epoxy resin (Polarbed 812, Polaron, Watford, U.K.). Sections were cut at room temperature on a glass knife with an ultramicrotome, placed on Formvar film covered copper slot grids, and covered with a second Formvar film to keep the sections in place. Adjacent sections were stained with methylene blue-basic fuchsin (Aparicio and Marsden 1969) and examined in the light microscope to aid in the identification of goblet cells.

X-ray microanalysis was carried out with a Kevex 7000 energy-dispersive spectrometer system mounted on a JEOL 100C electron microscope with an ASID-1 or an ASID-4B scanning attachment. Analysis of cell cultures or thick cryosections was carried out at 20 kV and the specimens were viewed in the secondary mode. Thin cryosections or sections of freeze-substituted tissue were analyzed at 80 or 100 kV and viewed in the scanning transmission mode.

Methods for quantitative analysis of thick and thin sections have been described in detail in Roomans (1980), Roomans (1981) and Müller and Roomans (1985b). Fully quantitative analysis of the cultured cells was not possible. The cells were cultured on a Formvar substrate, and since they generally are less than 2 µm thick, the electron beam does not only excite the cells but also the underlying substrate. The results for these specimens can therefore only be expressed as elemental ratios.

Cells and tissues of CF patients

Cultured fibroblasts are frequently used in the study of CF. In part, this is due to the relative ease with which this material can be obtained and investigated. Practical and ethical constraints limit the use of other, more relevant tissues of CF for investigation, whereas fibroblasts can easily be obtained from small skin biopsies. Cultured cells are well suited for biochemical studies, but it is difficult to culture exocrine cells, which would be the first choice in research on CF. Nevertheless, the study of cultured fibroblasts is not irrelevant to the study of CF. Although fibroblasts are not exocrine cells, they are secretory cells, and the secretory process in fibroblasts resembles that in exocrine glands. A number of biochemical abnormalities has also been demonstrated in cultured CF fibroblasts (e.g., Bardoń et al. 1984). Our X-ray microanalysis studies showed that CF fibroblasts had a relative calcium content about twice as high as that of fibroblasts from controls (Roomans et al. 1981; Ceder et al. 1982; Ceder and Roomans 1983). In Fig 2 the data for calcium are plotted relative to the sulfur concentration, which may be taken as a measure for the amount of protein. The choice of reference element is rather arbitrary, and phosphorus or potassium as reference elements (the two highest peaks in the spectrum) give a similar result. Despite the fact that the difference between CF and control cells is clearly significant, the data for CF and control cells overlap. At present, analysis of fibroblasts would not be suitable for diagnostic purposes. The spread of the data is to an appreciable extent caused by counting statistics and the fact that the contribution of the substrate to the spectrum is relatively large. Culturing the cells on very thin films, although technically more difficult, would probably give a substantial improvement.

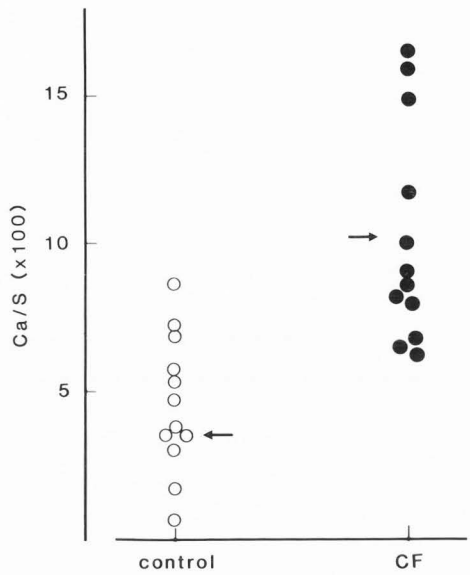

Fig 2. Relative calcium concentrations (Ca/S in %) in cultured fibroblasts from 12 controls (C) and 12 CF-patients, determined by X-ray microanalysis (Roomans et al. 1981; Ceder et al. 1982). Each point is the mean of 8-12 measurements. The mean for both groups is indicated by arrows.

The high calcium levels in CF fibroblasts confirm findings from the laboratory of Shapiro (Feigal and Shapiro 1979; Shapiro and Lam 1982), where calcium was analyzed with other techniques. In addition, we found that CF fibroblasts had a relatively low Na level (Roomans et al. 1981, 1983). Whether this is due to an intrinsic lower Na level, or to an increased loss of Na during the rinsing procedure preceding the freezing is difficult to know.

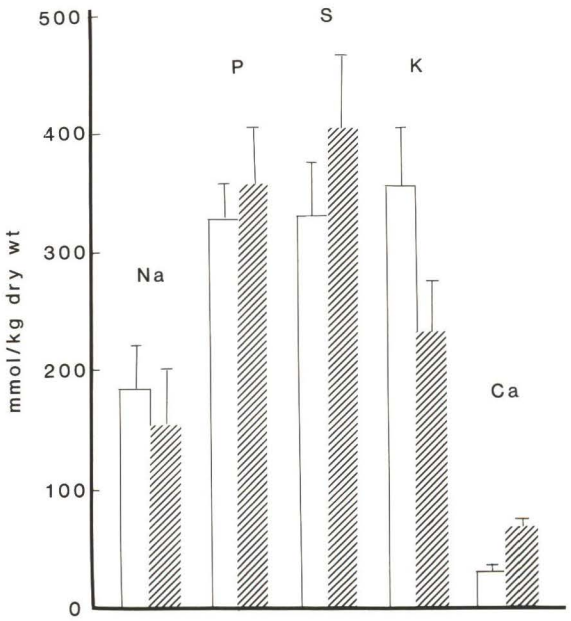

Fig 3. Elemental concentrations in bronchial goblet cells of three CF patients (hatched bars) and three controls (chronic bronchitis) (open bars). Thin bars indicate standard errors (n=3).

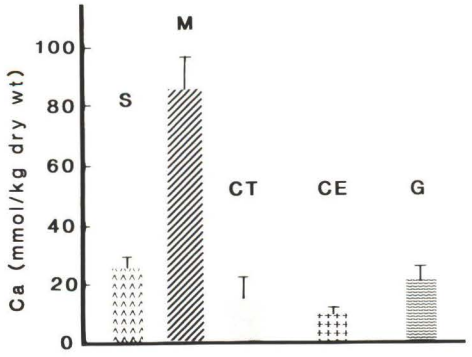

Fig 4: Ca concentrations in the serous cells of submucosal glands (S), mucous cells of submucosal glands (M), connective tissue (CT), ciliated epithelial cells (CE) and goblet cells (G) of a bronchial biopsy of a control (chronic bronchitis). Thin bars indicate standard errors (8-12 measurements per cell type).

X-ray microanalysis of goblet cells in bronchial epithelia of CF patients indicated that in CF patients the secretory granules of these cells had a rather high calcium content; in addition, a somewhat elevated sulfur content was noted (Fig 3) (Roomans et al. 1985). In order to avoid as much as possible secondary effects due to airway infections, patients with chronic bronchitis were selected as controls.

The elevated calcium concentrations in the secretory granules of the goblet cells could be a contributing factor to a higher calcium content of respiratory tract mucus. However, goblet cells are not the only cells secreting calcium into the respiratory tract. Detailed analysis of a control biopsy showed that the mucus cells of the submucosal gland have a much higher calcium content (Fig 4); this calcium is likely to be bound to mucus to be released into the airways. Since little is known about the relative contribution of various calcium sources to the final calcium content of respiratory tract mucus, the finding of elevated calcium levels in CF goblet cells must be interpreted with caution. It is tempting to correlate the finding of higher sulfur levels in CF goblet cells with reports on a higher degree of sulfatation of CF airway mucus (Boat et al. 1974). However, also in this case, caution must be exercised.

Both in fibroblasts and in goblet cells, the increase in cellular calcium levels must reflect an increase in bound calcium, since this forms the bulk of the cellular calcium. Conclusions about free calcium concentrations cannot be made on the basis of X-ray microanalytical measurements. In both cell types, calcium is mainly localized in the secretory granules. Increased calcium binding to the secretory proteins can be the result of compositional changes increasing the calcium-binding capacity of the proteins. It can also result from an increased uptake of calcium into the secretory compartment, possibly to protect the cell against an uncontrolled increase of cytoplasmic calcium levels.

Effects of the 'CF-factor' on ion transport

Circulating factors have for a long time been associated with CF, but no definite proof of their involvement in the disease has ever been obtained. Although progress is being made in the isolation of the factor (Bogart et al. 1984), its chemical identity is yet unknown. It is even possible that there exist not one but several 'CF-factors'.

Spock et al. (1967) reported the presence in serum of CF patients of a factor with ciliostatic properties. A number of laboratories has been able to reproduce this finding (although other laboratories have not) but the mechanism of the inhibition remains unclear. Possibly, the inhibition of ciliary motility is a secondary effect following hyperproduction of mucus (e.g., Kennedy and Allen 1980). The factor is not only found in serum, but also in urine of CF patients, and can be produced by CF fibroblast cultures. Mangos et al. (1967) reported the presence of a sodium transport inhibitory factor in the saliva of CF patients, but this finding has been difficult to reproduce by others.

Recently, however, other studies have linked the 'CF-factor' with abnormalities in ion transport. Bogart et al. (1982) reported that the 'CF-factor' induced potassium efflux from rat submandibular gland slices in vitro, and proposed a theory explaining this and other effects of the 'CF-factor' by the induction of an influx of calcium into the cells (Bogart et al. 1978, 1982).

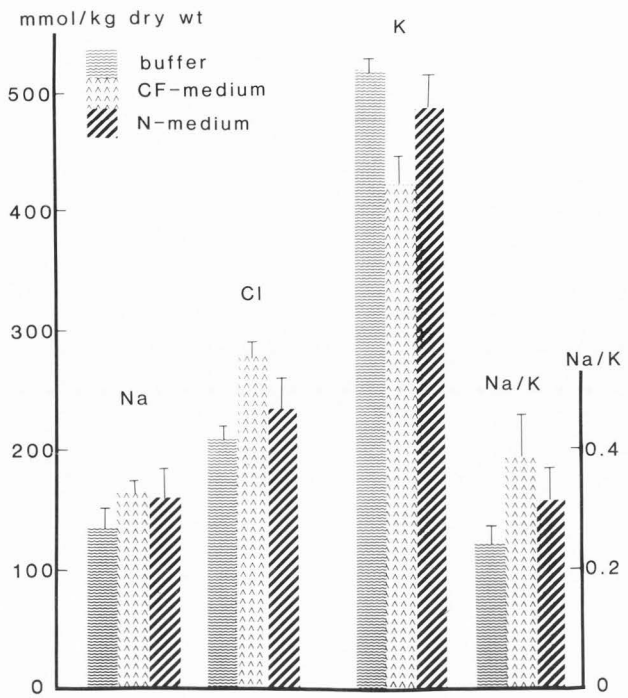

Fig 5: Effect of culture medium from CF fibroblasts (CF medium) or normal fibroblasts (N medium) on the elemental composition of rat submandibular acinar gland cells. Gland slices were incubated in buffer or in culture medium as described by von Euler et al. (1983). Incubation in culture medium causes an increase in cellular Na and Cl concentrations, a decrease in K concentrations and an increase in the Na/K ratio. The effect of CF medium is in all cases more pronounced than that of normal medium.

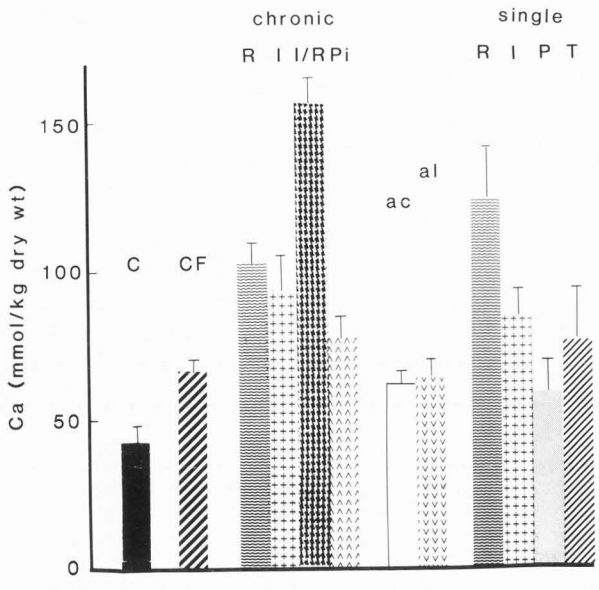

Fig 6: Calcium concentrations in mucous acinar cells of rat submandibular gland: (C) control, (CF) after chronic treatment with CF fibroblast medium (von Euler et al. 1985); chronic (R): after chronic reserpine treatment (Roomans et al. 1982b), (I) after chronic isoproterenol treatment (Müller and Roomans 1984a), (I/R) after chronic isoproterenol treatment combined with chronic reserpine treatment (Müller and Roomans 1984a), (Pi) after chronic pilocarpine treatment (Müller et al. 1985b); (ac) acidosis, (al) alkalosis; single: 24h after a single injection of reserpine (R), isoproterenol (I), prenalterol (P) and terbutaline (T) (Müller and Roomans 1985b; Müller et al. 1985a). The effect of the β_1-agonist prenalterol and the β_2-agonist terbutaline are qualitatively similar to those of isoproterenol, but less pronounced.

Using X-ray microanalysis, we could confirm the finding that the 'CF-factor' induced loss of potassium from acinar cells of rat submandibular gland slices incubated in CF serum or in culture medium from CF fibroblasts (von Euler et al. 1983). In addition, an increase in sodium and chloride concentrations was noted (Fig 5).

In subsequent studies it was noted that long-term exposure of normal fibroblasts to culture medium from CF fibroblasts resulted in an increase of the calcium concentration in the normal cells to about the value found in CF cells (Ceder and Roomans 1983). Chronic treatment (7 days) of rats with intraperitoneal injections of CF fibroblast medium also resulted in an increase of the cellular calcium concentration in the acinar cells of the submandibular gland (Fig 6).

Although these studies indicate that the 'CF-factor' indeed may induce an influx of calcium into sensitive cells, the mechanism of action of the 'CF-factor' remained unclear. In our first study, we attempted to distinguish between an 'agonist-like' effect of the 'CF-factor' and a 'membrane damage' effect, by comparing the effects of the 'CF-factor' with those of secretory agonists known to act via an increase in intracellular calcium concentration and those of an uncoupler (von Euler et al. 1983). In both cases ('agonist' and 'damage') one finds an increased sodium and decreased potassium concentration, resulting in an increased Na/K ratio. However, in a number of studies (von Euler et al. 1983, 1985; Roomans 1984; Roomans and Wei 1985; Wroblewski et al. 1983) we have found that cholinergic or α-adrenergic stimulation of various rat exocrine glands in vitro does not cause a significant increase of the cellular chloride concentrations over the time span used in these experiments. On the other hand, the effect of that 'CF-factor' resembles the uncoupler 2,4-dinitrophenol (von Euler et al. 1983) and the inhibitor of the Na-K-ATPase, ouabain (Kuijpers 1984) in that it causes an increase in the cellular chloride concentration.

Recently, von Euler and Wallace (1985) showed that the 'CF-factor' did not affect Ca-ATPase or the phosphatidylinositol metabolism (by which it would have affected calcium transport in a direct way), but that it inhibited

Na-K-ATPase activity in rat submandibular gland acinar cell membranes. As argued above, an 'ouabain-like' activity of the 'CF-factor' is quite compatible with the results of von Euler et al. 1983). Also a further consideration of the effects of ouabain is of interest: ouabain indirectly causes an increase of the cellular calcium concentration also in exocrine cells (Kuijpers 1984), which would bring the effect of the 'CF-factor' as a Na-K-ATPase inhibitor in line with the theory of Bogart et al. (1978, 1982). In addition, ouabain directly influences exocrine secretion: it reduces the secretion volume in the pancreas (Jansen 1980) and increases the chloride concentration in sweat (Quinton 1981). Furthermore, circulating inhibitors of Na-K-ATPase are known to occur in patients with essential hypertension (Hamlyn et al. 1982) but also in others (Crabos et al. 1984). On the other hand, it seems difficult to believe that a generalized inhibition of Na-K-ATPase could be a direct cause of the clinical symptoms associated with CF. CF patients do not suffer from hypertension and prolonged treatment with cardiac glycosides does not produce CF-related symptoms. A final conclusion about the relevance of the 'CF-factor' for the disease will therefore have to await further research.

Animal models for CF

Some tissues of interest cannot be studied directly in CF patients, either because biopsies cannot be obtained (major salivary glands) or because the tissue has undergone extensive secondary changes (pancreas). Based upon experience with other diseases where artificial models have provided useful information, attempts have been made to develop an animal model for CF. Even though the extent of its relevance is still a matter of discussion, the chronically reserpinized rat (Martinez et al. 1975a,b) appears to come as close as presently possible to being an animal model for CF. Other suggested animal models are the chronically isoproterenol-treated rat and the chronically pilocarpine-treated rat (Sturgess and Reid 1973; Mangos et al. 1981).

In the chronically reserpinized rat, the salivary gland ducts are dilated and at least partially obstructed by secretory material; the saliva has abnormally high concentrations of Na, Ca and protein (Martinez et al. 1975a,b). Reserpine is a noradrenaline depleter (Benmiloud and von Euler 1963) and is used as an anti-hypertensive drug. It has, however, a number of side effects that may be unrelated to its effects on the nervous system, and the mechanism by which chronic reserpine treatment produces CF-like changes in some of the exocrine glands of the rat is not known. In an attempt to clarify the mechanism behind the changes in exocrine glands caused by chronic treatment with reserpine, X-ray microanalysis was carried out on tissue of animals treated chronically with reserpine or with a single injection of this drug.

Chronic treatment with reserpine causes a marked increase in the calcium concentration of the acinar cells of the submandibular gland (Fig 6). This increase is caused by two factors: (1) an increase in the relative volume of cellular mucus, and (2) an increase in the local calcium content of the mucus (Roomans et al. 1982b; Müller and Roomans 1985a,b). In the submandibular acinar cell, the mucus granules have a calcium concentration much higher than that of the other cell compartments. Indeed, the calcium content of the cell is practically linearly related to the relative volume of the cellular mucus. If this volume increases, the cellular calcium level will increase even if the calcium content of the mucus *per se* does not increase. In the chronically reserpinized rat, this effect is strengthened by an increase in the local calcium content of the mucus - which can be shown in two ways: either by analysis of single mucus globules (Roomans et al. 1982b) or by calculating whether the increase in calcium content exceeds the increase in calcium based on the increase in relative mucus volume only (Fig 7).

A single injection with reserpine causes, over a 24h period, after an initial lag of about 4h, an increase in both cellular calcium level and relative cellular mucus content (Fig 8); although these increases are parallel, it can be shown that the increase in calcium content exceeds that expected on the basis of mucus increase alone (Fig 7); this is further confirmed by high-resolution analysis of mucus globules, which shows a more than three-fold increase in local calcium concentration (Müller et al. 1985a).

The causes of the increase in relative mucus content of the cells may be an inhibition of mucus extrusion. After an injection of the animal with reserpine, the responsiveness of the submandibular gland to β-adrenergic stimulation is decreased (Müller and Roomans 1985a,b) despite the fact that the number of β-receptors increases as a consequence of reserpine treatment (Cutler et al. 1981). The block may either lay in stimulus-secretion coupling or in the process of exocytosis.

Also the reason for the increase in local calcium concentration of the mucus granules remains to be clarified. The two possible explanations that come to mind are (1) increased calcium binding capacity of the mucus, and (2) increased uptake of calcium over the membrane of the granules, possibly as a protective mechanism to prevent unacceptably high calcium concentrations in the cytoplasm.

In this respect it is of interest that in contrast to the situation in the submandibular gland, the calcium concentration in the acinar cells of the parotid gland decreases in chronically reserpinized rats and chronically isoproterenol-treated rats (Fig 9) because of the formation of abnormal zymogen granules with a very low calcium content (Müller and Roomans 1984b). This may point to the possibility that the calcium-binding properties of the secretory granules are the factor determining the calcium content of the cell. However, although changes in chemical composition of mucus in reserpinized rats have been demonstrated (Mawhinney 1985a,b) direct proof of its increased calcium-binding capacity remains to be given.

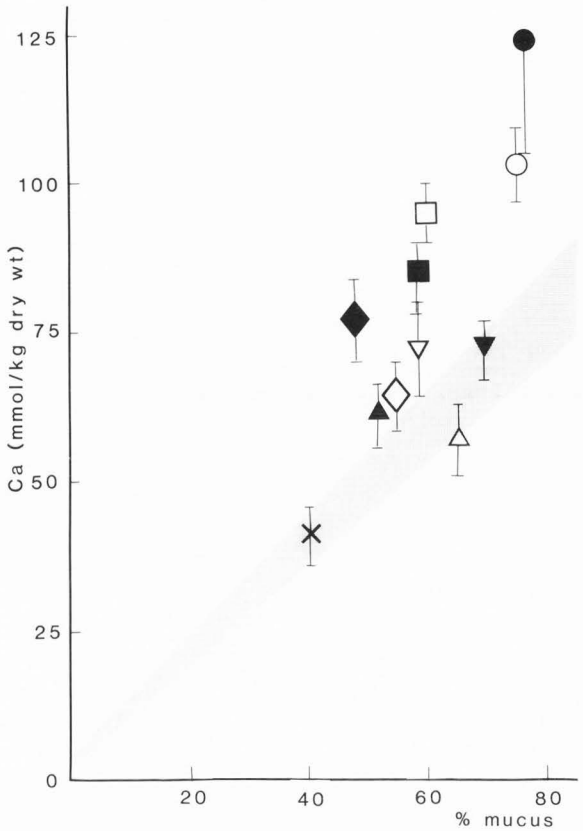

Fig 7: Plot of the cellular Ca concentration in acinar cells of rat submandibular glands (determined by X-ray microanalysis) versus the relative cellular mucus content (determined by morphometry on electron micrographs): control (×), acidosis (▲), alkalosis (◇), chronic vitamin D treatment (△), calcium gluconate (▽), calcitonin, 6h after a single dose (▼), chronic reserpine (○), chronic isoproterenol (□), chronic pilocarpine (◆), and 24h after a single injection with reserpine (●) or isoproterenol (■). The shaded area corresponds to the value expected if the calcium-binding properties of the mucus remain unchanged. The value is based on the value for the control gland, and the value for acinar cells that have been completely depleted of mucus by an injection with isoproterenol; an estimated error of 10% over the whole range of calcium concentrations is taken into account. Standard errors of actual data are indicated by thin bars. If a point is positioned above the shaded area, this indicates an increase in calcium concentration exceeding that expected on the basis of increased mucus content alone.

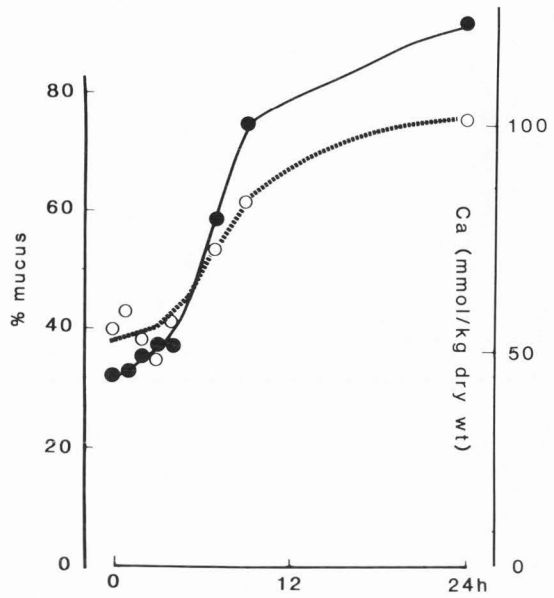

Fig 8: Changes in calcium concentration (●) and relative mucus content (○) of acinar cells of rat submandibular gland in the 24h-period following administration of reserpine (Müller et al. 1985a).

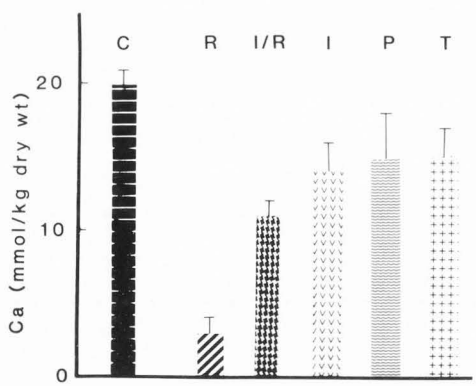

Fig 9: Calcium concentration in the acinar cells of rat parotid gland. (C) control, (R) after chronic reserpine treatment, (I/R) after combined chronic treatment with reserpine and isoproterenol, (I) after chronic isoproterenol treatment, (P) after chronic prenalterol treatment, (T) after chronic terbutaline treatment.

Also in chronically isoproterenol treated rats, the cellular calcium concentration in submandibular gland acinar cells is increased (Fig 6) due to an increase in the relative mucus content of the cells and an increased local calcium concentration in the mucus globules (Müller and Roomans 1984a, 1985b). After a single dose of isoproterenol, the cells are completely depleted of mucus, but after about 8h intracellular mucus is again present, and after that both the intracellular mucus and the intracellular calcium concentration increase to levels exceeding that of the control. The accumulation of intracellular mucus can readily be explained by desensitization of β-receptors after maximal stimulation by isoproterenol. Fig 7 shows that the newly formed

mucus is more calcium-rich than the originally present 'normal' mucus. Chronic pilocarpine stimulation (Müller et al, 1985b) gives qualitatively similar results (Figs 6 and 7).

Chronic reserpine treatment also leads to changes in activity of several enzymes of the glycolytic pathway; accumulation of phosphoenolpyruvate occurs, leading to acidosis in the acinar cells of the submandibular gland (Bardoń et al. 1985). Metabolic acidosis in rats can also be induced by exchanging the drinking water for a dilute solution of ammonium chloride during a period of a week. In these acidotic animals, the acinar cells of the submandibular gland contained more calcium than normal (Roomans and Bardoń 1984) (Fig 6) but the increase can completely be explained by the concurrent increase in the relative volume of intracellular mucus (Fig 7); the calcium-binding properties of the mucus thus were unaffected by the acidosis. Similar results were obtained in rats made alkalotic by exchanging the drinking water for 100 mM $NaHCO_3$ during a period of two weeks (Figs 6,7) (Roomans and Bardoń, unpublished results). The conclusion is that changes in cell pH, whether in the acidic or the alkaline direction, may inhibit the extrusion of mucus, but do not lead to increased calcium-binding by the mucus. The increased calcium concentration of the mucus in reserpinized rats can therefore not be explained by the reserpine-induced metabolic acidosis.

Regulation of serum calcium levels

Although serum calcium levels are reportedly normal in CF patients (Katz et al. 1985), a number of findings prompted us to have a closer look at the relation between the regulation of serum calcium levels and the functioning of the exocrine glands. Elevated calcium levels in saliva caused by or associated with hypercalcaemia have been reported (de Beer and Wilson 1932; Freeman and Welt 1965; Weinberger et al. 1974). In an *in vitro* preparation of human sweat glands, Prompt and Quinton (1978) showed that an increased calcium concentration in the bath (10 mM against normally 2.5 mM) resulted in a decreased rate of secretion, increased sweat calcium concentration, and a decrease in ductal reabsorption of sodium.

Acute hypercalcaemia in man can be experimentally provoked under controlled circumstances by calcium infusion with continuous control of the blood calcium level (Tørring and Sjöberg 1983). By such a 'calcium clamp' the free Ca^{2+} concentration in the blood can be raised by about 20% for a period up to several hours. However, no significant effect of the 'calcium clamp' on the concentration of calcium and other ions in parotid saliva, mixed saliva or sweat could be detected (Table 1) (Sagulin et al. 1985a, 1986). In rats, acute hypercalcaemia can be produced by an injection with a calcium salt solution. After two injections with calcium gluconate (35 mg/kg body weight), given with a 2h interval, the free Ca^{2+} concentration in the blood had increased from the control value of 1.33 mM to 1.55 mM. X-ray microanalysis of the submandibular gland showed an increase in the calcium concentration of the acinar cells, whereas morphometry showed an increase in the relative mucus content of these cells. According to Fig 7, the increase in calcium levels is not significantly higher than what would be expected on the basis of the increase in cellular mucus, and this would be consistent with an unchanged salivary calcium concentration, as also observed in the experiments on human volunteers.

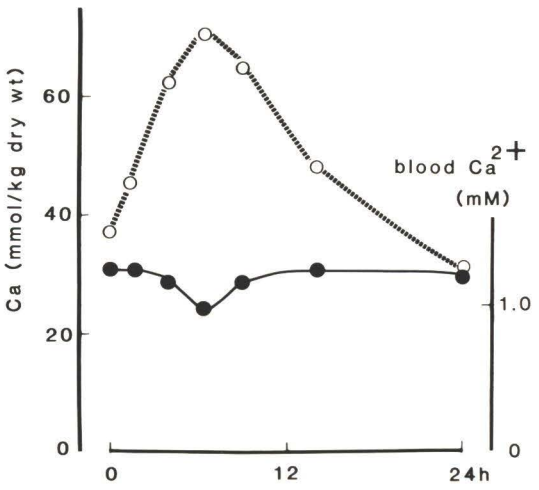

Fig 10: Effect of calcitonin on the calcium concentration in the acinar cells of rat submandibular gland (O) and on the level of ionized calcium in whole blood (●) during a 24h period (Sagulin et al. 1985b).

One method to produce chronic hypercalcaemia in experimental animals is by treatment with vitamin D or its metabolites. Rats injected for a period of 5 days with 1α-vitamin D3 (50,000 U/kg body weight) showed an increase in blood Ca^{2+} to 1.87 mM (versus 1.33 mM in the controls). X-ray microanalysis and morphometry of submandibular gland acinar cells showed an increase in cellular calcium concentration consistent with the increase in the concentration of (normal) intracellular mucus (Fig 7) (Sagulin et al. 1985c). In this respect, chronic hypercalcaemia does not appear to differ from acute hypercalcaemia.

The calciotropic hormone calcitonin has been implicated in cystic fibrosis (Kilbourn 1984). Hostyn and Hruskovic (1976) reported that the calcitonin-producing C-cells of the thyroid gland in CF patients appeared necrotic, which would suggest an abnormally low level of this hormone. On the other hand, high calcitonin levels have been found in patients with inflammatory lung diseases, due to involvement of the calcitonin-secreting Kulchitsky cells (K-cells) in the lung (Becker et al. 1981). Serum calcitonin levels and the frequency of K-cells in the lung of CF patients are now being investigated, and the results of this investigation should give a clue as to whether this hormone could be involved in CF.

It is however of interest to note, that

Table 1

Effect of a 'calcium clamp' on ionic composition of parotid saliva and sweat in man

	parotid saliva				sweat			
	norm		hyp		norm		hyp	
Na	15±	4	12±	3	49±	8	51±	6
Cl					38±	7	36±	5
K	24±	2	26±	2				
Ca	0.70±0.04		0.72±0.04		4.5±1.3		3.9±1.1	

A one-hour 'calcium clamp' was performed on healthy human volunteers (Sagulin et al. 1986; Tørring and Sjöberg 1983). The data give mean and standard error of 7-9 persons; all data in mM. No significant difference between normocalcaemic (norm) and hypercalcaemic (hyp) conditions could be noted.

calcitonin affects the exocrine glands: it modulates pancreatic secretion, gastric secretion and salivary gland secretion (Schmidt et al. 1971; Morley et al. 1981; Koelz et al. 1976). Injecting rats with calcitonin (salmon calcitonin, 25 IU/kg body weight) caused a transient increase in the calcium content of the submandibular gland acinar cells. The maximum effect is noted after about 6h, at which moment also the effect on serum calcium is maximal (Fig 10) (Sagulin et al. 1985b). The increase in cellular calcium concentration is paralleled by an increase in the relative mucus content of the cell (Fig 7), and preliminary data on the composition of submandibular saliva indeed indicate that its calcium content is not significantly affected by calcitonin. As previously discussed for reserpine and isoproterenol, the calcitonin-induced accumulation of intracellular mucus is due to an inhibition of mucus extrusion (Sagulin et al. 1985b). It is likely that this is a direct effect of calcitonin rather than an effect via the very minor change in serum calcium levels.

Conclusions

The experimental studies summarized in this review point to two separate mechanisms by which the calcium concentration of the acinar cells of the submandibular gland can be increased: (a) accumulation of intracellular mucus, and (b) increased calcium binding by intracellular mucus. The available evidence, obtained by a variety of methods (X-ray microanalysis at the cellular level supplemented by morphometry, X-ray microanalysis at the subcellular level, and chemical analysis of the ion content of the secreted fluid) points to the following conclusion:

In the case of hypercalcaemia (acute or chronic), calcitonin injection, acidosis or alkalosis only mechanism (a) is operative, whereas in the various 'animal models' of CF both (a) and (b) are operative. In the case of chronic treatment with the 'CF-factor' apparently only mechanism (b) is operative. Mucus accumulation is associated with an inhibition of secretion.

Mechanism (b) will result in an abnormal composition of the primary secreted fluid and probably also of the final secretion. Mechanism (a) will not generally lead to increased calcium levels in the secretory fluid, despite the fact that the total calcium content of the gland and of many glandular cells may be increased.

The mechanism behind the increased calcium binding by intracellular mucus is not completely clear. However, the available evidence suggests that this increase is due to changes in the secretory macromolecules rather than in calcium transport in the acinar cells. Otherwise, the difference in behaviour of submandibular and parotid gland cells in the chronically reserpinized and the chronically isoproterenol-treated rat is totally unexplainable. This does not mean that calcium transport in the acinar cells of these glands is not affected at all: Müller and Martinez (1986) showed for the chronically reserpinized rat that both influx and efflux of calcium in submandibular acini is abnormal. However, this abnormalty appears to be secondary to other changes in the cell.

In summary, three lines of evidence fail to confirm a primary role for calcium in abnormal exocrine gland function as observed in CF:

(1) the effects of the 'CF-factor' appear to be primarily on the transport of other ions: the effects on calcium are secondary,

(2) the experiments involving animal models of CF suggest that changes in calcium content of intracellular or secreted mucus are secondary to changes in secretory macromolecules,

(3) changes in serum calcium levels or in the serum levels of calciotropic hormones do not seem to affect the ionic composition of the fluid secreted by exocrine glands, although these factors may affect gland function.

Despite the fact that no support could be obtained for a primary role of calcium in CF, the secondary effects of increased calcium levels in the secreted fluids may still be of vital importance for the clinical problems associated with the disease.

Acknowledgements

The author gratefully acknowledges the cooperation of Gun-Britt Sagulin and Margareta Müller in the studies covered by this review. Tissue and cell cultures from CF patients were obtained through the kind collaboration of the Department of Pediatrics at Umeå University Hospital (Dr Hans Kollberg and Dr Ove Ceder) and the Department of Pulmonary Diseases at Huddinge Hospital (Dr Hans Gilljam). This research was financially supported by the Cystic Fibrosis Research Trust, the Swedish National Association against Heart and Chest Diseases,

the Swedish Association for Cystic Fibrosis and the Swedish Medical Research Council, and by instrumentation grants from the Wallenberg Foundation and the Swedish Natural Science Research Council.

References

Aparicio SR, Marsden P (1969) A rapid methylene blue-basic fuchsin stain for semi-thin sections of peripheral nerve and other tissues. J Micr 89, 139-141.

Bardoń A, Ceder O, Kollberg H (1984) Activities of some glycolytic enzymes in cultured fibroblasts from CF homozygotes, heterozygotes and normal controls. In: Cystic Fibrosis: Horizons. D Lawson (ed), J Wiley & Sons, Chichester, p 413.

Bardoń A, Müller RM, Ceder O, Roomans GM (1985) The chronically reserpinized rat. Decreased glycolytic activity in the submandibular gland. Biochem Med 33, 99-103.

Baur PS, Bolton WE, Barranco SC (1976) Electron microscopy and microchemical analysis of cystic fibrosis diploid fibroblasts in vitro. Tex Rep Biol Med 34, 113-134.

Becker KL, Nash D, Silva OL, Snider RH, Moore CF (1981) Increased serum and urinary calcitonin levels in patients with pulmonary disease. Chest 79, 211-216.

de Beer EJ, Wilson DW (1932) The inorganic composition of the parotid saliva of the dog and its relation to the compostion of the serum. J Biol Chem 95, 671-685.

Benmiloud M, von Euler US (1963) Effects of bretylium, reserpine, guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland. Acta Physiol Scand 59, 39-42.

Bijman J, Quinton PM (1984) Influence of abnormal Cl^- impermeability on sweating in cystic fibrosis. Am J Physiol 247 (Cell Physiol 16), C3-C9.

Boat TF, Kleinerman JI, Carlson DM, Maloney WH, Matthews LW (1974) Human respiratory tract secretions. I. Mucous glycoproteins secreted by cultured nasal polyp epithelium from subjects with allergic rhinitis and with cystic fibrosis. Am Rev Resp Dis 110, 428-441.

Bogart BI, Conod EJ, Gaerlan PF, Conover J (1978) The biological activities of cystic fibrosis serum. II. Ultrastructural aspects of the effect of cystic fibrosis sera and calcium ionophore A23187 on rabbit tracheal explants. Pediatr Res 12, 15-24.

Bogart BI, Picarelli J, Gaerlan PF, Denning CR (1982) Biological activities of cystic fibrosis serum. IV. Stimulation of the calcium mediated K^+ efflux from rat submandibular gland fragments. Pediatr Res 16, 223-226.

Bogart BI, Taylor T, Gaerlan PF, Denning CR (1984) Fractionation and partial characterization of CF serum ultrafiltrates. In: Cystic Fibrosis: Horizons. D Lawson (ed), John Wiley & Sons, Chichester, p 428.

Boucher RC, Knowles MR, Cotton C, Jackson Stutts M, Yankaskas JM, Gatzy JT (1984) On a unified theory of cystic fibrosis lung disease. In: Cystic Fibrosis: Horizons. D Lawson (ed), John Wiley & Sons, Chichester, pp 167-177.

Cabrini G, De Togni P (1985) Increased cytosolic calcium in cystic fibrosis neutrophils effect on stimulus-secretion coupling. Life Sci 36, 1561-1567.

Ceder O, Roomans GM (1983) Effects of culture medium on cystic fibrosis and normal fibroblasts studied by X-ray microanalysis. Ultrastruct Pathol 4, 305-309.

Ceder O, Roomans GM, Hösli P (1982) Increased calcium content in cultured fibroblasts from trisomy patients - comparison with cystic fibrosis fibroblasts. Scanning Electron Microsc 1982; II: 723-730.

Crabos M, Wainer IW, Cloix JF (1984) Measurement of endogenous Na^+, K^+-ATPase inhibitors in human plasma and urine using high-performance liquid chromatography. FEBS Letters, 176, 223-228.

Cutler LS, Boccuzzi J, Bottaro B, Christian CP, Martinez JR (1981) Effects of reserpine treatment on β-adrenergic / adenylate cyclase modulated secretion and resynthesis by the rat submandbular gland. Virchows Arch (Pathol Anat) 329: 185-198.

Doughney C, Dormer RL, McPherson MA (1985) Autonomic regulation of phosphatidylinositol (PI) breakdown in rat submandibular acinar cells. In: Proc 13th Annual EWGCF meeting Jerusalem, Israel, p 67. (Isr Med J, in press).

von Euler AM, Wallace MA (1985) Conditioned media from cultured cystic fibrosis fibroblasts inhibits Na/K ATPase activity. Life Sci 37, 2399-2407.

von Euler AM, Müller RM, Ceder O, Roomans GM (1983) Effects of cystic fibrosis serum and fibroblast culture medium on ion distribution in rat submandibular gland. Ultrastruct Pathol 5, 37-44.

von Euler AM, Ceder O, Kollberg H, Roomans GM (1985) Effect of chronic treatment with cystic fibrosis fibroblast medium on rat submandibular gland acinar cells. Exp Mol Pathol 43, 142-149.

Feigal RJ, Shapiro BL (1979) Altered intracellular calcium in fibroblasts from patients with cystic fibrosis and heterozygotes. Pediatr Res 13, 764-768.

Freeman RM, Welt LG (1965) Parotid fluid calcium and phosphate levels in patients with hypercalcemia. Proc Soc Exp Biol Med 120, 627-630.

Grinstein S, Elder B, Clarke CA, Buchwald M (1984) Is cytoplasmic Ca^{2+} in lymphocytes elevated in cystic fibrosis? Biochim Biophys Acta 769, 270-274.

Hamlyn JM, Ringel R, Schaeffer J, Levinson PD, Hamilton BP, Kowarski AA, Blaustein MP (1982) A circulating inhibitor of $(Na^+ + K^+)$ATPase associated with essential hypertension. Nature 300, 650-652.

Hostyn L, Hruskovic I (1976) Cysticka fibroza a bunky C stitnej zl'azy (Cystic fibrosis and C cells of thyroid glands). Cas Lék ces 115, 930-931.

Izutsu K, Johnson D, Schubert M, Wang E, Ramsey B, Tamarin A, Truelove E, Ensign W, Young M (1985) Electron microprobe analysis of human labial gland secretory granules in cystic fibrosis. J Clin Invest 75, 1951-1956.

Jansen JWCM (1980) Role of the paracellular pathway in pancreatic fluid secretion. Doctoral thesis, University of Nijmegen, The Netherlands, pp 45-58.

Katz S, Schöni MH, Bridges MA (1985) The calcium hypothesis of cystic fibrosis. Cell Calcium 5, 421-440.

Kennedy JR, Allen PL (1980) Cystic fibrosis serum effects on rabbit tracheal epithelium: an ultrastructural analysis. Pediatr Res 14, 1173-1179.

Kilbourn JP (1984) Composition of sputum from patients with cystic fibrosis. Curr Microbiol 11, 19-22.

Knowles M, Gatzy J, Boucher R (1983a) Relative ion permeability of normal and cystic fibrosis nasal epithelium. J Clin Invest 71, 1410-1417.

Knowles MR, Stutts MJ, Spock A, Fischer N, Gatzy JT, Boucher RC (1983b) Abnormal ion permeation through cystic fibrosis respiratory epithelium. Science 221, 1067-1070.

Knowlton RG, Cohen-Haguenauer O, Cong NV, Frézal J, Brown VA, Barker D, Braman JC, Schumm JW, Tsui L-C, Buchwald M, Donis-Keller H (1985) A polymorphic DNA marker linked to cystic fibrosis is located on chromosome 7. Nature 318, 380-382.

Koelz HR, Drack GT, Blum AL (1976) Calcitonin und Speicheldrüsen-Amylase-Sekretion *in vivo* und *in vitro*. Schweiz Med Wschr 106, 298-299.

Kollberg H, Bardoń A, Ceder O (1983) Cystic fibrosis, speculations on the pathogenesis. Acta Paediatr Scand Suppl 309, 41-47.

Kuijpers GAJ (1984) Transport mechanisms of pancreatic fluid and enzyme secretion. Doctoral thesis, University of Nijmegen, The Netherlands, pp 141-156.

Mangos JA, Donnelly WH (1981) Isolated parotid acinar cells from patients with cystic fibrosis. Morphology and composition. J Dent Res 60, 19-25.

Mangos JA, McSherry NR, Benke PJ (1967) A sodium transport inhibitory factor in the saliva of patients with cystic fibrosis of the pancreas. Pediatr Res 1, 436-442.

Mangos JA, Boyd RL, Bouchlas D, Farnham W (1981) Isolated parotid acinar cells from DL-isoproterenol treated rats. A cellular model for cystic fibrosis. J Dent Res 60, 80-85.

Martinez JR, Adelstein E, Quissel D, Barbero GJ (1975a) The chronically reserpinized rat as a possible model for cystic fibrosis. I. Submaxillary gland morphology and ultrastructure. Pediatr Res 9, 463-469.

Martinez JR, Adshead PC, Quissel DO, Barbero GJ (1975b) The chronically reserpinized rat as a possible model for cystic fibrosis. II. Composition and cilioinhibitory effects of submaxillary saliva. Pediatr Res 9, 470-475.

Mawhinney TP (1985a) Sublingual glycoproteins in control and reserpine-treated rats. In: Animal Models for Cystic Fibrosis: The Reserpine Treated Rat. JR Martinez, GJ Barbero (eds), San Francisco Press, San Francisco, pp 93-99.

Mawhinney TP (1985b) Effect of reserpine pretreatment on secretion of sulfated and sialylated glycoproteins by the rat trachea *in vivo* and *in vitro*. In: Animal Models for Cystic Fibrosis: The Reserpine Treated Rat. JR Martinez, GJ Barbero (eds), San Francisco Press, San Francisco, pp 125-137.

McPherson MA, Dormer RL, Al-Mutairy, Dodge JA, Goodchild MC (1985) Calmodulin-like activity in control and CF submandibular glands. In: Proc 13th Annual EWGCF meeting, Jerusalem, Israel, p 68. (Isr Med J, in press).

Morley JE, Levine AS, Silvis SE (1981) Intraventricular calcitonin inhibits gastric acid secretion. Science 214, 671-673.

Motta C, Garcia I, Touillon AM (1985) Lipids dynamics in cystic fibrosis fibroblasts. In: Proc 13th Annual EWGCF meeting, Jerusalem, Israel, p 82. (Isr Med J, in press).

Müller RM, Martinez JR (1986) Abnormal ^{45}Ca fluxes in dispersed submandibular acini of rats treated with reserpine. Acta Physiol Scand, in press.

Müller RM, Roomans GM (1984a) The chronically isoproterenol-treated rat in the study of cystic

fibrosis: X-ray microanalysis of the submandibular gland. Exp Mol Pathol 40, 391-400.

Müller RM, Roomans GM (1984b) X-ray microanalysis of the rat parotid gland after chronic sympathectomimetic stimulation. Exp Mol Pathol 41, 363-370.

Müller RM, Roomans GM (1985a) The reserpinized rat: X-ray microanalysis of salivary glands and pancreas. In: Animal Models for Cystic Fibrosis: The Reserpine Treated Rat. JR Martinez, GJ Barbero (eds), San Francisco Press, San Francisco, pp 51-62.

Müller RM, Roomans GM (1985b) X-ray microanalysis of exocrine glands in animal models for cystic fibrosis. Scanning Electron Microsc 1985; IV: 1583-1601.

Müller RM, Grundin TG, Roomans GM (1985a) Effects of reserpine and isoproterenol on elemental distribution in submandibular gland of rat. Acta Physiol Scand 123, 383-391.

Müller RM, Kuijpers GAJ, Bardoń A, Ceder O, Roomans GM (1985b) The chronically pilocarpine-treated rat in the study of cystic fibrosis: investigations on rat submandibular gland and pancreas. Exp Mol Pathol 43, 97-106.

Prompt CA, Quinton PM (1978) Functions of calcium in sweat secretion. Nature 272, 171-172.

Quinton PM (1981) Effects of some ion transport inhibitors on secretion and reabsorption in intact and perfused single human sweat glands. Pflügers Arch 391, 309-313.

Quinton PM (1983) Chloride impermeability in cystic fibrosis. Nature 301, 421-422.

Rogiers V, Crokaert L, Vis HL (1980) Altered phospholipid composition and changed fatty acid pattern of the various phospholipid fractions of red cell membranes of cystic fibrosis children with pancreatic insufficiency. Clin Chim Acta 105, 105-115.

Roomans GM (1980) Quantitative X-ray microanalysis of thin sections. In: X-ray microanalysis in biology. MA Hayat (ed). University Park Press, Baltimore, pp 401-453.

Roomans GM (1981) Quantitative electron probe X-ray microanalysis of biological bulk specimens. Scanning Electron Microsc 1981; II: 344-356.

Roomans GM (1984) X-ray microanalysis of rat exorbital lacrimal gland. Scanning Electron Microsc 1984; II: 889-895.

Roomans GM, Bardoń A (1984) Effect of metabolic acidosis on acinar cells of rat submandibular gland. Res Commun Chem Pathol Pharmacol 46, 155-158.

Roomans GM, Wei X (1985) X-ray microanalysis of resting and stimulated rat pancreas. Acta Physiol Scand 124, 353-359.

Roomans GM, Ceder O, Kollberg H (1981) Electrolyte redistribution in cystic fibrosis fibroblasts studied by electron probe X-ray microanalysis. Ultrastruct Pathol 2, 53-58.

Roomans GM, Wei X, Sevéus L (1982a) Cryoultramicrotomy as a preparative method for X-ray microanalysis in pathology. Ultrastruct Pathol 3, 65-84.

Roomans GM, Wei X, Ceder O, Kollberg H (1982b) The reserpinized rat in the study of cystic fibrosis - X-ray microanalysis of submandibular gland and pancreas. Ultrastruct Pathol 3, 285-293.

Roomans GM, von Euler AM, Müller RM (1983) Cystic fibrosis - ultrastructural and microanalytical studies. Scanning Electron Microsc 1983; II: 697-712.

Roomans GM, von Euler AM, Müller RM, Gilljam H (1985) X-ray microanalysis of goblet cells in bronchial epithelium of CF-patients. In: Proc 13th Annual EWGCF meeting, Jerusalem, Israel, p 77. (Isr Med J, in press).

Sagulin G-B, Tørring O, Sjöberg HE, Arver S, Appelgren B (1985a) Effects of a three-hour calcium clamp on calcium, phosphate, magnesium and zinc concentrations of human parotid saliva. Acta Physiol Scand 124, 1-4.

Sagulin G-B, Wroblewski J, Arver S, Roomans GM (1985b) Effects of calcitonin on elemental distribution and ultrastructure of rat submandibular gland acinar cells. Acta Physiol Scand 125, 649-654.

Sagulin G-B, Wroblewski J, Arver S, Roomans GM (1985c) Effects of calcitonin and hypercalcemia on rat submandibular gland acinar cells. In: Proc 13th Annual EWGCF meeting, Jerusalem, Israel, p 76. (Isr Med J, in press).

Sagulin G-B, Tørring O, Hellsing K, Roomans GM (1986) Saliva and sweat secretion in man during a one-hour calcium clamp. Acta Physiol Scand, in press.

Schmidt H, Hesch RD, Hüfner M, Paschen K, Creutzfeldt W (1971) Hemmung der exokrinen Pankreassekretion des Menschen durch Calcitonin. Dt Med Wschr 45, 1773-1774.

Schöni MH, Schöni-Affolter F, Jeffery D, Katz S (1985) Intracellular free calcium concentrations in mononuclear blood cells of patients with cystic fibrosis. In: Proc 13th Annual EWGCF meeting, Jerusalem, Israel, p. 65. (Isr Med J, in press).

Shapiro BL, Lam LFH (1982) Calcium and age in fibroblasts from control subjects and patients with cystic fibrosis. Science 216, 417-419.

Spock A, Heick HMC, Cress H, Logan WS (1967) Abnormal serum factor in patients with cystic fibrosis of the pancreas. Pediatr Res 1, 173-177.

Sturgess J, Reid L (1973) The effect of isoprenaline and pilocarpine on (a) bronchial mucus-secreting tissue and (b) pancreas, salivary glands, heart, thymus, liver and spleen. Brit J Exp Path 54, 388-403.

Tørring O, Sjöberg HE (1983) A calcium clamp technique in man. Acta Physiol Scand 119, 387-392.

Wainwright BJ, Scambler PJ, Schmidtke J, Watson EA, Law H-Y, Farrall M, Cooke HJ, Eiberg H, Williamson R (1985) Localization of cystic fibrosis locus to human chromosome 7cen-q22. Nature 318, 384-385.

Waller RL, Brattin WJ, Dearborn PG (1984) Cytosolic free calcium concentration and intracellular calcium distribution in lymphocytes from cystic fibrosis patients. Life Sci 35, 775781.

Weinberger A, Sperling O, De Vries A (1974) Calcium and inorganic phosphate in saliva of patients with primary hyperparathyroidism. Clin Chim Acta 50, 5-7.

White R, Woodward S, Leppert M, O'Connell P, Hoff M, Herbst J, Lalouel J-M, Dean M, Vande Woude G (1985) A closely linked genetic marker for cystic fibrosis. Nature 318, 382-384.

Wroblewski J, Müller RM, Wroblewski R, Roomans GM (1983) Quantitative X-ray microanalysis of semi-thick cryosections. Histochemistry 77, 447-463.

Wroblewski R, Roomans GM, Jansson E, Edström L (1978) Electron probe X-ray microanalysis of human muscle biopsies. Histochemistry 55, 281-292.

Discussion with Reviewers

B. Forslind: With respect to freezing of the samples, wouldn't propane dissolve lipids more than Freon?
Author: Although at room temperature both propane and Freon are lipid solvents, it is generally assumed that at typical temperatures for freezing no lipids are lost from the sample.

B. Forslind: In the model of the 'calcium theory' (Fig 1), couldn't there be decreased reabsorption of calcium in the ducts? Are the compositional alterations in membrane lipids primary or secondary to the effect of the mucus cover?
Author: Little is known about ductal modification of the calcium content of the primary secretion. It is assumed that there is either no (net) calcium transport in the duct, or, possibly, that duct cells may secrete calcium. Compositional alterations in membrane lipids are, according to Motta et al. (1985) present in fibroblasts, and would therefore not seem secondary to the effect of the mucus cover.

B.A. Afzelius: You mention that there is a possibility that Na is lost during the rinsing before X-ray microanalysis. Would it be possible that calcium also is lost, and even worse, be lost to different degrees in different cell types?
Author: The rinsing procedure (with cold ammonium acetate) aimed at replacing the culture medium by a volatile buffer containing no elements detectable by X-ray microanalysis. The procedure was checked as far as possible for ion loss (Ceder et al. 1982), and although we could not demonstrate any changes in Na/K ratio as a result of the rinsing, a minor loss of diffusible ions is not completely excluded by our investigations. With regard to calcium, however, our data receive independent support from studies of the group of Shapiro (Feigal and Shapiro 1979; Shapiro and Lam 1982) where only calcium was determined and rinsing with a more physiological buffer was carried out. We therefore conclude that it is unlikely that our calcium data are flawed.

B.A. Afzelius: In the study of human goblet cells you have used patients with chronic bronchitis as controls to the CF patients. How do these compare to healthy persons? Is there a relation between calcium levels and the degree of severity of the bronchitis or two species of bacteria in the mucus? In bronchitis it is commonly found that mucus secretion is increased.
Author: The samples were taken during bronchoscopy which was carried out as part of scheduled medical treatment of the patients. Since there is no need for bronchoscopy of healthy persons, bronchial biopsies from such persons are difficult to obtain. In addition, for this particular investigation, healthy persons may not be adequate as controls to find specific abnormalities related to cystic fibrosis. There have been several speculations about the importance of the increased calcium levels for the bacteria in the airways, e.g., that calcium would favour the occurrence of mucoid strains of Pseudomonas, or that it would inhibit the effect of some antibiotics. There are, however, no hard data to link calcium levels with severity of airway disease.

B.A. Afzelius: It is somewhat surprising to find that the secretory granules of submucosal glands have a calcium concentration that is more than four times higher than that of the goblet cells in the epithelium. Both cell types seem fairly alike. Do you have an explanation?
Author: The calcium content of secretory cells appears to be mainly determined by the chemical properties of the intracellular mucus. Subtle changes in those properties may result in markedly increased calcium levels, as is evident from our studies on the reserpinized rat.

A.B. Maunsbach: Would cultured sweat gland cells (Pedersen 1984) be a good system to study abnormal ion distribution in cystic fibrosis?
Author: Sweat glands have many advantages since these glands show a defect in ion transport regulation, but no secondary changes due to infection or necrosis. They also can be obtained with relatively little harm to the patients. In addition, the work of Pedersen (1984), that is now being repeated and extended in other laboratories, showed that it is possible to culture sweat gland cells, which is a prerequisite for many experimental studies that could not be performed *in vivo*. For X-ray microanalysis, sweat glands are, because of their relatively complicated architecture, not very easy, but gradually these technical problems are being resolved. Studies by X-ray microanalysis on sweat glands either *in vivo* or *in vitro* are being carried out or planned in a number of laboratories (e.g., Elder et al. 1985) and will doubtless become a fruitful area of research.

Å. Thureson-Klein: Have you investigated the effect of a β-antagonist on rat salivary glands?
Author: No, not yet, but this is evidently an experiment that is planned.

Additional References

Elder HY, Jenkinson DM, McWilliams SA, Wilson SM (1985) Intracellular concentrations of sodium, phosphorus, chlorine, and potassium in the ductal epithelium of unstimulated and active equine sweat glands. J Physiol *367*, 74P (abstract).

Pedersen PS (1984) Primary culture of epithelial cells derived from the reabsorptive coiled duct of human sweat glands. ICRS Med Sci *12*, 752-753.