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X-RAY MICROANALYSIS OF EPITHELIAL AND SECRETORY CELLS IN CULTURE

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Abstract

Cell cultures can be used to study ion transport processes. X-ray microanalysis of cell cultures at the cellular level gives interesting information that can complement electrophysiological and tracer studies. In this paper, methods for culturing and preparing a variety of epithelial and secretory cells (fibroblasts, insulinoma cells, bovine mammary epithelial cells, colon cancer cells) for X-ray microanalysis are presented. Results show that sometimes cell cultures are not homogeneous with respect to ion content or reaction to physiological stimuli. In colon cancer cell cultures, a "high K" and a "low K" cell subpopulation was found; these subpopulations also differed with respect to other elements. As examples of biological applications, chloride efflux was studied in fibroblasts and colon cancer cells, and strontium uptake in insulinoma cells. Chloride efflux from colon cancer cells is stimulated by cyclic AMP and vaso-active intestinal peptide (VIP), and can be inhibited by pretreatment of the cells with phorbol myristate acetate, which downregulates the cAMP-regulated chloride efflux mechanism.

Key Words: Cell culture, X-ray microanalysis, epithelial cells, fibroblasts, mammary gland, insulinoma, colon cancer, chloride transport, cystic fibrosis.

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Introduction

Cell cultures are commonly used to study a variety of problems in cell biology, physiology, and pathology. X-ray microanalysis allows the quantitative determination of the elemental content of the cells and can therefore be applied to studies of ion transport. In contrast to e.g., tracer studies and electrophysiological techniques which measure dynamic processes directly, X-ray microanalysis is a static technique. If several processes involving the same ion (element) occur between sampling, only the net sum of changes is detected. On the other hand, X-ray microanalysis allows simultaneous analysis of different ions (e.g., Na^+, K^+ and Cl^-) in one cell. X-ray microanalysis of thin sections allows determination of ion distribution at the subcellular level. Hence, X-ray microanalysis is a useful complementary technique in (patho)physiological studies of ion transport.

This paper is concerned with X-ray microanalysis of cultured cells at the cellular level. Analysis of freeze-dried, non-embedded, unsectioned cultured cells at the subcellular level is possible only when one is dealing with a cell type flat enough to allow visualization of individual organelles in the transmission (TEM) or scanning transmission (STEM) electron microscope. Analysis at the subcellular level carried out on thin sections of e.g., cell pellets, is very similar to analysis of thin sections of tissue in general. However, analysis of whole unsectioned cells at the cellular level may pose special methodological advantages and disadvantages.

Cells are cultured either on a solid substrate or on thin films. Culturing cells on a solid substrate is easier, but introduces two problems at the analytical stage: the substrate contributes to the continuum, decreasing the peak-to-background (P/B) ratio and making it more difficult to accurately measure elements occurring at low concentrations. It is also difficult to carry out absolute quantitative analysis of such cultured cells, since the continuum cannot be used as a measure for specimen mass. Both problems can be alleviated: the sensitivity

problem by analysis with a wavelength-dispersive system rather than an energy-dispersive system; the quantitation problem by use of the ratio method. In that case, it is assumed, that the concentration of the reference element, e.g., phosphorus, is a good indication of cell mass (Lechene, 1989) and is not affected by the experimental variables tested. Another assumption that can be made is that if the osmolality of the extracellular medium is not altered during the experiment, the ratio between two elements, e.g., Na and K may change while their sum is constant. Analysis of cells on thin films is more straightforward and can be carried out in the (scanning) transmission mode at high (100 kV and more) accelerating voltage. Quantitation techniques are the same as those used for thin sections and although the presence of extraneous continuum may constitute a problem, there are formalisms allowing fully quantitative analysis to be carried out (von Euler *et al.*, 1992). On the other hand, attachment of the cells to the thin films may constitute a problem and not all cells appear to grow easily on such substrates.

If the cells are not (embedded and) sectioned but freeze-dried, the culture medium has to be removed. If this is not done, remnants of the culture medium, containing a variety of elements, will after (freeze-)drying be present on top of and between the cells. Removal of the culture medium is generally carried out by rinsing the cells prior to freezing or drying. Several washing fluids have been used: (a) distilled water, (b) isotonic volatile buffers such as ammonium acetate, or (c) isotonic non-volatile solutions such as sucrose or mannitol. From a physiological point of view, the isotonic non-volatile solutions are to be preferred since they carry a minimal risk for osmotic effects. The drawback of this method is that remnants of the sucrose or mannitol cover the cells after (freeze-)drying. Often it is possible to easily remove sufficient amounts of the powdered substances to allow analysis (Sagström *et al.*, 1992). Rinsing with ammonium acetate should not give osmotic effects, but there is a risk that NH_4^+ ions are exchanged for K^+, Na^+, or H^+ ions. Rinsing with water avoids these problems, but may give an osmotic shock. However, the rinsing procedure takes no more than a few seconds. Experiments by the group of Lechene, who compared water, ammonium acetate and NaCl (the latter only with respect to the K content) showed no significant effects of water and ammonium acetate rinsing on the cellular K concentration, and no significant differences between ammonium acetate and water (Abraham *et al.*, 1985).

The present paper examines some methodological aspects of X-ray microanalysis of cultured cells. The method of growing cells on grids was tested on a variety of epithelial and secretory cells: skin fibroblasts, a primary culture of bovine mammary gland epithelial cells, an insulinoma cell line, and three colon cancer cell lines (T84, HT29 and Colo 205).

In the fibroblasts and in the colon cancer cell lines, cAMP-induced Cl^- efflux processes were tested. The cAMP-activated chloride channel has received much attention because of its involvement in the genetic disease cystic fibrosis (CF). The channel involved (also called the cystic fibrosis transmembrane conductance regulator, CFTR) is present in a number of epithelial cells. Conflicting evidence has been presented with regard to fibroblasts: some groups have presented evidence of a defective Cl^- transport in fibroblasts from CF patients, whereas others have found no difference between CF fibroblasts and control cells (Lin and Gruenstein, 1987; Rugolo *et al.*, 1986, Mattes *et al.*, 1987). The colon cancer cell lines HT29 and T84 used in this study have been well-characterized with respect to their ion transport properties. Both cell lines represent secretory intestinal epithelial cells and possess CFTR. The Colo 205 cell line had not yet been tested for its ion transport properties. Incubation with phorbol myristate acetate (PMA) has been shown to downregulate the expression of CFTR (Yoshimura *et al.*, 1991; Trapnell *et al.*, 1991). Hence, this treatment would confer CF-like properties on normal cells.

Materials and Methods

Cell Cultures

Fibroblasts. Skin fibroblasts (passage number ranging from 15 to 24) from CF patients and healthy controls were cultured in Eagles' minimal essential medium (MEM) (Gibco) supplemented with 10% calf serum (Flow) and penicillin/streptomycin.

Mammary Epithelial Cells. Bovine mammary epithelial cells were isolated and cultured as described in detail previously (Vult von Steyern, 1991). Briefly, tissue was obtained at a commercial slaughter house, and washed with 70% ethanol. The tissue was cut in small pieces, washed with Hank's balanced salt solution (HBSS) (Gibco), and placed in a digestive medium, i.e., HBSS containing collagenase type 1A from *Clostridium histolyticum*, DNAse type 1 from bovine pancreas, hyaluronidase type 1-S from bovine testes, α-chymotrypsin type II from bovine pancreas, elastase type I from porcine pancreas, nafcillin (Sigma), soybean trypsin inhibitor (Gibco), and bovine serum albumin (BSA) fraction V (Serva). The tissue was incubated for 3-4 h at 37°C or until the remaining tissue contained 5-30 cells per aggregate. During the last 2 min of stirring, EDTA was added to the digestive medium.

The cells were cultured in medium M199 (Gibco) with fungizone (2.4 μg/ml) (Gibco), BSA fraction V

(0.5%), gentamycin sulfate (50 μg/ml), insulin (10 ng/ml), prolactin (50 ng/ml) and cortison (2.5 μg/ml) (Sigma). During the first 24 h, bovine plasma fibronectin (Gibco) (2 μg/ml cells) was present to promote cell attachment. The cells were cultured on collagen gels in Petri dishes usually for 11 days, with a change of medium every 48 h.

Insulinoma Cells. The clonal insulin-secreting cell line Rin 5mF (Oie *et al.*, 1983) was used in this study. The cells were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, penicillin and streptomycin.

Colon Cancer Cells. Human colon carcinoma cell lines Colo 205 (ATCC CCL 222), HT29 (ATCC HTB38) and T84 (ATCC CCL 248) were used in this study. Colo 205 cells were cultured in Eagle's MEM supplemented with 8% fetal calf serum and 2% newborn bovine serum (Flow), HT29 cells were grown in Ham's F12 medium (Flow) with 10% fetal bovine serum, and T84 cells were grown in Ham's F12:Dulbecco's modified Eagle's medium (1:1) with 5% newborn calf serum. Penicillin, streptomycin, L-glutamine and Hepes were added to the medium.

Preparation of Cells for X-ray Microanalysis

Fibroblasts, bovine mammary epithelial cells, insulinoma cells, and colon cancer cells grown on plastic were trypsinized with 0.05% trypsin (Sigma) in 0.02% EDTA according to standard methods; as soon as the cells disengaged from the culture dish they were flushed with culture medium containing serum (which contains trypsin inhibitors), collected in a sterile centrifugation tube and pelleted at low speed. The cell pellet was gently agitated in fresh medium and the cells were seeded out on titanium grids (Agar Scientific Ltd.) which had been coated with a Formvar film and a thin carbon layer. The cells were allowed to attach and spread for 4-6 hours at 37°C in a humidified atmosphere of 5% CO_2/95% air in a culturing chamber. Fibroblasts were, in addition, seeded on collagen-coated polycarbonate membranes. The cell-covered grids were then quickly rinsed in standard Ringer's solution (140 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM glucose, pH 7.4) and placed upside down on drops of 5 mM 8-bromo-cAMP (Sigma) or 75nM vasoactive intestinal peptide (VIP) for various times at room temperature.

The incubation was stopped by a quick rinse in Ringer's solution. In most experiments, the cells were then rinsed in 0.15 M ammonium acetate in order to remove the salt-rich Ringer's solution. In initial experiments, washing untreated HT29 cells with distilled water was compared to washing with ammonium acetate, and fibroblasts were rinsed with ammonium acetate, distilled water, or mannitol. After the rinsing, the polycarbonate membranes or the grids were blotted on a filter paper and frozen in liquid nitrogen. While still in liquid nitrogen, the membranes or grids were placed in a cryostat and allowed to dry overnight at -30°C. The dried specimens were covered with a conductive carbon layer before analysis.

HT29 cells and T84 cells were also incubated in 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 24 hours at 37°C, prior to treatment with cAMP as described above. After dissolving PMA in DMSO (dimethyl sulfoxide, Sigma) a stock solution was prepared in Ringer's solution. Cells incubated for the same period as the PMA treatment (24 h) with or without equivalent amounts of DMSO alone, served as controls.

X-ray Microanalysis

X-ray microanalysis of cells on grids was performed at 100 kV in the scanning transmission mode in a JEOL 1200EX electron microscope with scanning attachment, or in the transmission mode of a Philips 400 transmission electron microscope with twin lens. Analysis of cells on polycarbonate membranes was carried out at 20 kV in the scanning mode of the JEOL microscope. Semi-quantitative analysis of the cells on polycarbonate membranes was carried out by relating the characteristic intensity of an elemental peak to the characteristic intensity of phosphorus. Fully quantitative analysis of the cells grown on grids was carried out by comparing the spectra from the cells with those from a standard, made of known concentrations of various mineral salts mixed with 20% gelatin and 5% glycerol, frozen and cryosectioned (Roomans, 1988). The cells were considered to be semi-thick specimens and analysis was therefore carried out by using the ratio of peak to the continuum in the same energy region (Roomans, 1988). It was established that this method did not give results that were significantly different from following the Hall method for thin specimens including a correction for extraneous background (von Euler *et al.*, 1992).

Spectra were acquired for 80 or 100 seconds. Only one spectrum was acquired from each cell. Unless otherwise stated, the statistical significance of differences in concentration between groups was determined by Student's t-test.

Results

Fibroblasts

In order to remove the medium in which the cells had been cultured, we used isotonic mannitol, isotonic ammonium acetate, and distilled water. Both mannitol and ammonium acetate left remnants on the freeze-dried cells. X-ray microanalysis showed that the highest K/Na

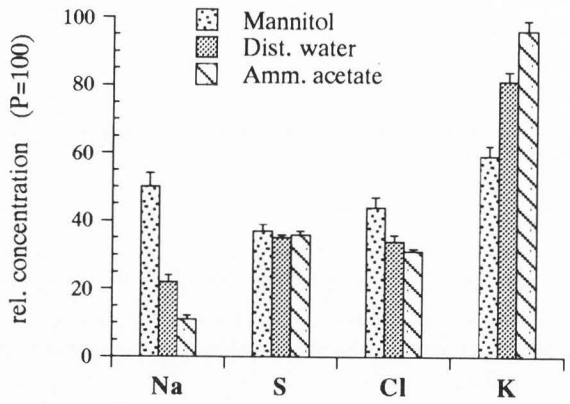

Fig. 1. Effect of rinsing fibroblasts with different rinsing fluids to remove the buffer. SEM analysis of fibroblasts cultured on polycarbonate membranes. P=100 denotes value scaled to phosphorus K_α=100 (modified from Roomans, 1991).

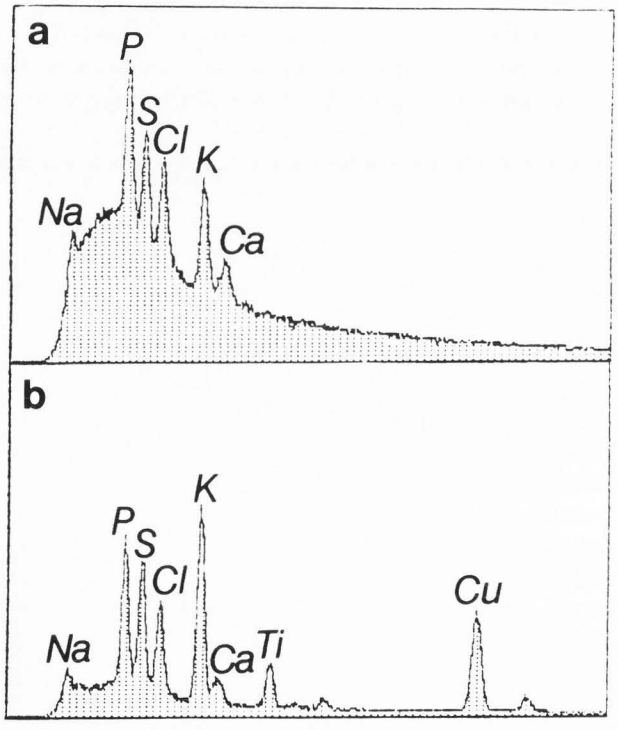

Fig. 2. Spectra of (a) a fibroblast grown on a polycarbonate membrane analyzed at 20 kV and (b) a fibroblast grown on a grid analyzed at 100 kV.

ratio was obtained after rinsing with ammonium acetate (Fig. 1), and this method was therefore used for subsequent experiments.

Cells were grown both on polycarbonate membranes and on Formvar-covered titanium grids. The cells grown on polycarbonate membranes were analyzed at 20 kV

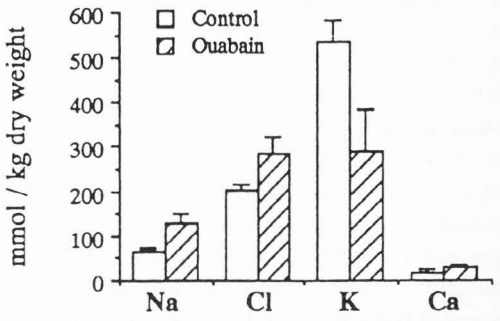

Fig. 3. Effect of ouabain (50 μM, 5 min) on Na, K, Cl and Ca concentrations in cultured fibroblasts (modified from von Euler and Roomans, 1991).

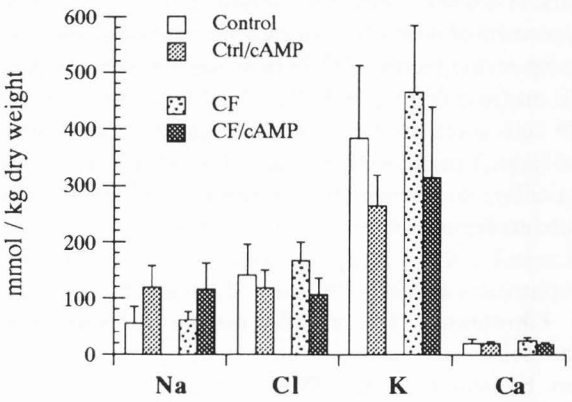

Fig. 4. Effect of cAMP (5 mM,1 min) on Na, K, Cl and Ca concentrations in cultured normal and CF fibroblasts (modified from von Euler and Roomans, 1991).

and even then a large part of the continuum was due to the substrate, whereas the cells grown on the thin films could be analyzed at 100 kV. The reduction of the contribution of the substrate and the higher accelerating voltage together resulted in a marked increase in the P/B-ratio (Fig. 2). In addition, the ammonium acetate remnants could be more easily be removed from the grids, and analysis of cells on a thin substrate allowed fully quantitative data to be obtained.

To check whether the cultured fibroblasts reacted in a normal way to external stimuli, the cells were treated with ouabain, an inhibitor of the Na^+-K^+-ATPase. After 5 min incubation, a significant increase in Na and Cl content and decrease of the K content of the cells could be observed (Fig. 3). This is as expected, and indicates that the preparative method used retains the normal physiological reaction of the cells to stimuli.

It has been speculated that fibroblasts would possess a cAMP-controlled Cl^- efflux mechanism. Exposure of the cells to cAMP results in a significant decrease of the cellular chloride content (Fig. 4). Chloride efflux was

noted both in cells from patients with cystic fibrosis and control cells. cAMP also induced a significant increase in Na and a decrease in K (Fig. 4).

Mammary Epithelial Cells

Cultured bovine mammary epithelial cells have an elemental content that is quite different from the other epithelial cells tested in this study. The main elements present are P and Ca, whereas the cellular concentration of Na, Cl and K is very low (Figs. 5 and 6a). Only in a subpopulation of the cells there seems to be a correlation between P and Ca at the cell level, in other cells the Ca level seems to be independent of the P concentration (Fig. 6b).

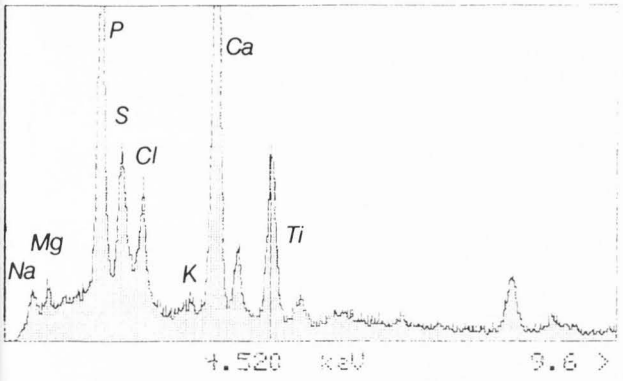

Fig. 5. Typical spectrum of a cultured bovine mammary epithelial cell.

Insulinoma Cells

A spectrum of a Rin 5mF insulinoma cell, exposed to 2.56 mM $SrCl_2$ in the presence of glucose and ATP, is shown in Fig. 7. Under these stimulatory conditions the cells take up Sr^{2+} ions (analogous to uptake of Ca^{2+} ions) to such an extent that the process can be studied by X-ray microanalysis.

Colon Cancer Cells

When a large number of cultured colon cancer cells were analyzed, it appeared that the cultures consisted of two subpopulations with respect to K. In all three cultures, "high K" and "low K" cells could be discerned, with a "demarcation line" at about 400-500 mmol/kg dry weight (Fig. 8). Typical spectra from a "high K" and a "low K" T84 cell are shown in Fig. 9. If the cell cultures are divided into "high K" and "low K" cells, it appears that there are also differences for other elements (Figs. 10). Consistently, "high K" cells have significantly higher P, higher S, higher Cl, and lower Ca concentrations than "low K" cells. For some elements, the difference between the two populations is very marked, even to the degree of complete separation of the subpopulations. In almost all cases, "borderline" cases

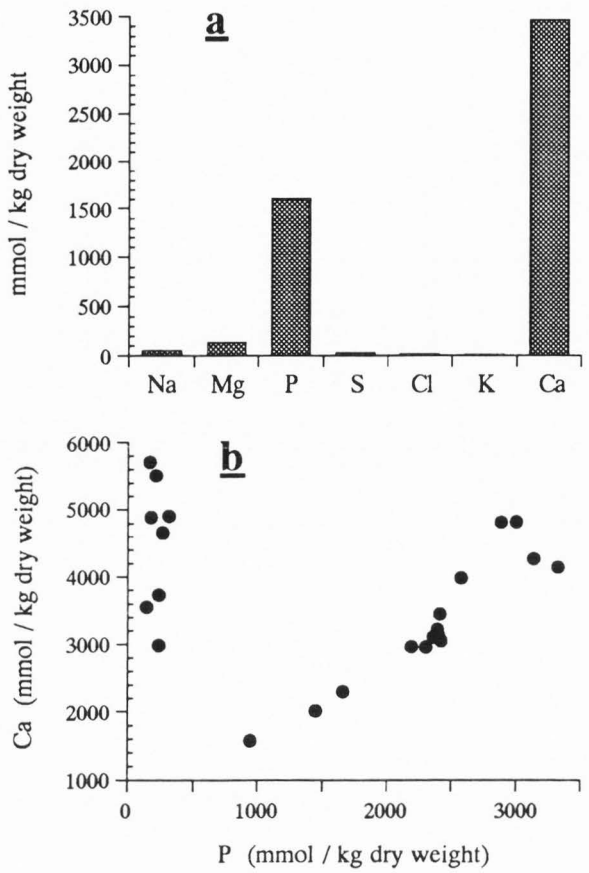

Fig. 6. (a) Average elemental concentrations determined by X-ray microanalysis of cultured bovine mammary epithelial cells (mean of 30 cells), (b) Concentration of P against Ca concentration in cultured bovine mammary epithelial cells.

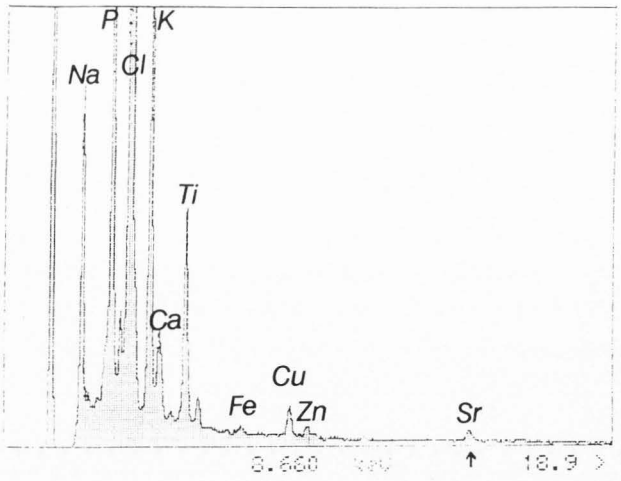

Fig. 7. Typical X-ray spectrum of a cultured insulinoma cell exposed to $SrCl_2$ in the presence of glucose and ATP.

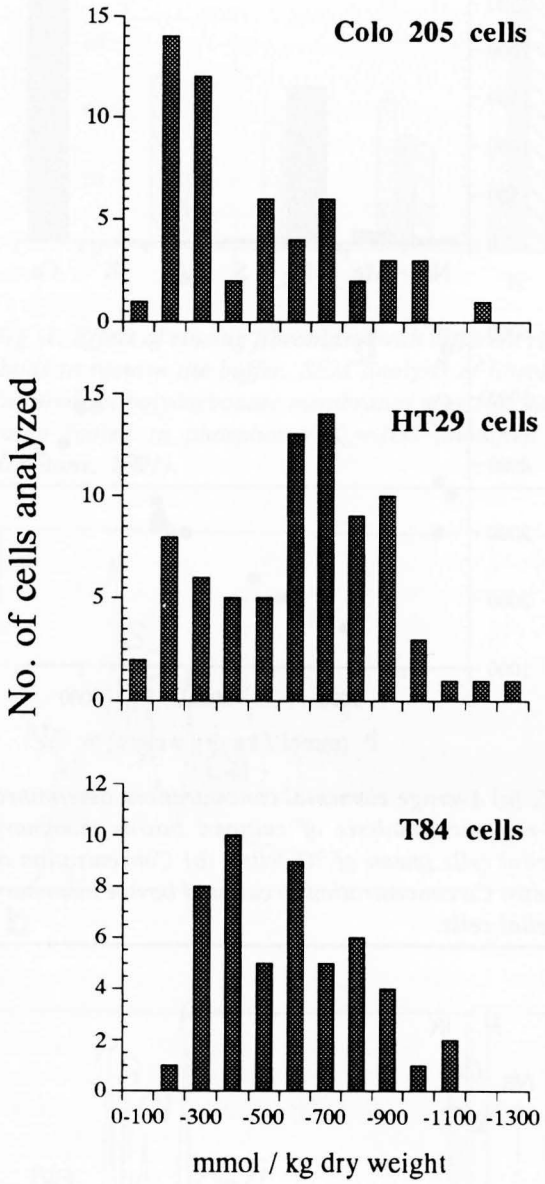

Fig. 8. Frequency distribution of K in Colo 205 cells, HT29 cells, and T84 cells, suggesting the presence of two subpopulations (modified from von Euler and Roomans, 1992).

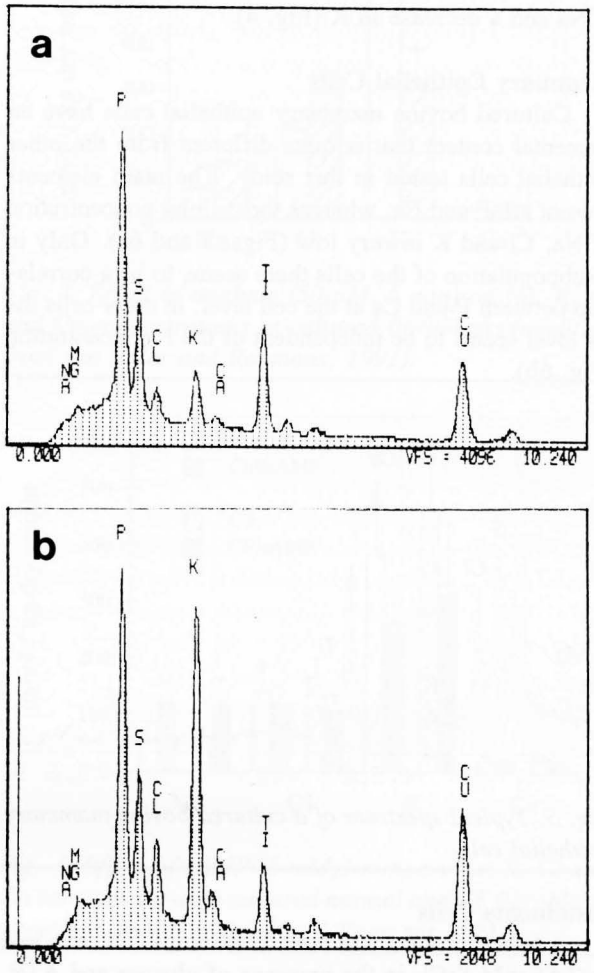

Fig. 9. Typical spectra of (a) a "low K" and (b) a "high K" colon carcinoma cell (Colo 205).

with respect to K can be assigned to one of the subpopulations based on other characteristics, e.g., calcium content.

After stimulation with cAMP, the Cl content in all three cell lines decreases already after 1 min, and even more clearly after 5 min. Since the cell culture consists of two subpopulations, results are preferably expressed as frequency distribution histograms (Fig. 11). It is evident that the "high Cl" cells (corresponding to the "high K" cells) secrete Cl^- ions in response to cAMP, whereas the "low Cl" ("low K") cells lose little or no Cl. Similarly, cAMP induces K secretion from the "high K" cells (Figs. 12 and 13).

Vaso-active intestinal peptide (VIP) that is assumed to act via cAMP, has effects similar to those of cAMP: significant decrease in cellular Cl and K content, no significant changes in Na (Table 1).

Pretreatment of the cells with PMA abolished the response of the cells to cAMP (Fig. 14). Since the cells were exposed to PMA for 24 h, the cells were cultured on the grids for 24 h instead of 5-6 h as in the previous experiments. Also after this period, a significant Cl^- secretion could be induced by cAMP.

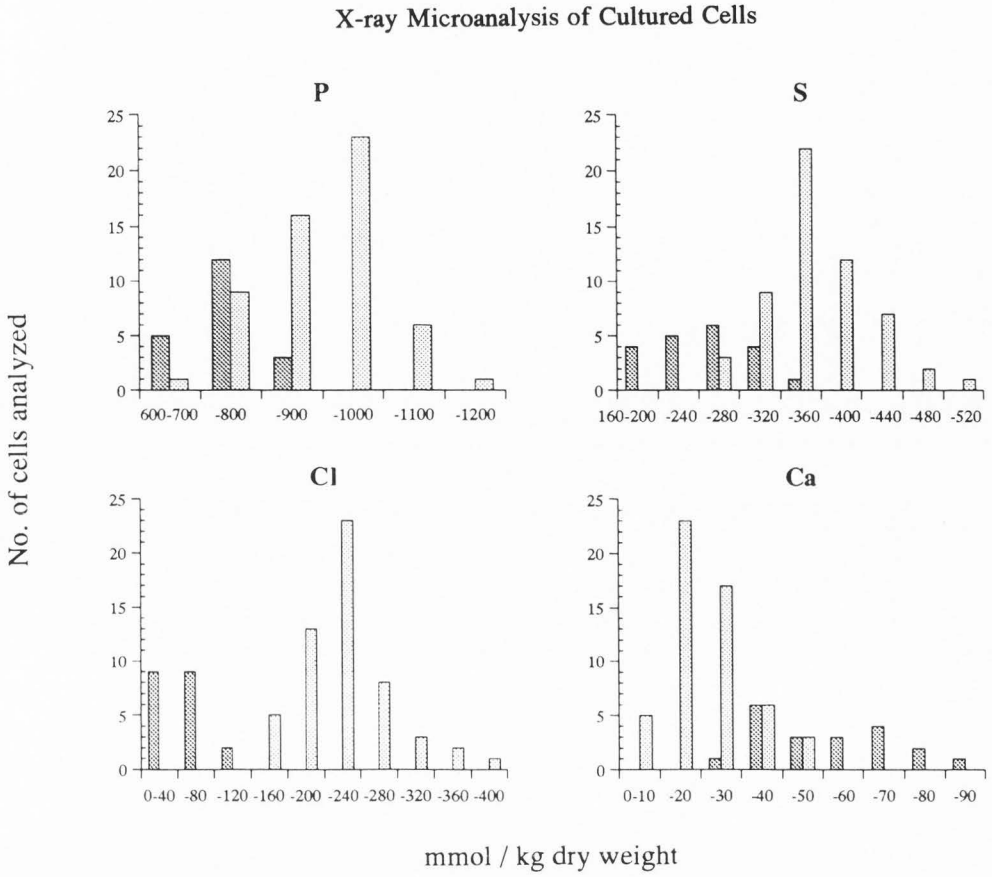

Fig. 10. Frequency distribution of P, S, Cl, and Ca concentrations in HT29 cells, divided into two populations according to their cellular K content; ▨ *K < 300 mmol/kg dry weight,* □ *K > 400 mmol/kg dry weight.*

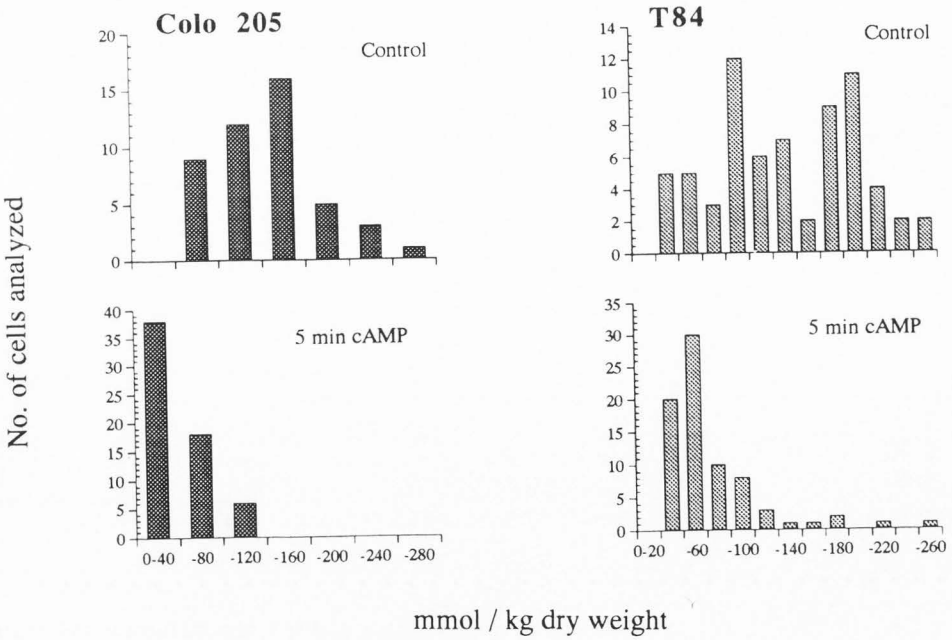

Fig. 11. Cl distribution in Colo 205 cells and T84 cells, before and after 5 min cAMP treatment (modified from von Euler and Roomans, 1992).

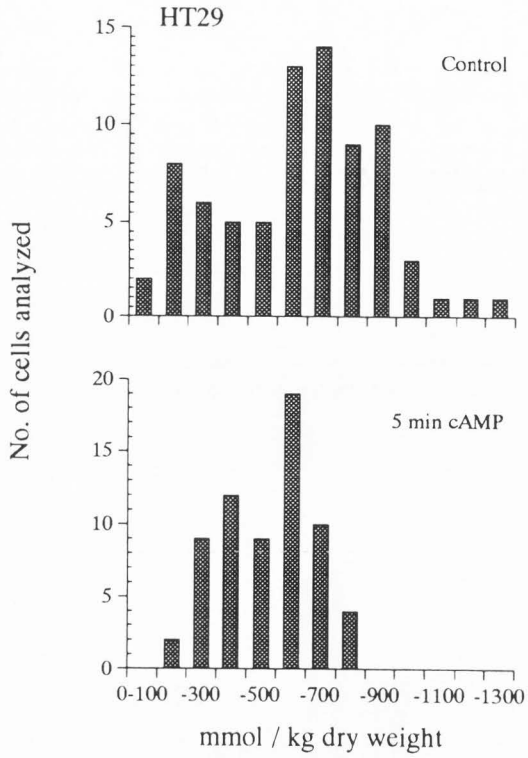

Fig. 12. K distribution in HT29 cells, before and after 5 min cAMP treatment (modified from von Euler and Roomans, 1992).

Table 1

Effect of VIP on cultured colon cancer cells ("high K" cells)

	control	+ VIP	
Na	7 ± 2	6 ± 1	ns
Cl	190 ± 12	130 ± 8	$p<0.001$
K	816 ± 56	689 ± 34	$p<0.05$
(n)	(22)	(20)	

Data in mmol/kg dry weight, mean and s.e.m.

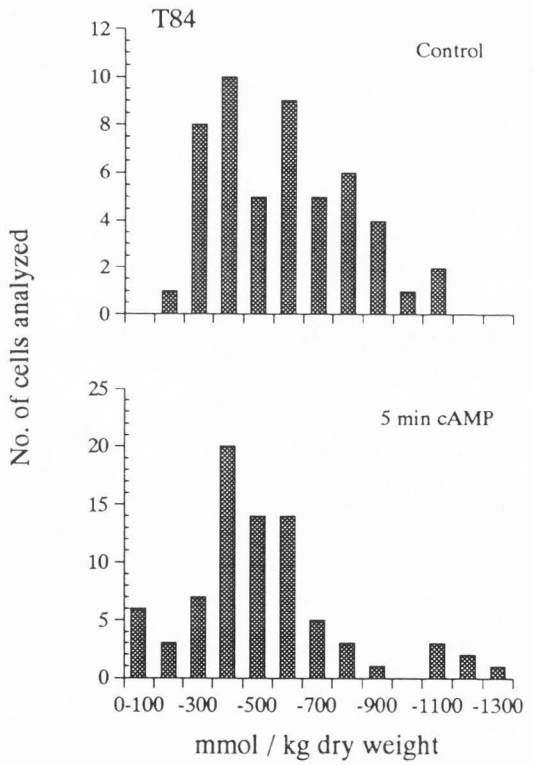

Fig. 13. K distribution in T84 cells, before and after 5 min cAMP treatment.

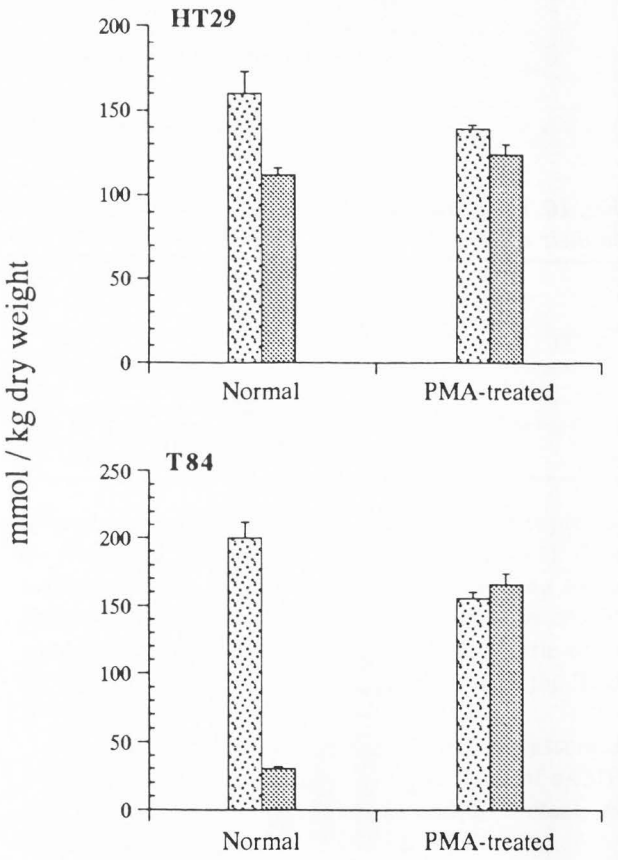

Fig. 14. Effect of PMA incubation on cAMP-stimulated Cl secretion by HT29 and T84 cells; ⊡ unstimulated cells, ▨ after 5 min cAMP stimulation (modified from von Euler and Roomans, 1992).

Discussion

The main advantage of culturing cells directly on grids is that it allows the analysis of single cells directly after treatment with different agents, without intermediate procedures such as centrifugation, encapsulation or embedding, that are time-consuming and may in themselves introduce artifacts (Warley, 1986). The main problem that is encountered in culturing cells directly on grids is that not all cells attach to the Formvar-coated grids. Cells that attach well to the plastic surface of a Petri dish may not do the same with the Formvar film. Attachment can in some cases be improved by coating the film with polylysine. In contrast, as proved to be the case with the bovine mammary gland cells used in this study, cells that require special growing conditions such as collagen-coated culturing dishes, may readily attach to and spread on the Formvar film-covered grid.

When adequate conditions for culturing the cells on grids have been established, an acceptable washing procedure has to be found. The washing procedure must not cause membrane damage with subsequent leakage of ions, but on the other hand, the culture medium must be effectively removed. It is possible that different cell types may require different washing procedures, and it is recommended to test various washing fluids with each new cell type tested. As argued by Roomans (1991) a high K/Na ratio and/or a high K/S ratio can be used as indications of a successful washing procedure. In the ideal case, data should be compared to sectioned cells.

The biological significance of the difference between "high K" and "low K" is not yet known. It could be argued that differences in K are due to rinsing-induced loss of K^+ ions. To assess the probability of such a leak, a plot was made of the correlation between the cellular concentration of S and P, respectively, with that of K. Fig. 15 shows such plots for the HT29 cells. If rinsing-induced leaking was a major factor, one would expect the S concentration to be independent of K concentration, since virtually all of the cell's S is bound in macromolecules and hence insensitive to the washing procedure. For P, one would expect a weak positive correlation with K, because part of cellular P is in the form of soluble ions, whereas another part is bound to macromolecules. However, the data show a stronger positive correlation between K and S than between K and P. This speaks against the hypothesis that the difference in K between "high K" cells and "low K" cells is due to leakage of K during the washing procedure. The "low K" cells also cannot be considered as damaged cells, since membrane damage would not only result in low K concentrations, but also in high Na and Cl concentrations. Contrary to this hypothesis, the "low K" cells have low Cl concentrations. Hence, the difference

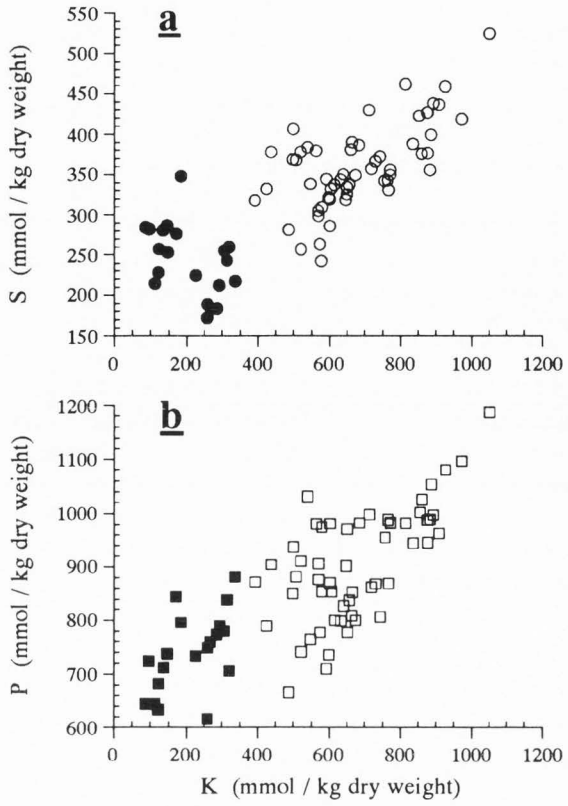

Fig. 15. Plot of (a) S versus K and (b) P versus K in HT29 cells; closed symbols represent "low K" cells, open symbols "high K" cells.

between the "high K" cells and the "low K" cells appears to have a biological basis. One possibility is that the cells represent different stages in the cell cycle; the cellular ion content is known to vary with the cell cycle (Stephen *et al.*, 1990). However, since the cells were used for the experiment relatively shortly after being trypsinized, one would expect the cells to be in the same stage of the cycle. Since the "low K" cells have high Ca levels, they may represent mucus-producing cells that have differentiated from the ion- and water-transporting "high K" cells. Since mucus has calcium-binding properties, mucus-secreting cells often have relatively high calcium levels (Roomans, 1986; Gupta, 1989). Electron microscopical studies by others have shown that colon cancer cell cultures may contain mucus-producing cells in addition (Semple *et al.*, 1978; McCool *et al.*, 1990).

While the explanation of the difference between "high K" cells and "low K" cells will require additional investigations, it is of interest to note that the two cell types react differently to physiological stimuli. In classical Ussing-chamber experiments, the epithelial

sheet of colon cancer cells would be considered as a homogeneous system, whereas X-ray microanalysis shows that this is clearly not the case, neither with respect to the ion concentrations in the resting state, nor with respect to ion fluxes resulting from stimuli. This implies that X-ray microanalysis adds extra information to studies of ion transport at the cellular level.

Deficient epithelial chloride transport is known to be the primary defect underlying cystic fibrosis (reviewed by Welsh, 1990 and Quinton, 1990). Conflicting evidence has been presented whether fibroblasts from CF patients also showed abnormal chloride transport (Lin and Gruenstein, 1987; Rugolo *et al.*, 1986, Mattes *et al.*, 1987) but our data do not support the notion of an abnormality in the cAMP-regulated chloride efflux mechanism in cystic fibrosis fibroblasts. In intestinal epithelial cells, chloride efflux is stimulated by cAMP and by substances that increase the level of cAMP in the cell, such as VIP. This efflux takes place *via* a channel of which the cystic fibrosis transmembrane regulator (CFTR) is an integral part. The expression of CFTR in these cells can be downregulated by treatment with PMA (Yoshimura *et al.*, 1991; Trapnell *et al.*, 1991), which gives the cells a "CF-phenotype". Our data show that PMA pretreatment indeed markedly decreases the efflux of Cl^- ions. Thus a similar situation as in CF epithelial cells is obtained (Sagström *et al.*, 1992).

Sr^{2+} ions can be used as "tracer" for Ca^{2+} ions in X-ray microanalysis (Krefting *et al.*, 1988; Wroblewski *et al.*, 1989) and allow the study of uptake of calcium ions by cultured cells. In the insulin-producing cell line Rin 5mF we studied the uptake of strontium as a tracer for calcium. Diabetes *per se* does not appear to induce changes in the cellular calcium concentration (Juntti-Berggren *et al.*, 1990, 1992), but the ion plays a role in the activation of insulin secretion (Juntti-Berggren, 1992) and the opportunity to study the movement of calcium or a substitute in cell cultures can thus give valuable information on whether or not calcium movements in diabetes are normal.

The cultured bovine mammary epithelial cells have an elemental composition that is strikingly different from that of the epithelial cells studied so far by X-ray microanalysis (LeFurgey *et al.*, 1988). The mammary gland is a modified sweat gland, and it could thus be expected that the epithelial cells contribute to the regulation of the ionic content of the milk. Very little is known about ion transport processes in the mammary gland, but it is hoped that the study of cell cultures can increase our knowledge about this process. The epithelial cells are filled with casein, which is the likely explanation for the high Ca content of the cells. The data indicate that two cell types may exist in the cell culture, one where Ca is associated with P, and one with a high Ca concentration despite a low P concentration.

Evidently, X-ray microanalysis of entire cultured cells cannot replace analysis of thin sections of cells. It does, however, provide in a relatively short time interesting and unique information on cell cultures at the cellular level.

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Discussion with Reviewers

A. Warley: Why do you change the substrate immediately before subjecting the cells to experiments / XRMA? Doesn't removal of the cells cause depolarisation or stimulate them into the division cycle and upset their ion profiles?

Authors: Cells are routinely grown in plastic dishes or culturing flasks, and have to be transferred to polycarbonate membranes or Formvar-coated grids prior to experiments. Cells which were allowed to grow for 24 hours on grids showed the same ion profiles as cells that were grown for only 4-6 hours on the grids before experiments.

A. Warley: Does the quantification procedure that you use make any allowance for absorption of the less energetic X-rays?

Authors: Routinely we use the P/B ratio for quantitation where the background is taken in the same energy region as the peak; this provides an intrinsic correction for X-

ray absorption. We found, however, that use of the Hall method with a single background region for all elements gave results that were not significantly different and conclude that the freeze-dried cells are so thin that absorption of X-rays is not a problem.

A. Warley: Freeze drying at -30°C uses a temperature which is much higher than most people would recommend. Do you think that use of this temperature might cause any problems?
Authors: We agree that freeze drying at -30°C might not be optimal, but the cells do not display any morphological damage and the Na/K ratios do not indicate membrane damage.

A. Warley: The high background observed in Fig. 2a is not at all surprising. Have you tried using a lower voltage, say 10 kV (as suggested in David Sigee's work)?
Authors: We agree that use of a lower accelerating voltage would increase the relative contribution of the specimen to the spectrum, and result in an increase of the P/B ratio. On the other hand, the signal would decrease which would require longer time of analysis. Therefore we feel that whenever possible, growing the cells on thin films is to be preferred, because this allows analysis of the cells at high accelerating voltage.

A. Warley: What raster area, or part of the cell, was used for analysis? If the analysis was confined to the cytoplasm it is not surprising that you might pick up spectra from secretory vacuoles etc., which would be expected to show a different element profile.
Authors: Analysis was carried out with the electron beam on the nucleus, but the analytical volume probably included cytoplasm as well.

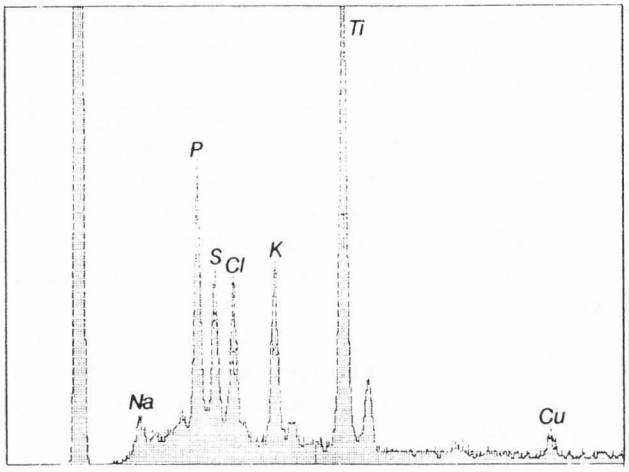

Fig. 16. Spectrum of a cultured insulinoma cell rinsed in 0.15 M ammonium acetate prior to freezing.

A. Warley: The spectrum from the cultured insulinoma cell (Fig. 7) shows very high levels of Na and Cl. Do you have any comments about this?
Authors: The spectrum in Fig. 7 is from a preliminary experiment on the insulinoma cells, showing the incorporation of strontium into the cells. The washing procedure had not yet been optimized, and we suspect that in Fig. 7 the high levels of Na and Cl are caused by buffer remnants. However, we include a figure from a recent experiment (without Sr uptake) with lower levels of Na and Cl (Fig. 16).

J.E. Beesley: How do you know the "abnormal" spectrum of a cultured bovine mammary epithelial cell (Fig. 5) is real and not induced by the washing regime?
Authors: There is no way that the washing procedure could be responsible for the high levels of Ca and P observed in these cells. Without measurements on thin cryosections it cannot be completely excluded that the washing procedure might influence Na, K or Cl levels, although we think this is less likely because Na and Cl levels are low.

L. Edelmann: Did you try to confirm the finding of "high K" and "low K" cell subpopulations by X-ray microanalysis of freeze-dried cryosections of cultured cells?
Authors: No, this has not yet been done.

A. Dörge: In Fig.1 it is shown that after washing fibroblasts with isotonic mannitol the cellular Na concentration was much higher than after washing with isotonic ammonium acetate or distilled water. Since the Cl concentration after mannitol washing was only slightly enhanced compared with the other washing fluids it seems very unlikely that this high Na concentration is caused by contamination by the incubation medium. Is there any other explanation for this?
Authors: It would be possible that Na^+ precipitates with other anions than Cl^- during or after rinsing with mannitol.

A. Dörge: Is there any indication that the dry weight content of the high and low K colon carcinoma cells is substantially different? If this is not the case, and assuming that K is an important cellular osmolyte, the question arises as to how the cells with extremely high or low K achieve osmotic equilibrium with the incubation medium?
Authors: We have not determined the relative dry weight concentrations. The question of osmotic equilibrium is, of course, intriguing but X-ray microanalysis does not give information on organic polycations or polyanions. In the low-K cells, mucins might be present.