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USE OF *IN VITRO* SYSTEMS FOR X-RAY MICROANALYSIS

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Abstract

The use of X-ray microanalysis in human pathology may require the use of cryoprepared tissue. Often it is impossible to carry out freezing of the tissue in an optimal way, and in addition, it is difficult to carry out experiments in living patients. The use of *in vitro* systems and cell cultures allows separation of the process of tissue removal and the freezing procedure, and also makes testing of pharmacological or toxic substances possible. In experiments with animal tissue it was shown that incubation in a physiological buffer induced significant changes in the concentrations of Na, K, and Cl. In general, the concentrations of Na and Cl increased, those of K decreased. Prolonged incubation of brain tissue (cortex and hippocampus) and of liver resulted in further changes of the cellular ion contents in the same direction. Incubation of pancreas and submandibular gland resulted in a limited reversal of the changes induced by dissection. The submandibular gland *in vitro* showed the same response to cholinergic stimulation as the gland *in situ*. The use of cell cultures for X-ray microanalysis is briefly reviewed and illustrated by an example of analysis of an immortalized sweat gland cell line. It was shown that these cells respond to stimulation by cAMP with loss of Cl and that this response was unaffected by the type of substrate the cells were grown on.

Key words: X-ray microanalysis, pathology, *in vitro*, *in situ*, cell culture, ion transport, brain, liver, exocrine glands.

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Introduction

In applications of X-ray microanalysis in human pathology, special problems with regard to specimen preparation are encountered if analysis of diffusible substances is to be carried out (Roomans, 1991). It is well known that analysis of diffusible substances requires the use of cryotechniques. The tissue should be frozen as rapidly as possible; dissection should be avoided as it can introduce ion shifts (Ingram and Ingram, 1983; von Zglinicki *et al.*, 1986; Tvedt *et al.*, 1989). *In situ* freezing is, however, generally not possible when human tissue is involved. Also, for practical reasons it may be difficult to carry our freezing in an optimal way in an operation theater. In addition, when biopsies from patients are taken, the patient may be under local or general anesthesia, which may include being treated with drugs affecting the circulatory system or muscle relaxants. In addition, the patient may be receiving drugs for the disease under investigation or unrelated conditions. Autopsy tissue shows major changes in the distribution of diffusible ions (Kuypers and Roomans, 1980; Roomans and Wroblewski, 1985) which poses major problems in the interpretation of results from this type of specimen.

In a previous paper (Roomans, 1991) it was suggested that possible ways to solve the problems with the use of biopsy material from human patients was to separate (both temporally and spatially) the biopsy procedure itself from the freezing procedure. This could be done by incubating the tissue or by culturing the cells prior to freezing. Both methods make it possible to freeze the sample under optimal conditions and, in addition, allow the study of the effects of pharmacological or toxic agents. This is something that is difficult, and often impossible, to do in a living patient. When *in vitro* systems are used, it is, however, important to know to which extent the *in vitro* system resembles the situation *in situ*. In the present paper, therefore, some aspects of the use of *in vitro* systems for X-ray microanalytical studies are investigated.

Cell cultures have been used quite frequently in biological X-ray microanalysis (Wroblewski and Roomans, 1984, Zierold and Schäfer, 1988). Much of the initial work was done by the group of Lechene (reviewed in Lechene, 1988). Roughly, cell cultures can be divided into four types: (1) primary cultures, (2) "spontaneously" growing non-cancer cells, (3) tumor cell lines, and (4) immortalized cell lines. As an example of the use of primary cultures, chloride transport was studied in primary cultures of respiratory epithelial cells (obtained from polyps) from patients with cystic fibrosis (CF) (Sagström *et al.*, 1992). It was shown that while cells from control subjects showed a decrease in Cl levels after stimulation with cAMP, the intracellular Cl concentration in cells from CF patients did not decrease after such stimulation. That the primary culture behaved in about the same way as respiratory epithelial cells *in situ* was shown in a comparison with hamster tracheal epithelium *in situ* in which also Cl decreased after β-adrenergic stimulation (Spencer and Roomans, 1989). An example of a "spontaneously" growing non-cancer cell line is the fibroblast. These cells can easily be cultured for a relatively large number of passages. Many studies on ion transport properties of these cells have been carried out (Abraham *et al.*, 1985). One example is a study of cAMP-induced chloride transport in which it was shown that this process was normal in CF-fibroblasts (von Euler and Roomans, 1991). Also tumor cells are easy to culture and have been used for a variety of studies, some with direct reference to the mechanism of tumor genesis or to treatment of tumors (Stephen *et al.*, 1990; Szolgai-Daniel *et al.*, 1991; Peschke *et al.*, 1992). Tumor cell lines can also be used to study normal cell physiology; colon cancer cell lines provide model systems in which cAMP-induced chloride transport can be studied (von Euler and Roomans, 1992) and shown to be similar to chloride fluxes in other secretory intestinal cells *in situ* (von Zglinicki and Roomans, 1993). Non-tumor cell lines can be "immortalized" by infection with Simian virus 40 (SV40). Such cells can be maintained over a relatively large number of passages. An example of the use of such a cell line is discussed in this paper.

Materials and Methods

Incubation experiments

For incubation experiments on brain tissue, NMRI (Naval Medical Research Institute) mice (11-13 weeks old, 30-35 g) were used. The animals were anesthetized with pentobarbital (85 mg/kg body weight). Part of the calvarium was removed, taking care not to damage the underlying tissue. In some animals, the cerebral cortex was frozen *in situ* by immersing the head of the animal directly into liquid propane cooled by liquid nitrogen. In other animals, the brain was dissected, and 1-1.5 mm thick slices of cerebral cortex and hippocampus were incubated. The dissection of the cerebral cortex took about 5-7 minutes (from opening the skull to the start of the incubation), that of the hippocampus 6-8 min. The incubation solution consisted of artificial cerebro-spinal fluid (ACSF) containing 120 mM NaCl, 3.1 mM KCl, 1.3 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM D-glucose at pH 7.4 (Ballyk and Goh, 1992), and was kept at a temperature of 36 ± 1 °C or 7 ± 1 °C. The incubation solution was oxygenated with 95% O_2/ 5% CO_2. The tissue was incubated for 2 h; samples were taken at half-hour intervals and frozen in liquid propane cooled with liquid nitrogen.

For incubation experiments on liver, pancreas, and submandibular glands, Sprague-Dawley rats (8-9 weeks old, 190-220 g) were used. The animals were anesthetized with pentobarbital (45 mg/kg). In some animals, the tissue was frozen *in situ* with liquid-nitrogen cooled brass clamps. In other animals the tissue was dissected and liver and pancreas were incubated in a Krebs-Ringers buffer, containing 140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM HEPES, 1 mM $MgCl_2$ supplemented with 5 mM D-glucose at pH 7.4. Submandibular gland slices were incubated in Krebs-Ringers bicarbonate buffer containing 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.7 mM $CaCl_2$ supplemented with 2.8 mM D-glucose, 5 mM β-hydroxybutyrate, 10 mM nicotinamide, 10 mM inosine, and 0.5 mM adenine at pH 7.4. The tissue was incubated for 2 h at 36 ± 1 °C and oxygenated with 95% O_2 and 5% CO_2; samples were taken at half-hour intervals and frozen as described above.

The effect of cholinergic stimulation on submandibular glands *in situ* and *in vitro* was compared in the following experiment. *In situ* stimulation was carried out by injecting the rat intraperitoneally with pilocarpine (20 mg/kg body weight); the glands were frozen *in situ* 2 min after stimulation as described above. *In vitro* stimulation was carried out by adding carbachol to the incubation medium to a final concentration of 20 μM at 30 or 120 min after the start of the incubation.

For analysis either 16-μm thick (Wróblewski *et al.*, 1978; McMillan and Roomans, 1990) or 4 μm-thick (Wroblewski *et al.*, 1983) cryosections were cut on a conventional cryostat, mounted on a carbon support (the thinner sections were mounted over a Formvar-film covered hole in the support) and coated with a thin carbon layer to prevent charging in the electron microscope.

Cell cultures

An ion transporting human sweat gland cell line NCL-SG3 (Lee and Dessi, 1989) was used in this study. The cells were grown in William's E medium (Gibco) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, L-glutamine (2 mM), 10 μg/ml insulin, 10 μg/ml transferrin, 5 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 10 ng/ml sodium selenite and 20 mM HEPES. Cells grown on plastic were trypsinized and then seeded on Formvar-film covered titanium grids (75 or 100 mesh, Agar Aids), plastic coverslips (Thermanox), glass coverslips (Agar Aids) or Transwell inserts (Costar). The cells were allowed to attach and spread for 37°C in a humidified atmosphere (5% CO_2 and 95% O_2). In stimulation experiments, the cells were, after rinsing with Krebs-Ringers buffer to remove the culture medium, stimulated with 5 mM 8-bromo-cAMP in Krebs-Ringers buffer. The incubation was stopped by a quick rinse in 0.15 M ammonium acetate (von Euler *et al.*, 1993). The specimens were frozen in liquid propane cooled by liquid nitrogen (-180°C) and freeze-dried overnight in vacuum at -80°C. Before analysis, the specimens were coated with a conductive carbon layer.

X-ray microanalysis

The 16 μm-thick cryosections and the sweat gland cells cultured on a solid substrate (glass, plastic, membrane filter) were analyzed in a Philips 525 scanning electron microscope in the secondary electron mode at 20 kV with a LINK AN10000 energy-dispersive spectrometer system. The 4 μm-thick sections were analyzed in a JEOL 1200EX TEMSCAN in the scanning transmission mode at 100 kV with a Tracor 5500 energy-dispersive spectrometer system. The sweat gland cells cultured on Formvar-film covered titanium grids were analyzed in a Philips 400 electron microscope in the transmission mode with a LINK QX200 energy-dispersive spectrometer system.

Quantitative analysis was carried out based on the ratio of characteristic counts to background intensity in the same energy region (P/B-ratio) (Roomans, 1988; von Euler *et al.*, 1992). P/B-ratios obtained on the samples were compared with those obtained on standards consisting of a gelatin/glycerol matrix containing mineral salts in known concentrations (Roomans, 1988). In the analysis of sweat gland cells grown on solid substrates, however, only semi-quantitative data (elemental ratios) were collected since a large part of the background signal originates from the substrate.

Statistical significance between groups was determined by analysis of variance (ANOVA) or Student's t-test.

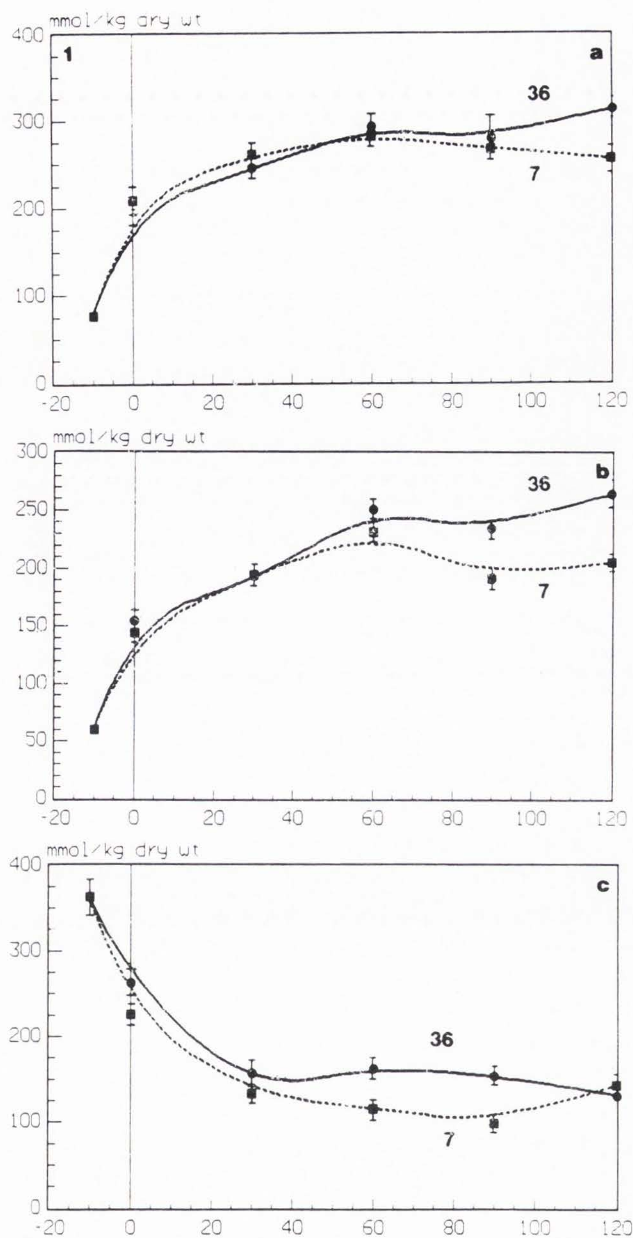
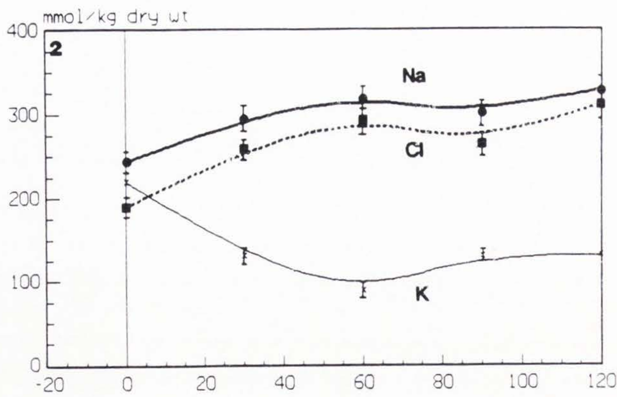
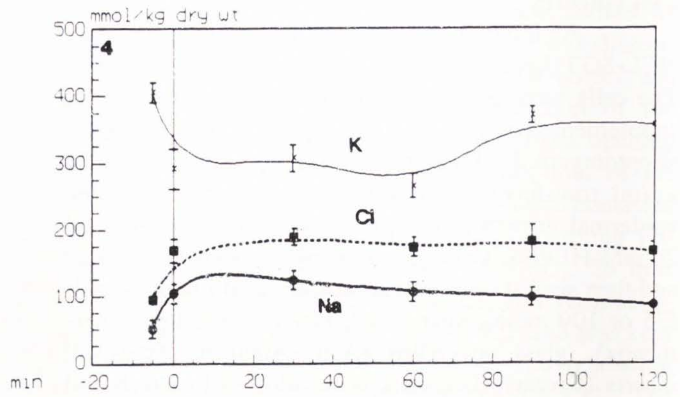
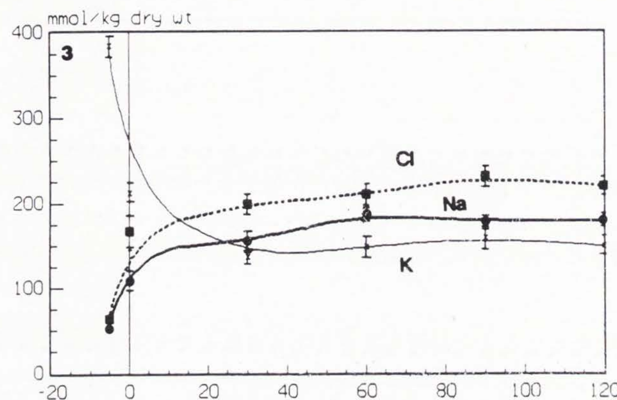


Fig. 1: Effect of incubation on the concentration of (a) Na, (b) Cl, (c) K in mouse cerebral cortex compared to the *in situ* concentrations (given as negative time point). Incubation time on the horizontal axis (in minutes). The data at zero minutes were obtained by briefly (a few seconds) incubating the dissected tissue in buffer. Drawn line represents data for 36°C, broken line represents data for 7°C. Data based on two separate experiments for each temperature, with 10 cells analyzed in each experiment for each time point. Mean and standard errors (bars) given.







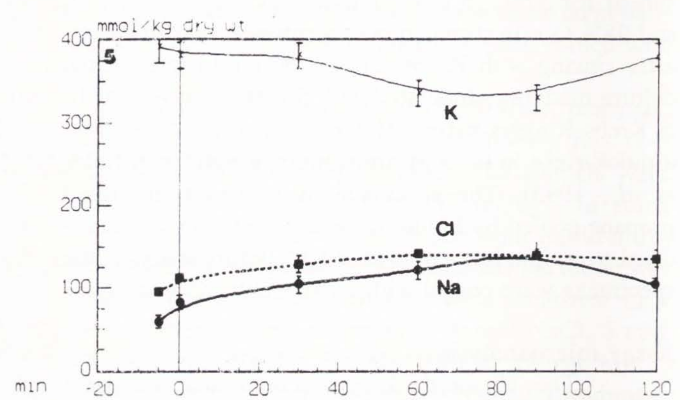


Figure 2: Effect of incubation on the concentration of Na, Cl and K in mouse hippocampus. (For a description of the experiment, see legends of figure 1). Data based on duplicate experiments, with 10 cells measured in each experiment for each time point. Mean and standard errors (bars) given.

Figure 3: Effect of incubation on the concentration of Na, Cl and K in rat liver. Data based on triplicate experiments, with 8 cells measured in each experiment for each time point. The *in situ* situation is given in the negative time point, the zero time point was incubated for a few seconds. Mean and standard errors given.

Figure 4: Effect of incubation on the concentration of Na, Cl and K in rat exocrine pancreas. Other legends as in figure 3.

Figure 5: Effect of incubation on the concentration of Na, Cl and K in rat submandibular gland. Other legends as in figure 3.

Results

Dissection and incubation of mouse cerebral cortex resulted in a significant increase in Na ($p < 0.001$) and Cl ($p < 0.001$) and a decrease in K ($p < 0.001$) compared to tissue frozen *in situ* (figure 1a-c). During prolonged incubation at 36°C, a further increase in Na and Cl and a decrease in K was noted. The increase in Na and Cl was somewhat less marked at the lower incubation temperature of 7°C. Since the hippocampus is not immediately accessible, no *in situ* values could be obtained. During incubation, concentrations of Na and Cl increased, concentrations of K decreased (figure 2).

Dissection and incubation of rat liver caused highly significant changes in the concentrations of Na, Cl and K ($p < 0.001$ for all elements) compared to the *in situ* concentrations. The concentration changes proceeded during the first 30 min of the incubation, but then the concentrations seemed to stabilize. However, the final K/Na ratio was below 1 as compared to a value of about 7 *in situ* (figure 3). Similar changes as in liver were caused by dissection of the pancreas: the changes in Na, K and Cl were highly significant ($p < 0.001$) (figure 4). In this case, a normalization of the concentrations after the first half hour of incubation was noted. In the submandibular gland, dissection-induced changes were less prominent (figure 5) and ion concentrations appeared to return to near-normal during the incubation. *In vivo*, submandibular gland acinar cells responded to cholinergic stimulation with no significant change in the cellular Na concentration and a decrease in Cl and K concentration (figure 6). This response is maintained in

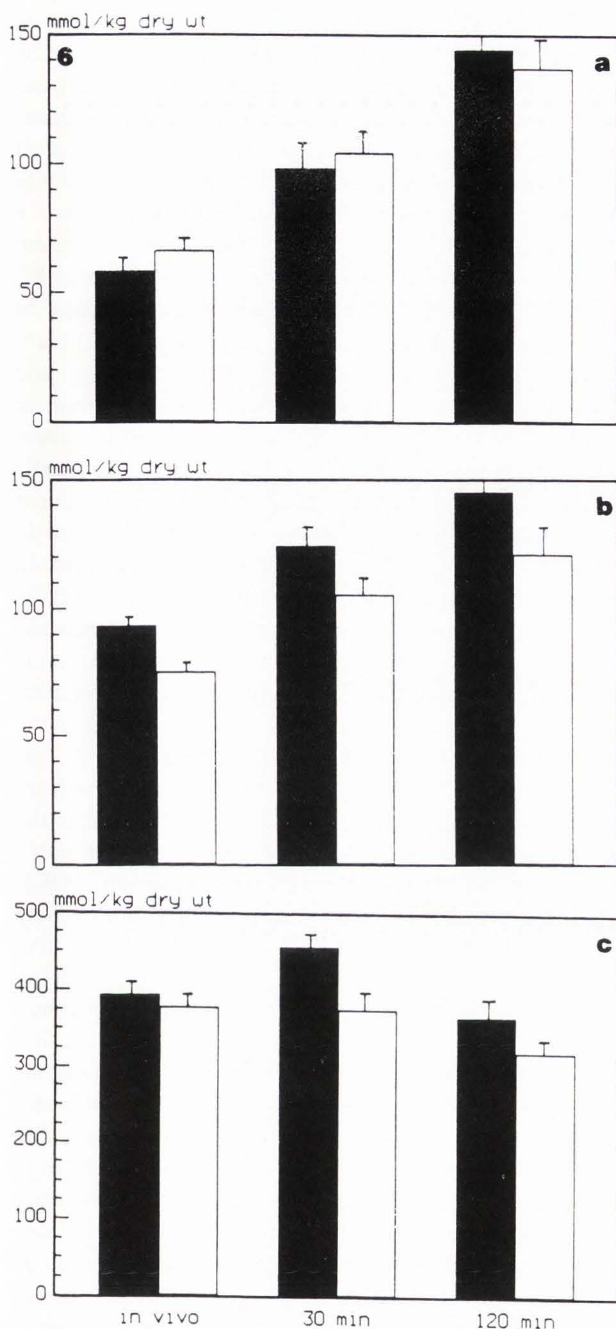


Figure 6: Effect of cholinergic stimulation on the concentrations of (a) Na, (b) Cl, (c) K in rat submandibular gland acinar cells: *in vivo*, after 30 min incubation, and after 120 min incubation. Solid bars, controls (unstimulated); open bars, stimulated (see Materials and Methods). Mean of triplicate experiments with 8 cells measured in each experiment. Mean and standard errors (bars) given.

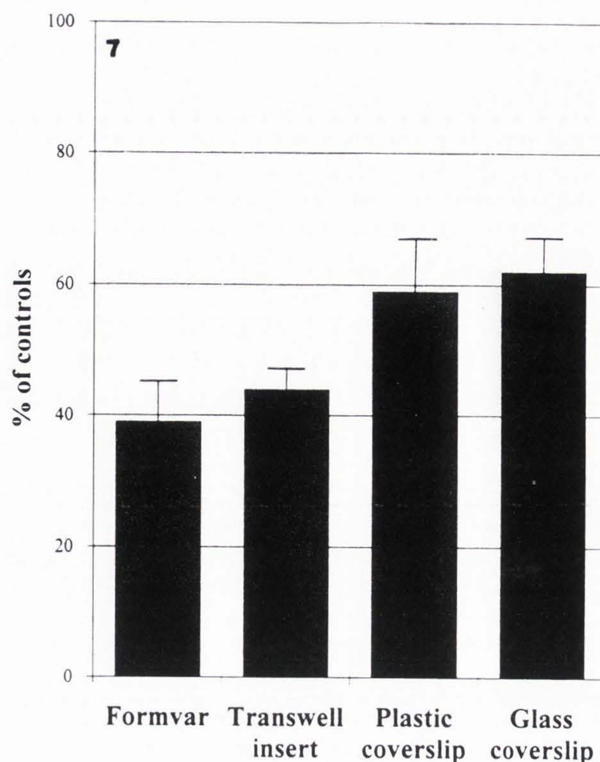


Figure 7: Cl/S ratio in NCL-SG3 cells cultured on different substrates. To show the effect of cAMP stimulation, data are expressed as % of the Cl/S ratio in unstimulated cells on the same substrate. Mean and standard error (bar) of 20-30 cells given.

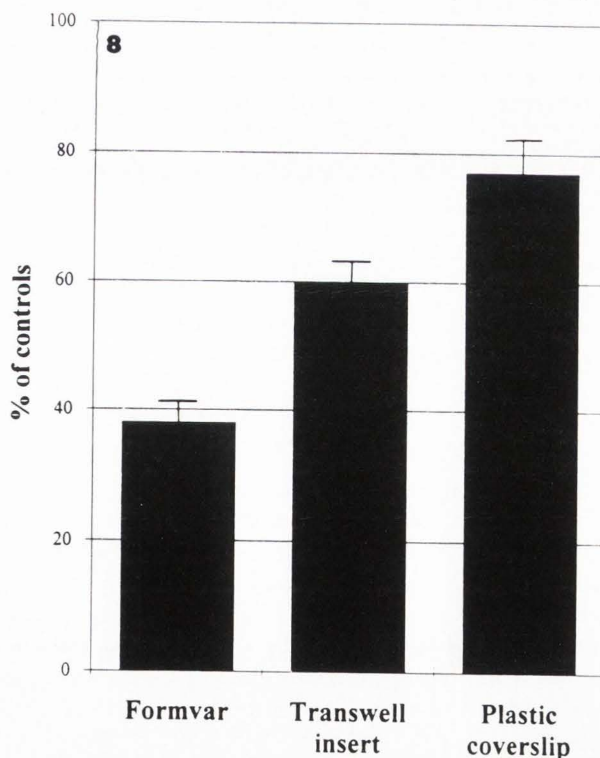


Figure 8: K/S ratio in NCL-SG3 cells stimulated with cAMP. Other legends as in figure 7.

the *in vitro* experiment both at 30 min and 120 min of incubation.

Cultured cells can be grown on different substrates. In an experiment to investigate whether the response to cAMP stimulation is affected by the substrate on which the cells were grown, the SV40-infected sweat gland cell line NCL-SG3 was grown on different substrates and stimulated with cAMP. Measurements of the Cl/S and the K/S ratio showed that qualitatively, the response of the cells to cAMP (decrease in Cl and K) is similar for all substrates (figures 7 and 8).

Discussion

The results of the incubation experiments show that *in situ* freezing is a prerequisite for obtaining elemental concentrations close to the living state. This is particularly evident for excitable tissue, but also in liver and pancreas a highly significant increase in Na and Cl and a decrease in K could be noted. Rapid ion shifts due to dissection have previously been demonstrated in liver by von Zglinicki *et al.* (1986) and Tvedt *et al.* (1989) and *in situ* freezing of muscle was already suggested by Ingram and Ingram (1983). In the present experiment, the tissue was both dissected and briefly (a few seconds) exposed to the incubation buffer. Further studies (Hongpaisan and Roomans, in preparation) indicate that the exposure to the buffer, rather than the dissection *per se*, is responsible for the changes. Despite the use of incubation methods that are standard for biochemical studies of brain tissue, elemental concentrations do not return to *in vivo* values, but the situation deteriorates further. Leakage of ions over the cell membrane appears to be somewhat reduced by lowering the temperature of the incubation medium from 36°C to 7°C. This may be due both to a slowdown of cell metabolism and to a decreased diffusion rate. Unfortunately, the results are not very encouraging with respect to the use of incubated tissue for studies of the nervous system, and since *in situ* freezing is excluded in the case of human pathology, this severely restricts the possibilities for X-ray microanalysis of human nervous tissue. In published microanalytical studies of human brain tissue subjected to dissection (Malmqvist *et al.*, 1988; Inamura *et al.*, 1990) the values for the elemental concentrations obtained may not be equal to the *in vivo* values. Still, pathological changes might be observed despite the superimposition of the damage done by dissection, but such investigations are limited to cases where the pathological process has induced major changes in ionic composition.

The situation is better for pancreas and submandibular gland, where the elemental concentrations stabilize during incubation, and even show a tendency to normalization. Although the absolute concentration values differ from the *in vivo* situation, the system can quite well be used to investigate physiological processes involving ion transport. As an example, the effect of cholinergic stimulation on the submandibular gland was studied. The observed changes in ion content are a consequence of several ion transport processes: secretion of Cl^- ions by apical chloride channels, secretion of K^+ ions by basolateral potassium channels, and uptake of Na^+, K^+ and Cl^- ions, presumably by a basolateral Na^+-K^+-Cl^--cotransporter (Petersen, 1980; Poulsen and Kristensen, 1982; Novak and Young, 1986; Pirani *et al.*, 1987). The doses of cholinergic agonists used were calculated to give maximal stimulation of the acinar cells of the gland, and there is a clear qualitative agreement between the changes in ion concentrations observed in the *in situ* experiments and those observed *in vitro*. In another recent study (Rezapour *et al.*, in preparation) we have shown that when strips of human myometrium obtained at caesarean section were incubated, ionic changes after stimulation with oxytocin and progesterone measured by X-ray microanalysis corresponded with the physiological reaction of the tissue to these stimuli.

Sweat gland duct cells resorb chloride ions from the primary sweat through a chloride channel in the apical cell membrane and excrete the chloride through a channel in the basolateral membrane (Quinton, 1990). This transcellular chloride transport is stimulated by cAMP. It appears that chloride secretion is faster than chloride uptake, so that stimulation by cAMP results in a net loss of Cl from the cell. We have found it advantageous to culture cells for X-ray microanalysis on thin supports (von Euler *et al.*, 1993). Quantitative analysis is more straightforward because there is no solid substrate contributing to the continuum, and sensitivity can be improved since analysis can be carried out at high accelerating voltage. However, the Formvar-coated titanium grids are very different from normal cell culture substrates, which are used in biochemical and physiological studies. It is therefore important to establish that the response of the cells to physiological stimuli is not qualitatively changed by the substrate. The results in the present study show that this is not the case for cAMP.

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Discussion with Reviewers

A.J. Morgan: Can the authors explain why the electrolyte composition of excitable tissues changes dramatically in comparison, for example, to the composition of pancreas and submandibular glands after dissection, and does not "recover" during *in vitro* incubation? Why is the change irreversible?

Authors: At present, we can only speculate. It is not unlikely that the excitable cells depolarize as a result of dissection, which would give rise to influx of Na and efflux of K in addition to fluxes due to metabolic inhibition or anoxia. *In vivo*, the ionic environment of the brain neurons is closely regulated by glia cells and it is an open question whether this regulation functions *in vitro*.

B.L. Gupta: Was it not possible to measure the dry weight and hence water fractions of the samples? In addition to the changes in Na, Cl and K, the anoxia during dissection is also known to cause volume changes. Such information would have been very useful.

Authors: We did not measure water content, but have no doubt that it increases with increasing Na and Cl concentrations. We are currently carrying out a morphological study confirming volume changes during incubation; the results will be published elsewhere.

K. Zierold: Your data show the dramatic effect of sample excision from the intact tissue on the intracellular electrolyte content. Can you imagine more appropriate incubation solutions and conditions for the recovery of dissected samples or biopsies in order to restore cellular function and intracellular electrolyte distribution closer to the state of the intact tissue?

Authors: A theoretical possibility would be to store the tissue in a solution with a K/Na ratio closer to the intracellular ratio (instead of in a solution resembling the extracellular environment). This method is, e.g., used in the storage of tissue for transplantation (Wahlberg *et al.*, 1986). We are now investigating the effect of such incubation solutions on the intracellular ion content.

A.J. Morgan: Whilst accepting that the responses of cells to physiological stimuli are not qualitatively changed by the nature of the substrate upon which they were grown, it is apparent that there are substrate-related quantitative differences? How are these explained?

Authors: In the experiments described in this study, short stimulation times (about 1 min) were used. Diffusion rates, both of the stimulant and of the secreted ions, may be important under such conditions, and it can be speculated that the solid substrates impede diffusion, compared to e.g., a Formvar film.

K. Zierold: Have you studied the effects of ammonium acetate on the elemental content of the cultured sweat gland cells?

Authors: Washing with ammonium acetate gives results that are not significantly different from those obtained if cultured cells are rinsed with distilled water (von Euler *et al.*, 1993).

K. Zierold: Have you observed differences in the intracellular element content of the cultured cells depending on the degree of attachment of the cells or the state of confluency, respectively?

Authors: The rinsing procedure certainly results in the loss of poorly attached cells, so we selectively analyze well-attached cells. We have not investigated the relationship between the state of confluency and the elemental content of the cells.

Additional Reference

Wahlberg J, Southard JH, Belzer FO (1986) Development of a cold storage solution for pancreas preservation. Cryobiology **23**: 477-482.