# X-Ray Microanalysis of Endocrine, Exocrine and Intestinal Cells and Organs in Culture: Technical and Physiological Aspects

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# X-RAY MICROANALYSIS OF ENDOCRINE, EXOCRINE AND INTESTINAL CELLS AND ORGANS IN CULTURE: TECHNICAL AND PHYSIOLOGICAL ASPECTS

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## Abstract

In the present study methods for preparation of cultured cells and organ cultures for analytical electron microscopy are investigated. These methods allow qualitative and quantitative analysis of mobile ions in combination with biochemical or morphological studies. Cultured cells can be easily prepared for analytical microscopy and therefore use of *in vitro* systems for X-ray microanalysis has increased over the last few years. Two major, anhydrous preparation techniques, by which loss or redistribution of ions is minimized, were used: (1) Cells were cryosectioned and analysis carried out on freeze-dried sections obtained from frozen cell monolayers, pelleted cells or organ cultures. (2) Cells cultured on supports compatible with elemental analysis were frozen after removal of experimental media by rinsing, freeze-dried and analyzed.

The first technique was applied to the studies of the elemental content of isolated Langerhans islets and thyroid follicles cultured in collagen gel. The second was used in studies of the ionic changes in enterocytes.

Data obtained from organotypic cell cultures and cultures of single cells were compared with analytical data obtained from sections of corresponding tissues, where isolation, culturing and steps in processing such as removal of culture or experimental medium were omitted. It was shown that often culture systems fully acceptable to physiologists have an elemental composition different from that of tissue *in situ* and can not be regarded as fully normal tissue.

**Key words:** Cell culture, ions, microanalysis, endocrine organs, intestine, preparation methods.

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## Introduction

Strict control of experimental conditions is the main reason why cell and organ cultures are so widely used in biomedical research and also why they are more and more frequently used in microanalytical studies. Cell cultures can be easily prepared for analytical purposes, simultaneously with preparations for biochemical and morphological purposes. *In vitro* systems allow a study of the effects of different factors such as toxic substances, teratogens, growth factors, irradiation as well as changes in the ionic content of the extracellular milieu. Several cellular processes have been shown to be regulated by changes in the extra- and intracellular content of inorganic ions. Also by fluorometric methods and microelectrode techniques one can measure the changes in ionic content as a result of interaction of the ligand with its receptor. However, in such studies only one ion can be studied at a time.

Development of preparation techniques of cells and organs in culture for microanalysis during the last decades contributed to the frequent use of *in vitro* systems in combination with microanalytical applications. Two main preparation techniques can be distinguished, depending on the way the cells or organs are to be analyzed: (1) analysis of cryosections and (2) analysis of cells grown on specimen supports which are also used during analysis. If the analysis is going to be performed on cryosections there is no need for a rinsing procedure prior to cryofixation. Cells in the form of a pellet can easily be used. Cells cultured on supports that function at the same time as specimen holder must, however, be rinsed prior to cryofixation as elemental content of the culturing medium will interfere with measurement of the elemental content of the cells. The present paper deals with the methods of preparation and analysis of cultured cells performed on entire cells which were not cryosectioned prior to analysis. We also tried to illustrate changes in elemental composition of cells in organotypic cultures due to isolation, culturing and other steps in processing. Knowledge of such changes is important for researchers carrying out physiological experiments on this type of preparation.
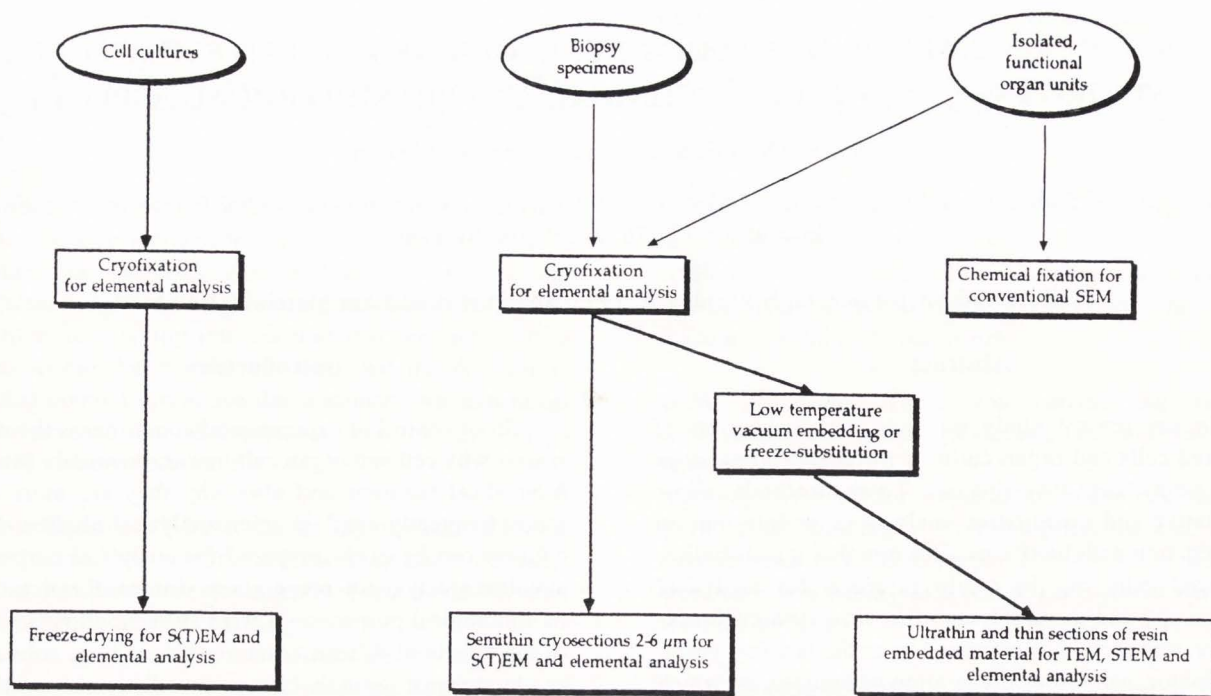
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**Fig. 1.** Processing routes used in the present study.

Examples of preparation and analysis of cultures of different cell types as well as of organotypic cultures will be presented (Fig. 1). The aim of this study was also to show the existence of changes in elemental composition of such systems that are often neglected by the physiologist, whose only criteria often are the response of the cells to the given stimuli.

## Materials and Methods

### Material

*In vivo* studies were performed on male Sprague-Dawley rats, *ob/ob* mice and domestic pigs. *In vitro* studies were performed on thyroid follicle cultures from domestic pigs, isolated Langerhans islets from *ob/ob* mice and on the rat intestinal cell line IEC-6.

### Freezing and sectioning of specimens for X-ray microanalysis

In the present study, prior to freezing excess water (medium) was quickly absorbed with a filter paper. Tissue samples, organotypic cultures on Nucleopore support and cultured cells on transparent support were cryofixed by plunging into Freon 22 cooled by liquid nitrogen or liquid nitrogen alone. Sections of frozen tissues (2-6 $\mu$m thick) were cut on a cryostat at -30°C. They were picked up on specially designed carbon specimen holders for X-ray microanalysis (Wroblewski

*et al.*, 1983). In such cryosections it was possible to analyze separately nucleus and cytoplasm. For light microscopical examination, adjacent sections were mounted on glass slides and stained with hematoxylin-eosin.

Freeze-drying of cultured cells was done in a conventional freeze-drier at < -85°C for 48 hours and subsequently the cells were coated with carbon. We found that films on a grid withstand freezing and freeze-drying better than films stretched over a large hole in the carbon plate. In some cases frozen specimens of pancreas and of isolated Langerhans islets were embedded in Lowicryl K11M and HM23 using a low temperature vacuum embedding system (Wroblewski and Wroblewski, 1984). Samples are freeze-dried and embedded in Lowicryl and polymerized at low temperature which preserves the elemental distribution.

### Electron microscopy and X-ray microanalysis

The cultured cells on titanium grids and carbon specimen holders, cryosections and Lowicryl sections designated for X-ray microanalysis were examined in a JEOL 1200 CX electron microscope equipped with a scanning attachment and a TRACOR energy dispersive X-ray spectrometer. The specimens were examined in the scanning transmission mode at either 100 or 120 kV. The total counting time (live-time) was 50 -100 seconds. Quantitative analysis was carried out using salt-gelatin

**Table 1.** Requirements for growth support of cultured cells.

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- ○ non toxic
- ○ easy to sterilize
- ○ cellophilic (hydrophilic)
- ○ resistant to 70 % ethanol, UV and salts
- ○ heat conducting
- ○ compatible with analysis

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**Table 2.** Types of supports for XRMA of cultured cells

*Solid supports*
- ○ coverslips (glass + Thermanox)
- ○ silicon chips
- ○ culture dishes
- ○ titanium and gold stubs
- ○ pure graphite and beryllium plates

*Thin transparent supports*
- ○ Formvar or Pioloform film on Parafilm
- ○ Formvar or Pioloform film coated grids of titanium, gold, carbon or plastic
- ○ Formvar or Pioloform film stretched over a hole in a carbon plate

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standards (Wroblewski *et al.*, 1983). The cultured cells designated for topographical studies were examined in a Philips 515 scanning electron microscope.

Analyses of Lowicryl sections ($0.1 \ \mu m$ < thick) allowed visualization of organelles down to the size of ribosomes. In $\beta$-cells it was possible to see nuclei, the Golgi apparatus and numerous dense vesicles of varying density and size (20-100nm).

## Conventional scanning electron microscopy

Cells were cultured on plastic cover slips (Thermanox) placed on the bottom of 12-well plates (Fig. 2 and Fig. 4). 48 h after irradiation, the cells were fixed in glutaraldehyde in phosphate buffer followed by $OsO_4$, dehydrated in a graded series of ethanol and Freon 113, and critical point dried. Samples were thereafter coated with gold. Scanning electron microscopy was then performed at an accelerating voltage of 20-30 kV.

## Technical Aspects of Cell Culture Preparation

### Specimen support

The specimen support must be chosen with respect to the planned mode of operation of the electron microscope (Table 1). A solid support is usually chosen for analysis in the scanning mode. A thin support must be chosen when the image is formed by transmitted electrons as in scanning transmission (STEM) and transmission (TEM) electron microscopy. In both cases however, the support should not contribute to the analytical spectra due to characteristic peaks. The contribution of the support to background (continuum) radiation should be minimal.

A thin layer of collagen or other extracellular matrix proteins can be used to cover the holder prior to cell culturing. The elemental contribution of such layers to the spectrum is negligible due to the limited thickness.

### Solid supports

For analysis in a scanning electron microscope solid supports are mainly used (Table 2). A spectroscopically pure carbon planchet is a typical solid support and cells can be grown directly on its surface. However, it is advisable to coat with e.g., a Formvar film. The elemental composition of such films has to be checked. Some culture dishes can be dissolved in organic solvents and films produced from that material. This can be a solution for those who want to have similar growing conditions for cells aimed for X-ray microanalysis and for other investigations. One has, however, to take into account that some of the materials are difficult to dissolve and that some culture dishes have an extra coat and that this layer often is essential for cell adhesion and growth. Cells can also be cultured in original culture dishes or pieces of dishes. However, charging due to the insulating characteristics of the plastic can be a serious problem. The image can be improved by imaging with backscattered electrons instead of secondary electrons. Chlorine-free plastic coverslips inserted into the Petri-dishes can also be used. Advantages and drawbacks of the use of solid supports are summarized in Tables 3 and 4.

### Transparent supports

For analysis by transmission and scanning-transmission electron microscopy, grids or specially designed carbon specimen holders can be used. Similar to solid supports, grids should not be of toxic material or have other adverse influences on the growing cells. Grids are covered with a plastic films (Fig. 2 a-c) and grid materials include carbon, nylon, titanium and gold (Table 2). Titanium grids have been widely used. The titanium peak does not generally interfere with the peaks of interest and titanium is a biocompatible material. Titanium grids are also stronger and less sensitive to mechanical force during preparation procedures than gold grids. Usually growing the cells on grids does not create special problems. The supporting film (Formvar or Pioloform) should be carbon coated, if possible glow
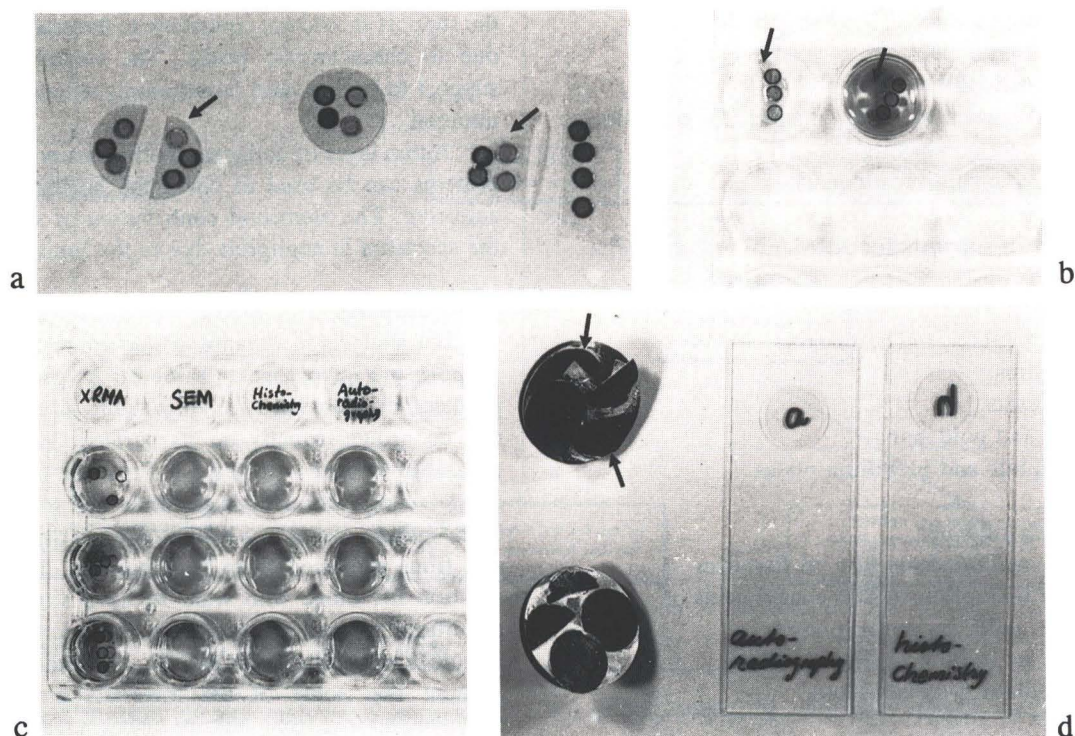
**Fig. 2 a-b.** Transparent and solid supports. **a.** The grids were coated with Formvar film by placing them in triplicates or quadruplicates onto squares of film floating on the surface of distilled water. The film with the grids was then removed from the water with a piece of Parafilm or Termanox coverslips (arrows). **b.** The grids and Termanox coverslips were placed in the bottoms of dry culture dishes and droplets of cell suspension were deposited on the Formvar film covering grids (arrow). After the cells had attached to the growth support, more medium was added to the dishes (asterisk). **c.** For scanning microscopical, histochemical and autoradiographical investigations droplets of cell suspension were deposited in the same way on empty Termanox coverslips in adjacent wells of 12 hole plates. **d** Mounted on a common specimen holder Termanox coverslips with cells processed for conventional scanning microscopical investigations (arrows) and coverslips used for autoradiography and histochemistry mounted on light microscopical slides.

discharged (to make it hydrophilic) and sterilized before culture. Breakage of the film can also occur on grids, but is often limited to a few windows of the grid. Carbon specimen holders are an alternative to grids. They are easy to handle and allow numerous cells to be cultured on the film stretched over a 3 mm hole. Analysis can be performed in all modes of microscopy: scanning electron microscopy (SEM), TEM, and STEM). The extraneous background is negligible and fully quantitative analysis can be performed without additional correction for contribution of the extraneous signal. Breakage of the film could, however, be a much more serious problem than when grids are used as support. Another alternative which we used was to peel off the film with the cells from the Thermanox coverslips and sandwich it in the JEOL carbon specimen holder. This alternative was developed to solve problems

with breakage of the film on carbon holders which in most cases was due to the rinsing solution left under the carbon plate. Advantages and drawbacks of use of transparent supports are summarized in Table 3 and 4.

**Removal of medium, rinsing solutions**

Handling of the cells in culture after the experiment and prior to freezing is a crucial step in the preparation. Medium surrounding the cells (culture medium or experimental medium) has to be removed prior to freezing, to avoid contribution to the analytical results. Several rinsing solutions have been used. The criteria for "rinsing solutions" is that they should not cause elemental changes in the cells and their composition should not interfere with the elements of interest within the cells (Table 5). Rinsing solutions should not disturb the visualization in the electron microscope nor add to

the mass of the analyzed cells.

Until now there is no rinsing solution which accomplishes all above criteria. Furthermore, it has been shown that different types of cultured cells react differently to rinsing solutions. Therefore it is necessary to experimentally determine which constitution, osmolarity, and temperature of rinsing solution does not cause elemental changes within the cells. In such experiments numerous cells should be analyzed as cells in culture are not in exactly the same phase of the cell cycle. Analytical results with small deviations might indicate that the rinsing solution causes changes to the entire cell population rather than the possibility of a uniform elemental composition of the entire cell population. However, comparison with cryosectioned cells is the most adequate control and should be applied if possible.

Distilled water, or volatile buffers, such as ammonium acetate or sucrose solutions of the same osmolarity as the culture medium or the experimental buffers can be used (Wroblewski and Roomans, 1984). A sucrose solution has a very small effect on the ionic composition of the cells, but, if not fully removed it can cover the cell monolayer and its thickness will deteriorate the image. The presence of sucrose on top of the cells will also affect the final elemental concentration due to its contribution to the mass of analyzed volume. Ice-cold distilled water has been shown to be ideal rinse solution for several cell types (Lechene, 1989, Wroblewski and Roomans, 1984, Sagström *et al.*, 1992, von Euler *et al.*, 1992, Östlund *et al.*, 1993).

## Comparison of Analytical Data Obtained on Cultures of Single Cells with Cells *in Situ*

### Intestine

Effects of radiation were studied in *in vivo* and *in vitro* systems (Jalnäs *et al.*, in preparation). The *in vivo* study was carried out on the rat small intestine and for the *in vitro* study the intestinal crypt cell-line IEC (Fig. 3a,b and Fig. 4) was used. Rat intestine and IEC-6 cells were irradiated with X-ray doses ranging between 1-16 Gy. Energy-dispersive X-ray microanalysis was used to detect the elemental changes in the cells. Cell morphology was investigated in the scanning electron microscope (Fig. 4), DNA-synthesis by autoradiography of $^3$H-thymidine incorporating nuclei and proliferation by cell counting.

*In vivo* studies. Male Sprague-Dawley rats, weighing approximately 350 g were used. Under anesthesia the distal part of the ileum was lifted out and exposed to X-irradiation at 250 kV and 15 mA. The rest of the animal was protected by a special lead-chamber.

**Table 3**. Advantages of transparent and solid supports for XRMA of cultured cells

*Thin transparent support*
- good visibility during culture
- high speed of cryofixation
- correlation possibilities (XRMA-autoradiography, histochemistry, SEM on co-cultured specimens)
- preview possibilities in LM (prior to EM)
- visualization and analysis in all modes of electron microscopy
- higher spatial resolution
- higher sensitivity
- possibilities to use low and high accelerating voltages without support contribution

*Solid support*
- easier to handle
- ideal for SEM observations

**Table 4**. Drawbacks of transparent and solid supports for XRMA of cultured cells
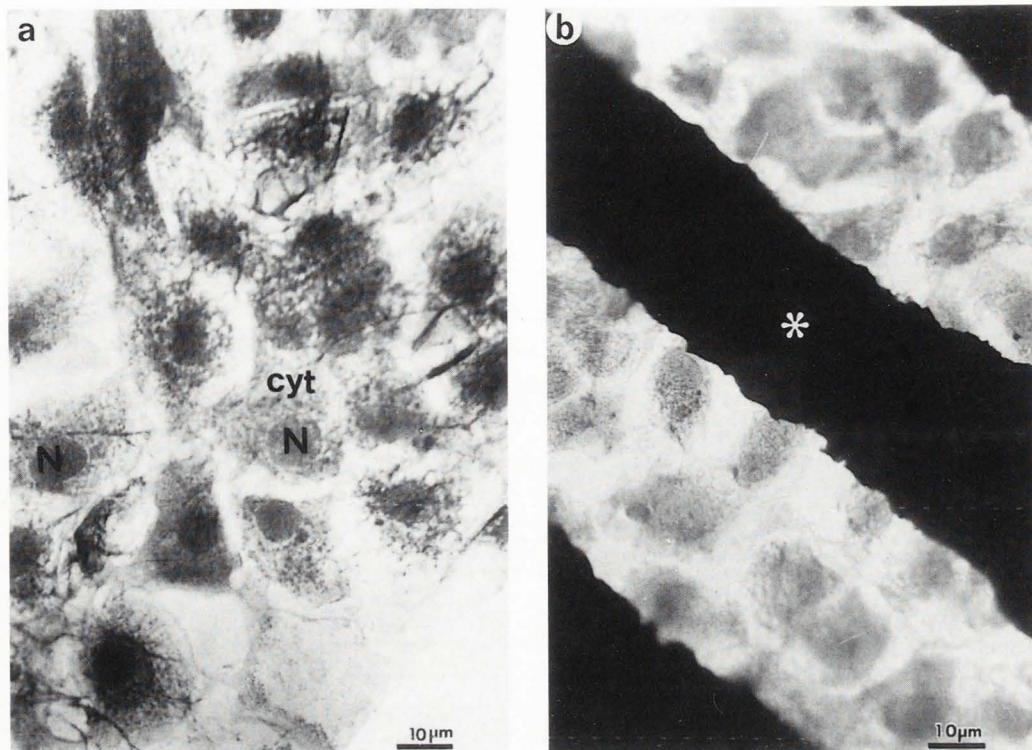
*Thin transparent support*
- fragile
- supporting film breakage

*Solid support*
- difficulties in observation of cells during culturing
- lower sensitivity
- low freezing speed
- X-rays from solid support
- low count rate at low accelerating
- poor spatial resolution
- limited visualization possibilities during analysis
- insulation problems causing charging during observation and analysis

**Table 5**. Requirements for rinsing solutions

- should not contain detectable elements
- should not cause loss of elements
- volatile (if possible)
- low viscosity

**Fig. 3 a-b.** Transparent supports. STEM micrograph of intestinal epithelial cells IEC-6, cultured on a Formvar film stretched over a hole in the carbon plate **a** and on titanium grid **b**. Several cells are present within each grid window. The nucleus (N) and cytoplasm (cyt) are visible, grid bars are marked with an asterisk. The Formvar film outside the cells is almost free of the visible extracellular matrix. No remnants of culture medium can be seen. Broken Formvar/carbon support can be seen in the lower right hand corner of Fig. 3a.
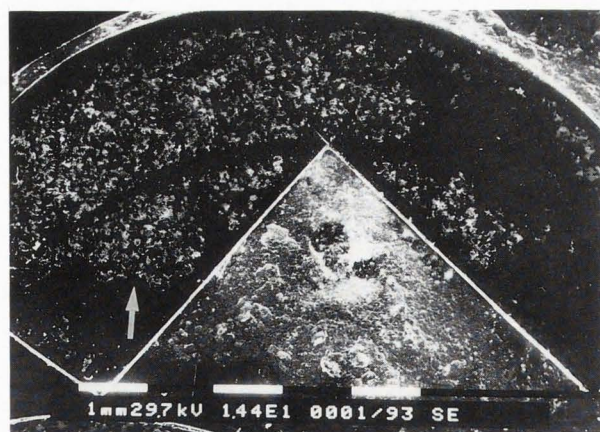


**Fig. 4.** Solid supports. Scanning electron micrograph of the Termanox coverslip with IEC-6 cells (arrows). The same preparation is visualized in Fig. 2d.

Immediately after irradiation the bowel was pulled back intra-abdominally and the incision was closed with stitches. The radiation doses varied from 9-21 Gy. Ten days after irradiation the rats were anesthetized and portions of ileum were quickly dissected out. The samples were plunged into liquid Freon 22, cooled by liquid nitrogen. Normal ileum was taken from the part proximal to the irradiated area and from control rats. Samples were stored in liquid nitrogen.

Sections of frozen intestine (2-6 $\mu$m thick) were cut on a cryostat at -30°C. They were picked up on specially designed carbon specimen holders for X-ray microanalysis. In such cryosections it was possible to analyze separately nucleus and cytoplasm. For light microscopical examination, adjacent sections were mounted on glass slides and stained with hematoxylin-eosin.

Analysis of crypt cells from irradiated intestine (Fig. 5a) showed that already at 9 Gy elemental changes occurred in the cytoplasm of these cells. Changes became more prominent at 12 Gy, with significantly lowered phosphorus and potassium concentrations.

*In vitro* studies. The rat intestinal cell line IEC-6 (Quaroni and May, 1980) was obtained from the American Type Culture Collection (Rockville, MD) at passage 14. The cells were propagated in 75-cm²-flasks from Costar (Cambridge, MA) in DMEM (Dulbecco's
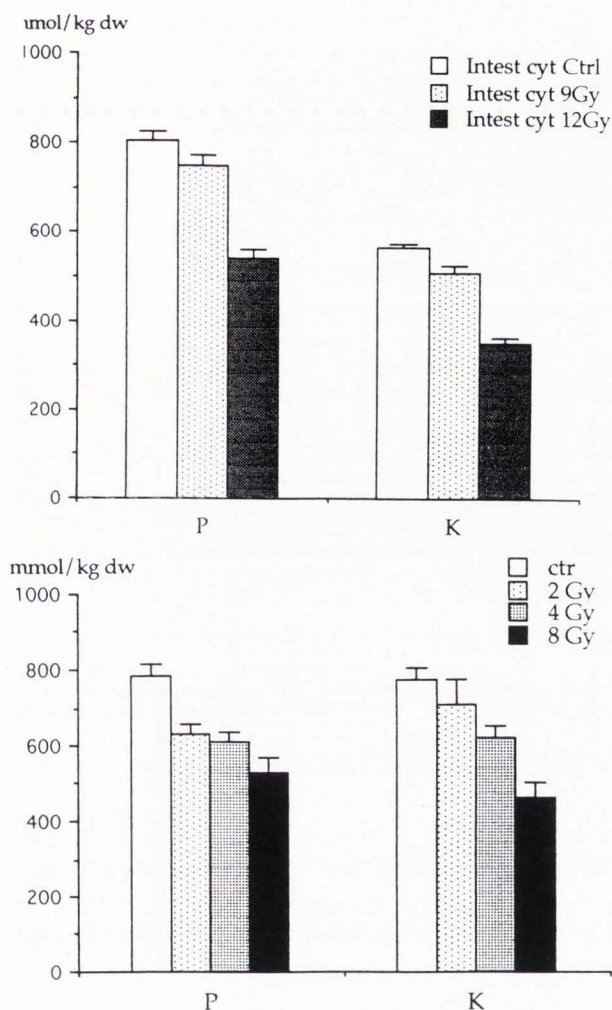
**Fig. 5 a-b.** Effects of irradiation on elemental concentrations of intestinal cells in vivo and *in vitro*. **a** Concentration of elements studied in sections of small intestine and **b** in intestinal crypt cells of cultured IEC-6 cells. Elements are marked with their chemical symbols. Data are expressed as absolute concentrations in $mmol/kg_{dw}$. Mean and standard error of mean are given.

Modified Eagle's Medium) containing 5 % FCS (fetal calf serum), 10 $\mu g$/ml insulin, 25 U/ml penicillin and 25 $\mu g$/ml streptomycin (standard medium) and maintained at 37°C in water saturated atmosphere with 5 % $CO_2$. The cells were harvested by exposure to a Trypsin-EDTA solution. The experiments were performed during passages 15-25.

Cells cultured on different growth supports (depending on type of analysis) were irradiated at the same time (Fig. 2). The radiation source and protocols were the same as in the *in vivo* experiments. The irradiation dose varied from 1-16 Gy. During irradiation precaution was taken to keep the temperature constant (37°C) through-

out the experiment. Control cells were treated identically except for irradiation.

For X-ray microanalysis, the cells were grown directly on titanium grids (Agar Aids) or specially designed carbon plates (Wroblewski and Wroblewski, 1993). The grids and the carbon plates were coated with Formvar film. The film was evaporated with carbon which renders it cellophilic and more stable under the electron beam. The grids and carbon plates (sterilized by UV-illumination or rinse in 70 % ethanol) were placed on the bottom of dry culture dishes and droplets of the cell suspension were deposited on the Formvar film. After the cells had attached to the growth support, more medium was added to the dishes (Fig. 2b). This procedure gives a higher cell density on the support and is of interest when the number of cells is limited.For X-ray microanalysis the cell-covered grids and carbon plates were quickly rinsed in redistilled water (4°C) 48 h after irradiation, blotted with filter paper and rapidly frozen in Freon cooled by liquid nitrogen (Wroblewski and Wroblewski, 1993). After freezing the cells were freeze-dried and slowly brought to room temperature and atmospheric pressure. Prior to microanalysis cells were evaporated with carbon.

X-ray microanalysis of the cultured cells (Fig. 5b) revealed that increasing doses of irradiation caused in a dose dependent manner, a decrease of phosphorus and potassium concentration. Already at 2 Gy, the concentration of phosphorus was significantly lowered, while the level of potassium was reduced first at 4 Gy.

Our results indicate that *in vivo*, in the crypt cells, the increasing doses of irradiation led to lowered potassium and phosphorus concentrations. Corresponding ion shifts were found in the irradiated IEC-6 cells (Fig. 5a,b). Proliferation rate and DNA-synthesis became lower with increasing dose of irradiation in IEC-6 cells. No mitotic figures could be found in crypts of irradiated small intestine (Jalnäs *et al.*, in preparation). The study clearly demonstrates that changes caused by irradiation in the ionic composition in the crypt cells in vivo were the same as in the intestinal cell line *in vitro*. This indicates that cultured IEC-6 cells can be used for investigation of side effects of radiation to the small intestine.

## Comparison of Analytical Data from Organotypic Cultures with Cell and Organ systems *in Situ*

### Endocrine pancreas

The endocrine pancreas consists of small endocrine glands, the Islets of Langerhans, which are approximately 1 % of the pancreatic volume and are scattered randomly within the glandular substance of the exocrine
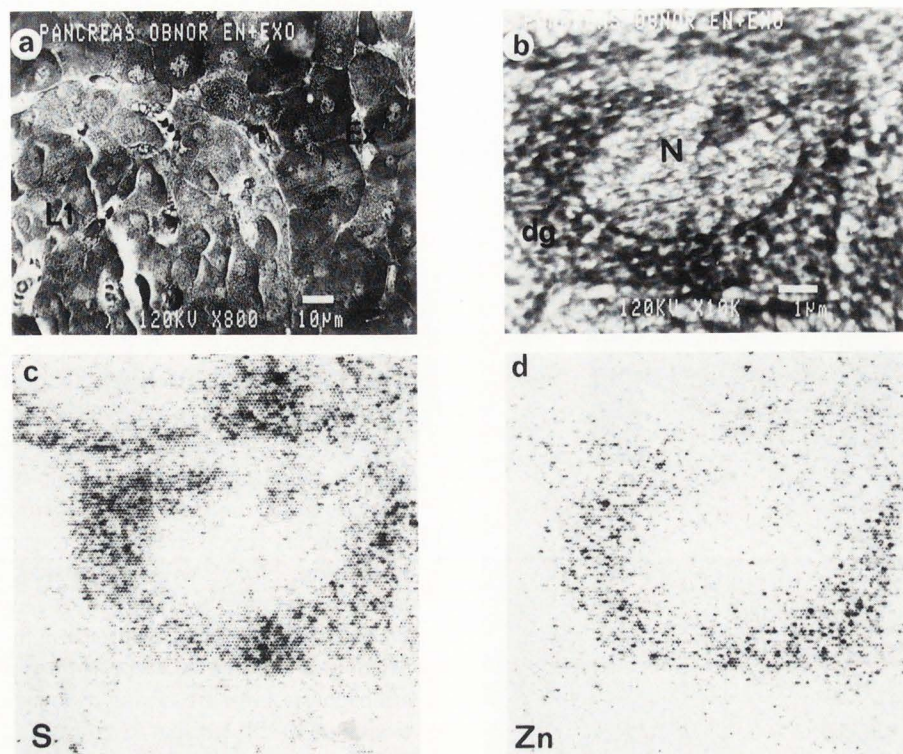
**Fig. 6 a-d. a** Scanning transmission micrograph of an unstained cryosection of normal mouse pancreas showing the Langerhans islets (LI) and the exocrine pancreas (Ex): Note lower electron density of the Langerhans islets and density of the capillaries. At higher magnification of the cell marked with an arrow is shown in **b**. In $\beta$-cells, the nucleus (N) and dense granules (dg) are easily identified. **c-d**. Elemental distribution maps of (c) sulphur (S) and (d) zinc (Zn) in $\beta$-cell in the Langerhans islets.

pancreas (Fig. 6a). Islet $\beta$-cells are responsible for insulin synthesis and secretion. The present study was undertaken to study to which extent isolation and culturing affect the elemental content of $\beta$-cells. For this reason, obese hyperglycemic *ob/ob* mouse were used. These mice exhibit islet hypertrophy and $\beta$-cell hyperplasia (more than 90% of the islet cells are of the $\beta$-type). Numerous investigations testify to the fact that these $\beta$-cells are functionally normal (Arkhammar *et al.*, 1987) and have shown stimulus-secretion coupling pathway resulting in insulin release. Several studies of this kind were performed using isolated islets and by changing the concentration of e.g., glucose and ions in the experimental media. The present study was performed on isolated islets and islets *in situ* within the exocrine pancreas (Fig. 6).

Using semithin cryosections it was possible to selectively measure elemental content in the nucleus, cytoplasm and in areas rich in the dense granules representing insulin granules in $\beta$-cells. The spreading of the electron beam in the rather thick sections prevents, however, accurate analysis of these small granules. The

presence of high levels of S, Zn and Ca in the cells within the islets indicated that analyzed cells were of the $\beta$-type since $\beta$-cells are known to contain relatively much sulphur, zinc and calcium. Zinc is mainly localized in the core of secretory insulin granules where also calcium and sulphur are present (Hutton, 1989; Nordlund *et al.*, 1987; Foster *et al.*, 1993). Cells with no Zn were characterized as non $\beta$-cells and were not included in present study.

***In vivo* studies on endocrine pancreas.** *ob/ob* mice weighing approximately 150 g and normal mice without obese gene were used. The pancreas was quickly dissected out and the samples were plunged into liquid Freon 22, cooled by liquid nitrogen. Samples were stored in liquid nitrogen. Sections of frozen pancreas (2-6 $\mu$m thick) were cut on a cryostat as described above for the intestine (Fig. 6).

***In vitro* studies on isolated Langerhans islets.** Langerhans islets were isolated from pancreas of *ob/ob* mice as described by Nilsson *et al.* (1987). Islets were incubated for 4 hours in culture medium containing 3mM glucose and 1.28 mM $Ca^{2+}$. After incubation the
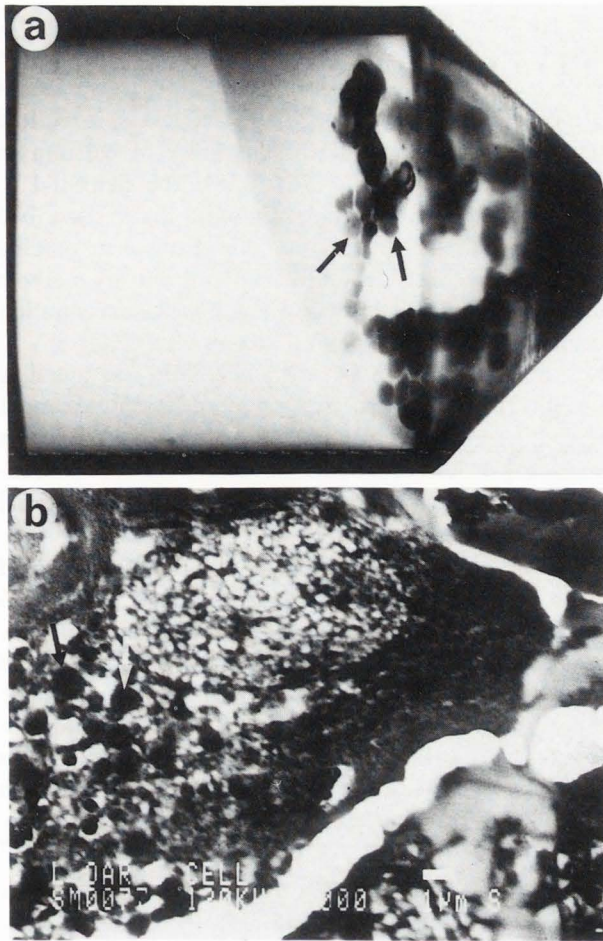
**Fig. 7 a-b. a** Micrograph of Lowicryl embedding showing numerous Langerhans islets (arrows) processed according to low temperature vacuum embedding process. The diameter of single islets is about 1mm. **b**, Scanning transmission electron micrograph of dry cut Lowicryl section showing $\beta$-cells with secretory granules (arrows).

Langerhans islets were placed on a strip of aluminum foil (5x15 mm). The incubation medium was soaked off with filter paper and the islets were coated with a drop of Tissue-Teks cryoembedding OCT compound (Miles Scientific, USA). The foil with the islets was frozen in Freon 12 cooled by liquid nitrogen and stored either in liquid nitrogen or in freezer at -80°C. OCT-embedded tissues can be stored for months without signs of drying as OCT compound prevents desiccation.

Langerhans islets without cryoembedding compound were frozen separately and used for low temperature vacuum embedding in Lowicryl (Wroblewski and Wroblewski, 1984). In Lowicryl sections, which are much thinner, granules were clearly seen and X-ray

analysis could be performed with higher spatial resolution than within semithin cryosections (Fig. 7).

**Thyroid follicles**

The thyroid follicle is a functional unit of the thyroid gland. The follicle is spherical and composed of a single layer of epithelial (follicular) cells that encloses the follicle lumen which is filled with colloid containing prohormone (thyroglobulin). Iodinated thyroglobulin is synthesized by follicular cells and stored in the lumen until it is taken up by endocytosis followed by lysosomal degradation resulting finally in release of thyroid hormones. As both exo- and endocytosis are taking place in the same cell the normal polarity of follicle cells is of crucial importance for normal function.

**Thyroid follicles *in vitro*.** Thyroid follicles were isolated from pig thyroid gland by perfusing the gland with collagenase through a blood vessel. Free isolated follicles were then seeded and cultured in 12 hole plates filled with collagen gel (Westermark *et al.*, 1991).

Prior to freezing, gels have to be cut out from 12 hole plates. After the gels were cut out excess of water was removed with a filter paper. The gels will shrink and roll up and can then be picked up and frozen directly using small tweezers.

**Organotypic cultures.** The most crucial preparation step in the preparation of the thyroid follicles and Langerhans islets was to find a way to catch free floating units and cryofix these. This could be successfully done with the titanium grids which were functioning as a fishing net. However, grids gave problems in the sectioning stage since hitting the grid could be disastrous to the knife edge. Sometimes the collecting procedure was not completed quickly enough in the experiments with controlled, short exposure times (such as 10-20 seconds) to certain substances in the incubation medium.

As an alternative to the titanium grids we used a small rim of a Nucleopore filter which could be used as a collection tool as well as a support for the Langerhans islets during the incubations. The Nucleopore filter and the islets can be simultaneously frozen and cryosectioned or processed through the entire low temperature vacuum embedding process or the freeze-substitution. The Nucleopore filter is totally infiltrated by the Lowicryl resin and does not cause any problems during sectioning or analysis.

For cryosectioning, the Nucleopore filter method was discontinued. Instead, a thin aluminum foil with a small droplet of Tissue-Teks OCT compound was used. The tissue was incubated in the experimental medium and at the end of the incubation time it was sucked into the very narrow tip of a micropipet and placed on a

droplet of OCT compound earlier positioned on the aluminum foil. The foil was then immediately cryofixed. OCT compound serves as matrix for the islets and also gives stability to the entire specimen, thus allowing the cutting of large sections with numerous islets. Due to the insulating properties of OCT compound, this procedure will slightly reduce the freezing speed compared to the situation in specimens mounted on titanium grids or on the rim of Nucleopore. However, the specimens are easier to mount in the cryostat without risk of thawing. OCT compound does not cause any elemental changes in the specimen as compared to tissue cryofixed and cryosectioned without any support.

## Comparison of Analytical Data from Organotypic Cell Cultures and Cultures of Single Cells with Cell and Organ Systems *in Situ*

Data obtained from organotypic cell cultures and cultures of single cells were compared with analytical data obtained from the same cell type within sections, where isolation, culturing and steps in processing such as rinsing had been omitted. In this investigation a comparison was made between porcine thyroid follicles analyzed in the sections of the gland and the isolated follicles cultured in collagen gel. Similarly, a comparison was made between $\beta$-cells in sections in pancreas of *ob/ob* mice and $\beta$-cells within isolated cultured islets (Fig. 8).

In porcine thyroid follicles analyzed within sections of the thyroid gland and in the isolated follicles cultured in collagen gel we selectively analyzed follicular cells and colloid fluid. The elemental concentrations measured in follicles are given in Fig. 9 a,b. In follicular cells besides changes in mobile elements (Na, Mg, Cl, K, Ca and I) there are also significant changes in phosphorus. It is likely that elemental changes partly reflect the altered functional state of the cell membrane but the elevated phosphorus content may also indicate a disturbance in endocytosis and exocytosis. High concentrations of phosphorus were shown to occur in dense granules (vesicles) which were also rich in sulphur and iodine and therefore probably active in endocytotic processes (Wroblewski *et al.*, 1991). Sulphur levels are slightly lower indicating lower protein synthesis and/or lower rate of sulfation of thyroglobulin in the Golgi apparatus (Herzog, 1985) prior to exocytosis and iodination. The ratios between sulphur and iodine in isolated follicles and in the colloid are higher than in the gland *in situ* (Fig. 10) most likely due to lower a iodination rate of the thyroglobulin. The comparison of the colloid fluid mirrors the processes in the follicular cells. P and Mg, which are lower in follicular cells of
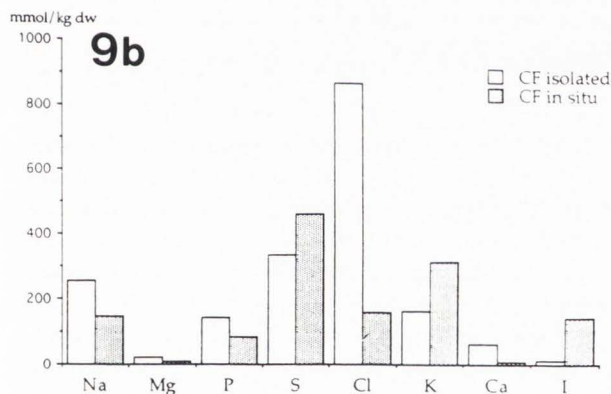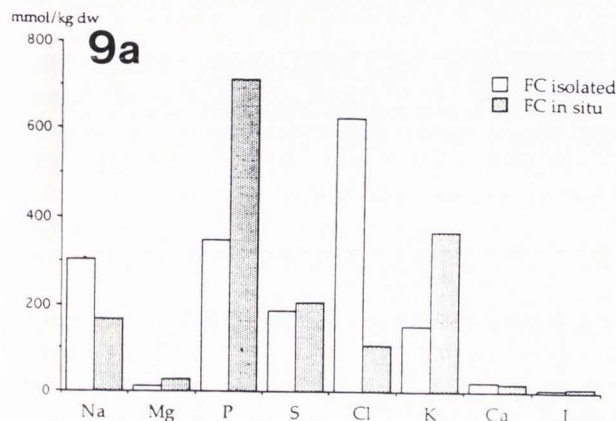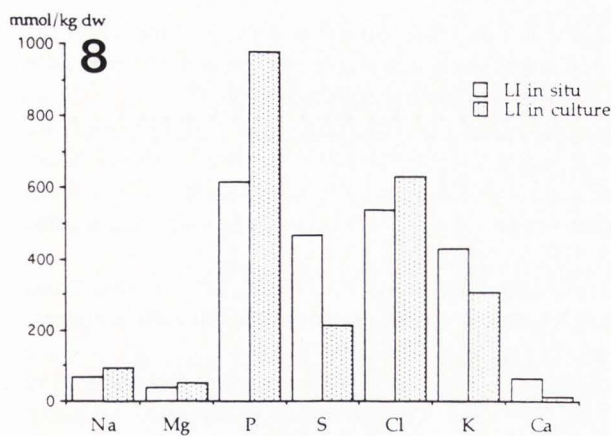
the isolated follicles are elevated in the colloid. Colloidal Ca, Na and Cl were also elevated which could partly reflect cellular events but could also be due to the increased intercellular permeability due to structural changes in tight junctions. High Ca levels in colloid could also be due to the high affinity of calcium to thyroglobulin (Haeberli *et al.*, 1978). The elemental changes in the isolated and cultured follicles described above are similar to post mortem changes in porcine thyroid. It is likely that follicular cells in organotypic cultures are not able to maintain a high K concentration in the cells and in the colloid lumen.

A decrease in K in cells and colloid was found as a result of post mortem changes after 20 minutes and 1 hour. The matrix (collagen gel) in which the follicles were grown showed S and K but at much lower concentrations than found in the connective tissue of the basal lamina in vivo.
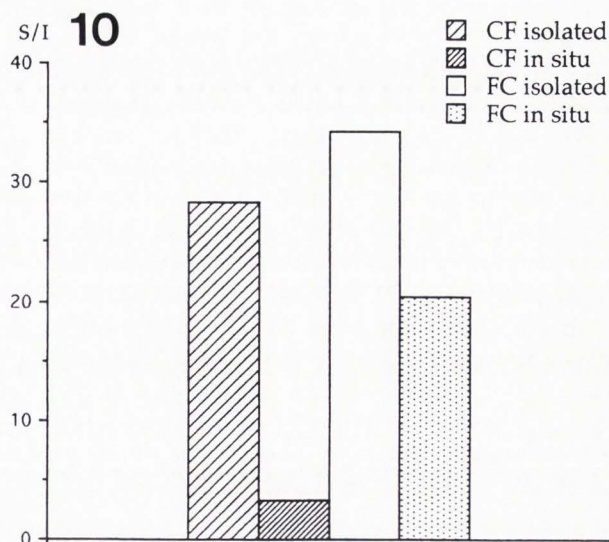
Langerhans islets isolated from the pancreas of *ob/ob* mice were cultured for a recovery period of 6 hours prior to cryofixation. No rinsing was applied prior to cryofixation. The islets were cryosectioned in the same way as the entire pancreas obtained from the same mouse strain. In the present study Zn was taken to be a marker for the insulin containing cells - $\beta$-cells since there are no other organelles in pancreatic cells with such high zinc concentrations (Fig. 6 a-d). X-ray microanalysis revealed a minor increase of Na, Mg, P and Cl in $\beta$ cells of isolated islets and an decrease in S, K and Ca compared to the *in vivo* situation. The decreased S/P ratio in isolated islets is similar to the changes found in isolated thyroid follicles. The changes in P and Mg (increased in isolated islets) are opposite, however, to findings in the follicular cells *in vivo* and *in vitro*. It is possible that lowered sulfur levels can be attributed to decreased synthesis and partial secretion of insulin content from the $\beta$-cells. Normally secretion of the insulin in beta cells is triggered by depolarization of the cell membrane followed by calcium influx. The fall in calcium level in isolated $\beta$ cells is difficult to explain but it is possible that some of the calcium was released together with insulin. Data obtained from cryosections agreed with data obtained from low temperature vacuum processed Langerhans islets embedded in Lowicryl resin (not shown).

The elemental changes in organotypic cultures used in numerous laboratories showed the need of controlling many parameters before organotypic systems can be used in physiological experiments. This does not necessarily preclude the use of such systems since it has been shown that cells in organotypic systems of thyroid follicles and Langerhans islets are sensitive to different types of stimuli. It is important to be aware that the

**Fig. 8.** Comparison of elemental concentrations of β-cells analyzed *in situ* in the cryosections of entire pancreas and in cryosections of the organotypic cultures of isolated Langerhans islets. Elements are marked with their chemical symbols. Data expressed as absolute concentrations in mmol/kg$_{dw}$. Thin bars represent the standard error of mean. o denotes a statistically significant difference ($p < 0.05$).

**Fig. 9 a-b.** Elemental composition of thyroid follicular cell cytoplasm (FC) and of colloid fluid (CF) in *"in situ"* preparations as compared with isolated thyroid follicles. Data obtained by analysis of 2-6 μm cryosections. Data are expressed as absolute concentrations in mmol/kg$_{dw}$. Thin bars represent the standard error of mean.

o denotes a statistically significant difference ($p < 0.05$).

**Fig. 10.** Sulfur to iodine ratio in colloid in follicles *in situ* and in isolated thyroid follicles cultured in collagen gel.

elemental changes affect the normal function of the cell as well as its reaction to different stimuli. The cause of the elemental changes may be rather complex and elemental changes might be due to several factors such as anesthesia, anoxia during dissection, post-mortem changes, isolation and culturing procedures. The recovery process of organotypic cultures should be critically studied. We found that shortening of the "recovery time" of Langerhans islets in organotypic cultures from 24 to 6 hours resulted in cultures with an elemental content closer to islets *in situ* and with the same response to different stimuli.

## Conclusions

Changes in the elemental composition of cultured cells and organotypic cultures can be measured by X-ray microanalysis (XRMA) which, combined with biochemical or morphological techniques, contributes to an understanding of the role of ions in physiological and pathological processes. XRMA in the electron microscope allows besides visualization simultaneous detection and quantitative analysis of several elements, even if it does not provide information on whether the element occurs as a free ion or in a bound state. Cell cultures can be relatively easily prepared for XRMA without the

use of costly equipment such as a cryostat or cryoultramicrotome.

Cells for XRMA should be grown on a support that is compatible with cell culture conditions and with XRMA, and is inert to the cells. Cultures grown on transparent supporting film allow observation in all modes of light and electron microscopy and are therefore preferable. Our results showed that cultured cells and organotypic cultures provide a useful and reproducible experimental model for the study of cell physiology and the mechanisms of drug and irradiation treatment. XRMA of whole cultured cells may in many cases replace use of animals and time- consuming preparation of thin cryosections. Organotypic cultures can be used to study elemental changes in systems that earlier have been mainly used in physiological studies.

## Acknowledgements

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## Discussion with Reviewers

**B.L. Gupta:** In the irradiated intestinal cells (Fig. 5), there is loss of phosphorus as well as of potassium. Could it be due to an osmotic swelling of the cells? Influx of sodium and loss of potassium can often cause osmotic swelling.

Microanalysis of cells and organs in culture

**Authors:** We could not detect any visible swelling of the irradiated crypt cells *in vivo* and *in vitro*. However, swelling was noted in the intestinal villus cells *in vivo*. As our data are based on the dry weight, we can not exclude that some of the described elemental losses in the irradiated cells may be due in part to higher water content in the cells.

**L. Edelmann:** Fig. 7b shows considerable shrinkage artefacts which - according to own experience - may be due to incomplete freeze-drying at low temperature. The questions: How long did you freeze-dry, at which temperature and did you try to improve the structure preservation by longer freeze-drying at low temperature?
**Authors:** Electron micrographs of dry cut Lowicryl sections of pancreatic β-cells show a rather moderate compression. The rate of compression is best demonstrated by the oval form of the nuclei while compression can not be detected in the secretory granules. Some of the granules had been slightly displaced during sectioning. We found that lowering of the cutting temperature of Lowicryl embeddings might be used if too soft blocks were produced or when we wanted to cut thinner sections or to diminish compression artefacts. As we have noticed the differences in the shape of nuclei in the subsequent sections cut from the same block, we feel that artifacts to which you refer, are compression artefacts caused by cutting rather than artefacts caused by incomplete freeze-drying. We found that the rate of compression often increases with decreased section thickness.

We used a previously described (Wroblewski and Wroblewski 1984) freeze-drying and low temperature embedding process (LTVEP) where frozen samples were transferred into the chamber of the modified freeze-drying apparatus in plastic vials filled with $LN_2$ (Nunc cryotubes). Freeze-drying was started at about -90°C (temperature of the freeze-drying chamber) and thereafter continued at -70°C for 10-12 hours. The condenser temperature was set at -120°C. The following 48 hours the temperature was slowly raised to -50°C. The vacuum in the freeze-drier was kept constant at $10^{-6}$ Torr. After completed freeze-drying the embedding medium was introduced to the plastic vials with the specimens without breaking the vacuum or changing the temperature in the cryochamber. The samples were impregnated with resin overnight under the conditions described above. Lowicryls (HM23 and K11M), were polymerized by illumination with UV light (360 nm) at -50°C. Longer freeze-drying at -50°C or increase of temperature to -20°C did not improve the structure preservation.

**L. Edelmann:** Did you obtain differences in the elemental composition of cryosections and of sections of freeze-dried and embedded preparations of the same material? Please give data if avaible.
**Authors:** Our earlier publications (Wróblewski *et al.* 1985, Wroblewski and Wroblewski 1984, Wróblewski *et al.* 1987) contain comparison of analytical results obtained from the tissues processed by means of freeze-substitution or freeze-drying followed by embedding in Lowicryl or in Araldite resin with thin or semithin cryosections. The validity of low temperature embedding techniques in studies of mobile ions has been demonstrated using tenotomized rat skeletal muscles known to have high sodium and chlorine and low potassium levels compared with healthy muscles (Wroblewski and Edström, 1984). The characteristic elemental shifts found in cryosectioned tissues were also found in freeze-dried and low temperature embedded.

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