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A new method culturing renal cells under permanent superfusion and producing a luminal-basal medium gradient

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Renal cells in culture represent excellent experimental tools to investigate specific physiological and cell biological functions of the nephron [1–6]. However, with the limitations of present cell culture techniques, these in vitro methods still inadequately mimic an ideal renal microenvironment.

Renal cell culture is commonly achieved by diluting the isolated nephron cell type in a suitable culture medium. The cells are then transferred to polystyrene tissue culture plates. Most of the cells attach to the impermeable culture plates, where they divide until a more or less confluent monolayer is achieved. If cultures are utilized for transport investigations, filter inserts for improved polar differentiation can be used. Compared to tissue culture plates it is proposed that filters mimic a more physiological situation since they are semipermeable. In many cases they are useful in inducing the cells to establish a polarized epithelial barrier. However, experiments with filter inserts are limited, because only a relatively small number of different filter materials for optimal cell attachment is offered. Finally, the environment within the culture dishes is far from being physiological. 1) The medium remains unexchanged and unstirred over an extended period of time ranging from one to several days. During this period uncontrolled conditions can develop within the cultures. 2) By the same argument, if hormones are introduced to the medium their bioavailability is barely adjustable. The concentration of hormones may be modified by the cells or by the medium in an unknown manner. 3) The questionable attachment of cells in a culture dish, together with the small choice of biocompatible support materials, and the uncertain composition of an unstirred medium are accompanied by a further problem. It is well known from continuous cell lines, and especially from primary cell cultures, that cells lose many of their morphological, physiological and biochemical characteristics as a result of dedifferentiation [1–3].

With the combination of technical limitations of cell culture experiments on the one hand and the problem of dedifferentiation on the other, it would not be surprising if results in vitro do not reflect exactly the conditions in vivo. Therefore the primary intention of this work was to develop a new cell culture system for anchorage-dependent cells in which a kidney-specific situation could be mimicked with simple laboratory equipment. The new technique provides several options: i) the selection of a specific support for optimal cell attachment, ii) the continuous superfusion of cells for the whole culture period, and iii) the exposure of cultured cells to a luminal-basal fluid gradient.

The culture experiments were started with newly developed cell holder sets (Fig. 1) which can be placed in different culture containers. The cell holder sets provide the basis for our culture devices. They are suitable for mounting small or large diameter, flexible or inflexible, support materials for cell attachment. A wide range of different support or cell attachment materials can be used, varying from biological membranes such as the renal capsule or chorion allantois membrane, to non-biological supports, for example, cellulose nitrate or polycarbonate filters [7]. Two types of cell holder sets were constructed (Fig. 1 b and c). First, support materials can be trimmed to the standard coverslip diameter of 13 mm with a punching tool (Fig. 1 a and b). The support material is then fixed in place by rings made from plexiglass. This is done by mounting the support material on a plexiglass ring into which a second tightly fitting ring is pressed. This manipulation completes the mounting procedure. We named the cell holder set MINUSHEET. Secondly, a modified cell holder set up for the following experiments with renal collecting duct cells is seen in Figure 1c. It permits the mounting of small sized supports including those derived from in vivo specimens, such as the flexible capsula fibrosa of neonatal rabbit kidney [8]. Both types of cell holder sets (Fig. 1 b and c) can be placed into all commercially-available cell culture plates (Fig. 2). Cell biological experiments can be started if media are added to the MINUSHEETs and the cells are allowed to attach to the support material.

The cell holder sets are constructed for specific cell culture containers allowing optimal medium exchange (Fig. 3a).

1.) The flat geometry of the cell holder sets allow them to be stacked (that is, in a basket) (Fig. 3b). After inserting the basket into the cell culture container, an immediate superfusion of cells with media is possible (Fig. 4). It is obvious that in this type of culture container the same medium is superfused at the apical and basal side of the cells.

2.) Modifications of the cell culture container allows bilateral gradient superfusion of in vitro epithelia to be achieved (Figs. 5 and 6). Using hypotonic or hypertonic media (Fig. 6), the epithelia can be exposed to gradients over various periods of time, thereby keeping the culture conditions as close as possible to the physiological environment within the kidney.

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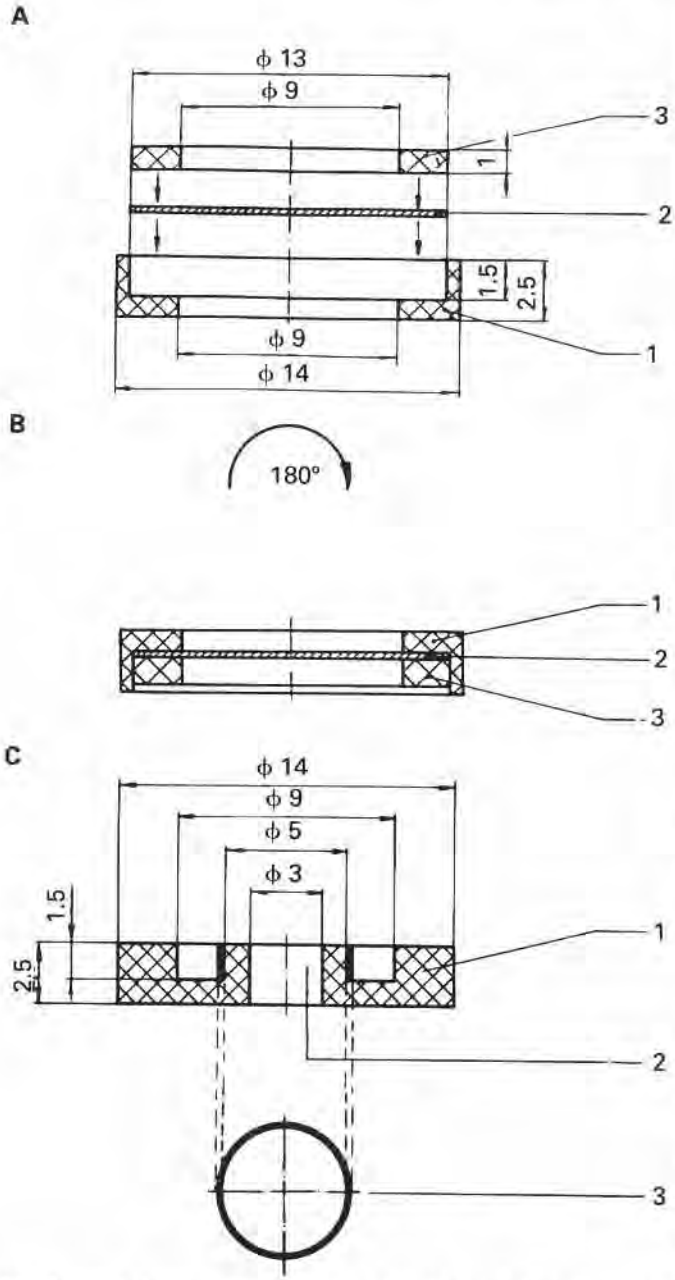

Fig. 1. a-c. *Schematic illustration of cell holder sets for cell support membranes of large diameter and inflexible material* (**a, b**) *and small diameter flexible materials* (**c**). a. Into the set up ring (1), the biocompatible support material (2) for cell attachment is installed. Then the tightly fitting ring (3) is pressed into the set up ring (1). b. By pressing the parts 1, 2, 3 against each other, the MINUSHEET is completed. After inverting, the tool is ready for use. c. For small and flexible support materials, the set up ring (1) has the same outer dimensions as in (a), Pos. (1). Flexible supports such as the capsula fibrosa of neonatal rabbit kidney can be mounted on the cylinder (2). Like the skin on a drum, the material is fixed in place with an overlapping ring (3). Sizes are given in mm.

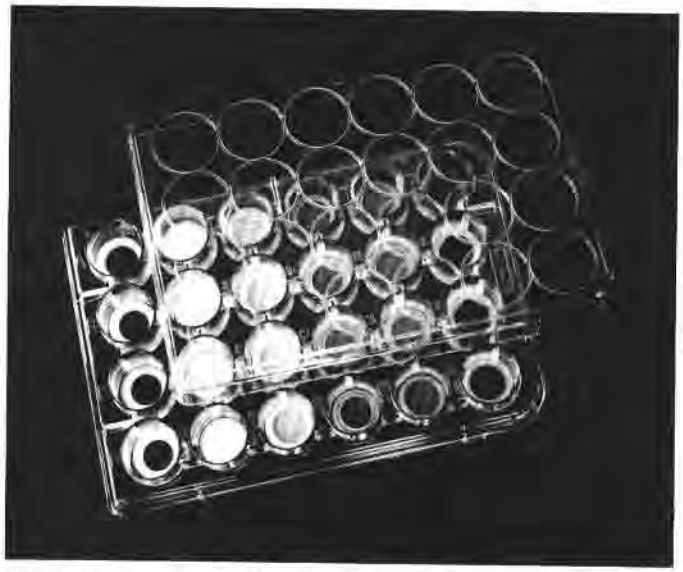

Fig. 2. *Photograph of a 24-multiwell tissue culture plate with mounted cell holder sets.* The figure depicts the wide versality of cell holder sets which can be discerned from six different interchangeable support materials marked by different color codes. The cell holder sets are used to improve the attachment characteristics of the plastic culture dish.

The superfusion cell culture system requires some basic components which include (Fig. 4): two glass bottles, two screw caps (for media bottle 10.01, Nr. 47622, Endotronics, Inc., Minneapolis, Minnesota, USA), a roller pump normally used for liquid chromatography (0.5 to 10 ml/hr), a perfusion cell culture container for the MINUSHEETs and a heat exchanger such as a circulating water bath or an aluminum heating block. Most of these pieces of equipment can be found in an ordinary cell biological laboratory.

Protocol for a representative cell culture experiment

Sterilization of MINUSHEETs. The cell holder sets (Fig. 1 b and c), with or without a suitable support material, are sterilized by soaking in 70% ethanol for ten minutes. The sheets are rinsed twice for 10 minutes each in sterile, isotonic phosphate-buffered saline (PBS), and then transferred to standard 24-well tissue culture plates (Greiner, Nürtingen, Germany; Fig. 2).

Cell pre-culture. Thin cortical explants from the kidney of newborn New Zealand rabbits [8] were mounted into the modified cell holder set (Fig. 1c). The explants consisted of a piece of capsula fibrosa with adherent nephrogenic blastema containing S-shaped bodies and collecting duct anlage. During cultivation of these explants in Iscove's Modified Dulbecco's Medium (IMDM/HEPES) containing 10% fetal calf serum, we observed an outgrowth of cells from the collecting duct anlage [8]. The collecting duct cells spread over the outer surface of the explants. Within 24 hours after starting the culture, the entire surface of the explant was completely covered by a single layer of epithelium. Pre-culture for 24 hours was carried out in a Heraeus tissue incubator (Hanau, Germany) at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Superfusion culture conditions. Twenty-four hours after starting the pre-culture the MINUSHEETs were removed from the culture plate with a pair of fine forceps and transferred to the basket cell culture container (Fig. 3b). Six MINUSHEETs were placed into the basket which was then inserted into the cell culture container. Through a basal cannula inlet, and an

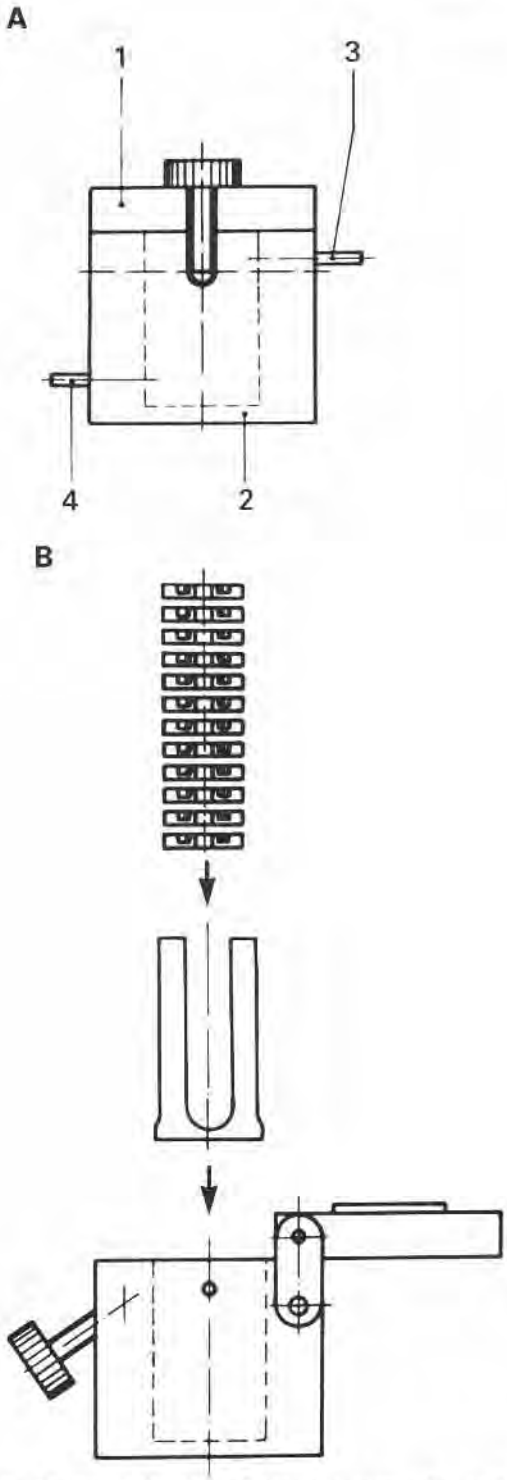

Fig. 3a, b. *Technical drawing of the side view of a cell culture container for superfusion culture.* **a.** The chamber consists of: 1 = lid; 2 = base; 3 = outlet cannula; 4 = inlet cannula for the medium. **b.** Schematic illustration of stacked MINUSHEETs. The pile of sheets is inserted into a basket. The cell culture container is loaded by setting the basket with the sheets into the opening. Then the lid is closed.

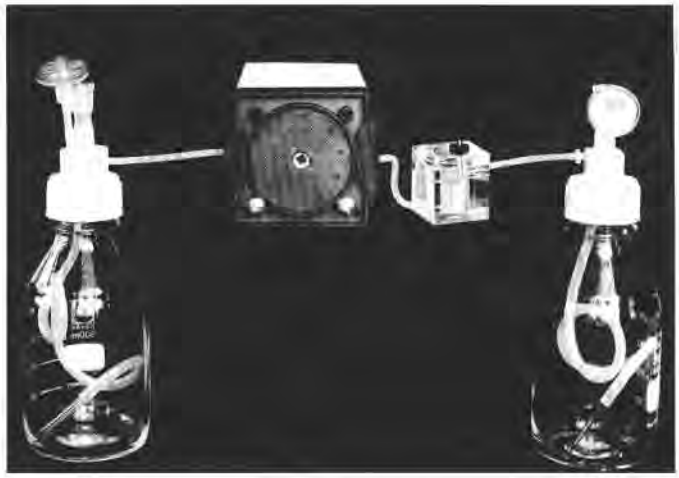

Fig. 4. *Photographic illustration of the perfusion system.* It consists of a set of media bottles, a peristaltic pump for column chromatography (1 ml/hr) and the cell culture container (see Fig. 4) connected with silicone rubber tubes.

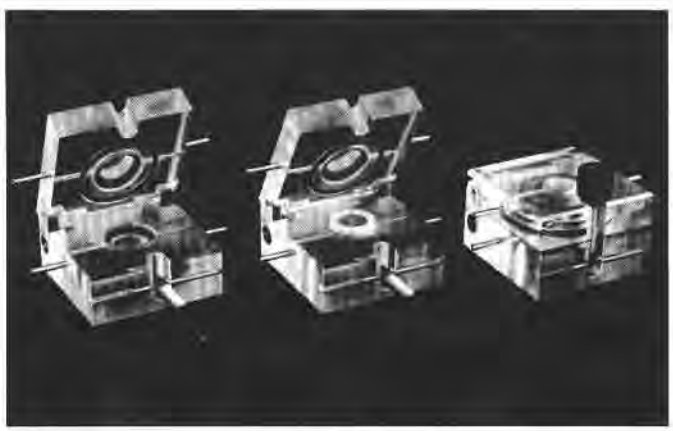

Fig. 5. *Photographic illustration of mounting a MINUSHEET in the gradient superfusion chamber: left side—open chamber, middle—positioning of a sheet into the chamber and right side—closing of the chamber by fastening the frontal lever screw.* The specific construction makes it possible to culture cells under a luminal-basal medium gradient. Single sheets with the mounted epithelium are easily placed by a pair of forceps in the gradient superfusion chamber. Iso-, hypo- or hypertonic media can be used either at the apical or basal side of the epithelium through the inlet and outlet cannulas in the lid and base.

apical outlet on the cell culture container (Fig. 3a), medium can be superfused for various periods. During the present experiment, the cells (Figs. 7 and 8) were superfused from the apical and basal side for 13 days each with Iscove's modified Dulbecco's medium (IMDM), 25 mM HEPES and a 1% mixture of antibiotic/antimycotic solution. The cell culture container was warmed up to 37°C by a circulating waterbath. Media bottles, culture containers and waste bottles were connected by silicone rubber tubes (1 mm inner diameter) mounted by standard luer fittings. The superfusion rate was 1 ml/hr. IMDM/HEPES containing 1 ml/100 ml antibiotic-antimycotic mixture was used as the basal medium for controls. Aldosterone (1×10^{-7} M), arginine vasopressin (1×10^{-6} M), and/or insulin (1×10^{-6} M) were added to the culture medium in the different experimental series. Total culture time was 14 days (1 day pre-culture, 13

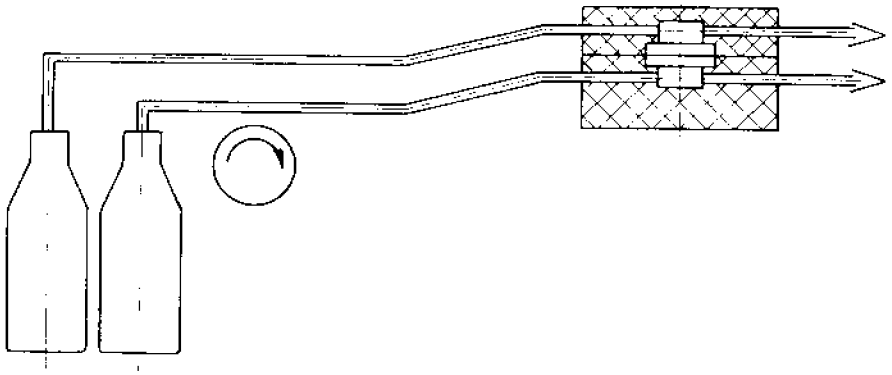

Fig. 6. *Schematic illustration of an gradient superfusion experiment.* The gradient superfusion chamber permits two different media at the apical and basal side of the cultured epithelium within the chamber.

days perfusion culture). Culture media and additives were obtained from Gibco-BRL Life Technology (Eggenstein, Germany). Aldosterone (Aldocorten) was donated by Ciba-Geigy, Wehr (Baden, Germany). Arginine-vasopressin (AVP) and insulin were obtained from Sigma Chemie GmbH (Deisenhofen, Germany).

Histology

For histological examination mounted collecting duct epithelia (Figs. 7 and 8) subjected to long-term superfusion were fixed for 20 minutes in IMDM containing 3% glutaraldehyde. Following the initial fixation step, the epithelia were removed from the cell holder sets by lifting the mounting ring with forceps (Fig. 1c; Pos. 3). The epithelia were then postfixed with 1% osmium tetroxide in PBS (pH 7.4) for 15 minutes. The tissue was washed in PBS and dehydrated in a graded series of alcohols, passed through propylene oxide, and embedded in Epon. Semithin sections were stained with Richardson solution, while ultrathin sections were stained with uranyl acetate and lead citrate. The specimens were examined with a Zeiss electron microscope (EM 902). Incubations with peanut lectin to identify intercalated cells were performed as described earlier [4]. In total, more than 180 sets of epithelia were analyzed by morphological and histochemical methods.

Cell culture results

In the present experiments, the differentiation of embryonic collecting duct cells from the ampulla of neonatal rabbit kidneys into terminal-differentiated principal and intercalated cells was investigated by morphological and histochemical techniques. In the first control series, the embryonic renal collecting duct epithelia were superfused with 1 ml/hr IMDM for 14 days (Fig. 7a) with no hormonal supplements. The resulting collecting duct epithelium was comprised of a regular prismatic shape and a relatively high degreed polar differentiation, as revealed by light microscopy (Fig. 7a). The apical side of the epithelium was in contact with the culture medium, while the basal side was orientated to the support. As revealed by electron microscopy, a basement membrane was consistently developed (data not shown). Near the apical surface numerous vacuoles were evident. Only one cell type could be documented.

In contrast, a heterogenous cell population was observed after aldosterone administration (Fig. 7b). Precisely orientated vertical sections revealed that the height of the epithelia was decreased by about 25% compared to controls (Fig. 7a). The cultured epithelial cells exhibited a cuboidal shape. In contrast to the control epithelia (Fig. 7a), the aldosterone-treated specimens (Fig. 7b) revealed cellular heterogeneity, with numerous lightly and some darkly labelled cells. This may reflect the differentiation of principal and intercalated cells. In the third series, where aldosterone, AVP and insulin were all applied (Fig. 7 c and d), the majority of cells appeared as darkly labelled cells. Most of the darkly labelled cells also stained positively at the luminal surface with fluorescent PNA-lectin (Fig. 8). This result indicated that the β-cell type of intercalated cells [9] had differentiated in these culture. Transmission electron microscopy generally showed that all of the epithelia exhibited morphological features typical of collecting duct principal (not shown) and intercalated cells (Fig. 7d). Even the concentration of mitochondria at the apical pole of intercalated cells in vivo was mimicked in culture. Indeed, no differences in morphology were discernible between the in vitro samples and specimens obtained directly from the kidney.

The most striking finding of the above experiments was that cultured collecting duct cells derived from undifferentiated ampullary cells can be induced to differentiate into lightly and darkly labelled cells by an appropriate superfusion culture system and hormonal supplement. Appointly aldosterone, in combination with insulin and AVP, acted in our experiments to induce differentiation (Figs. 7 c and d, 8). Under conventional culture conditions, however, these hormones never induced a similar differentiation process.

The new and relatively simple superfusion technique may allow the bridging of the gap between cell culture and the in vivo situation, thus allowing manipulations to be performed on physiologically relevant cells which could never be achieved in situ. The apparent chance to mimic the in vivo situation by this kind of tissue culture technique certainly have a major impact in cell culture technology for the future.

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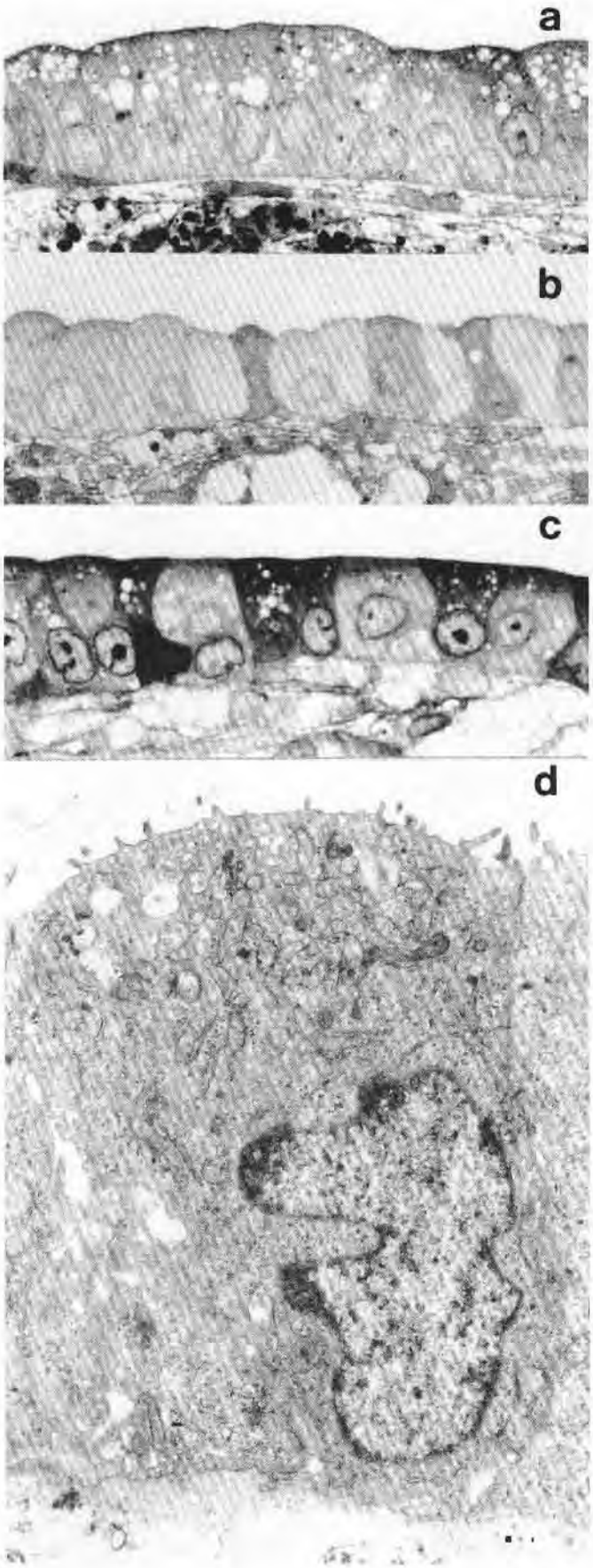

Fig. 7. a–d. *Photographic illustration of renal collecting duct epithelium cultured for 14 days under superfusion conditions.* **a–c.** Light microscopy, and d, electron microscopy; ampullary precursor cells without hormonal treatment (a), aldosterone treated (b) and aldosterone, AVP and insulin treated (c) specimens. Embryonic precursor cells (a) are found to be differentiated into lightly and darkly labeled cells by the use of hormone supplemented superfusion media (b and c). **d.** The electron micrograph reveals all characteristics of a polarized collecting duct epithelium. In contrast to lightly labelled cells, darkly labelled cells have numerous mitochondria concentrated at the apical cell pole. Lightly and darkly labelled epithelial cells resemble the Principal and Intercalated cells in the collecting duct of the adult kidney, respectively. a, b, c × 500; d × 6000.

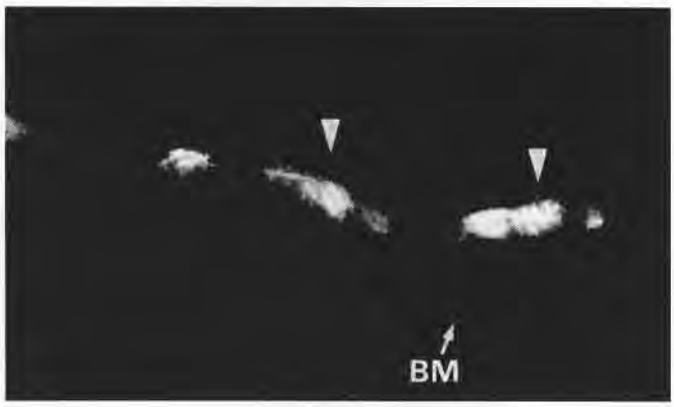

Fig. 8. *PNA-lectin binding on renal collecting duct epithelium cultured for 14 days under superfusion conditions with aldosterone, AVP and insulin containing IMDM.* Numerous cells are labelled at their apical cell poles with fluorescent PNA-lectin (arrow head), thus indicating that the β-cell type of intercalated cells differentiates under these condition. BM, basement membrane of the epithelium. × 500.

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