Construction of an apparatus for perfusion cell cultures which enables in vitro experiments under organotypic conditions

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The value of cultured cells in cell biological, pharmaceutical or biotechnological research depends on the degree of terminal cell differentiation. In conventional Petri dishes or tissue culture plates it is often difficult to achieve culture conditions which resemble the in situ situation of intact tissue, as regards optimal cell adhesion, exchange of nutrients and metabolic products. These limitations prompted us to develop simple laboratory tools which optimize the environment of cultured cells. A perfusion apparatus with various culture containers and compatible cell holder sets was constructed which allows the simulation of organotypic conditions. (*i*) The cells can be kept on individual and interchangeable support materials for an optimal cell attachment. (*ii*) Culture medium can be perfused during the whole culture period. (*iii*) One type of the new culture container can be perfused with different media at the apical and basal side of the cells, thus mimicking the organotypic environment that applies for epithelial monolayers. Cell culture experiments with renal collecting duct epithelia exhibited an excellent morphological appearance showing typical features of principal and intercalated cells.

Introduction

Cell culture techniques are important tools for cell and molecular biology. However, cells kept in culture by the common techniques can lose features of differentiation, i.e., they dedifferentiate [2–7]. This loss of particular morphological, physiological and biochemical characteristics remains a crucial problem for cell culture techniques. Considerable efforts have been undertaken to establish in vitro models which are not only related, but are as close as possible to the in situ situation [1, 9–12].

The technical limitation of cell culture protocols using the conventional plastic Petri dish technique is in our opinion one of the main reasons for the dedifferentiation process. To overcome this situation, we developed a perfusion cell culture system for anchorage-dependent cells in which an organospecific situation can be obtained. The new technique provides the selection of an organospecific support for optimal cell attachment, the superfusion of cells for the whole culture period with media, and the simulation of luminal/basal fluid gradients with different media [13, 14]. In the present paper we report the construction of a new perfusion cell culture apparatus which includes various culture containers and compatible cell holder sets. In an initial series of experiments renal primary cultures were grown in this apparatus under optimized conditions.

Materials and methods

To mimick the natural situation, cell cultures should be maintained with continuous superfusion of medium. This technique requires improved culture equipment as well as accessory tools such as media bottles, silicone rubber tubes, pumps and specific cell culture containers. All components should be compatible and easily interchangeable for quick and uncomplicated management in order to minimize the risk of contamination and cell death. Combining all of these needs, we constructed an apparatus for the culture of anchorage-dependent cells on a laboratory scale. All of the main compartments were designed and constructed in our laboratory. Pending patents have been applied. MINUSHEET is a registered trade mark [13].

Perfusion cell culture apparatus

The perfusion cell culture apparatus consists of 4 separate compartments (Figs. 1, 2):
1) At the left side a cooling compartment for bottles of fresh culture medium, *2*) a pump station in the middle, *3*) the cell culture housing area which is heated at 37 °C, and *4*) at the right side a second cooling container for waste culture medium is installed.

The cooling compartment is sufficient to take 4 × 1000 ml glass bottles which can be loaded from the top. They are located at the left and the right side of the cell culture apparatus. The stainless steel containers have dimensions of 22 cm height, 11 cm width and 41 cm depth. A cooling temperature of 6 °C for the media is main-

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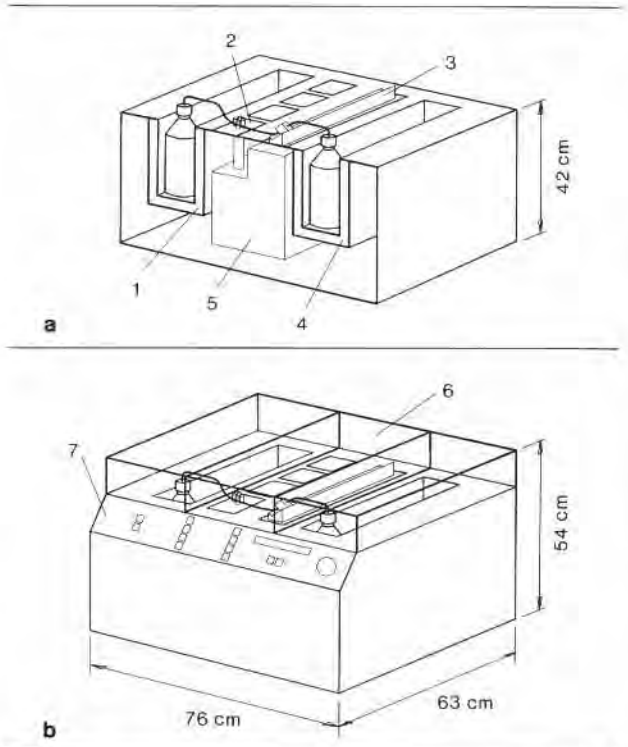


Fig. 1. Schematic illustration of the cell culture apparatus showing (**a**) longitudinal cross section and (**b**) view to the top and to the front. — **a.** The apparatus consists of four compartments: The cooling container for the medium bottles at the left side (1), the pump station (2), the cell culture housing area in the middle with one cell culture container (3), and the cooling container at the right side (4). Below the pump station and the cell culture housing area the electronic components (5) are housed. — **b.** The apparatus is covered by a plexiglass lid (6) covering and isolating the different compartments. By a front panel the microprocessor (7) is controlled.

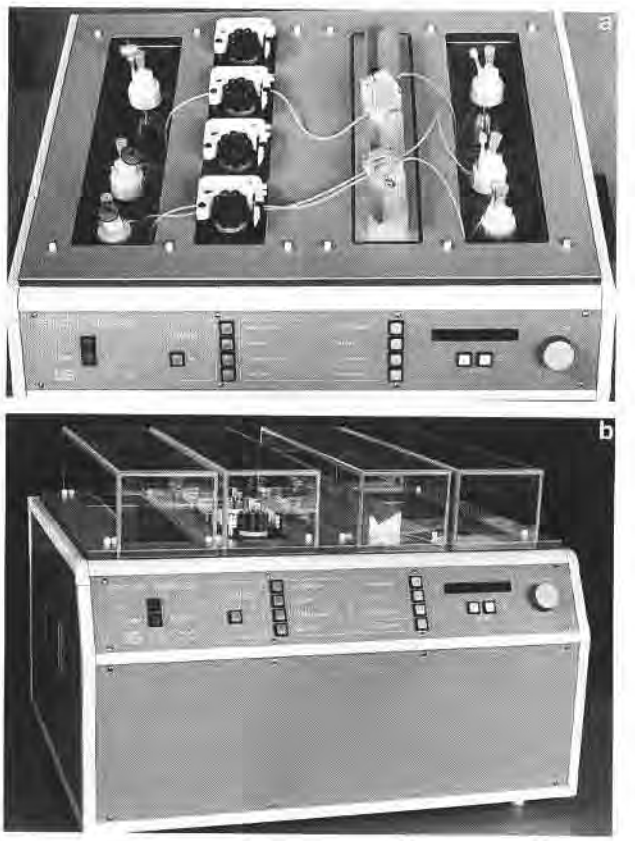


Fig. 2. Photographs of the cell culture apparatus. — **a.** Top of the apparatus. — **b.** Front view. At the left side, a cooling container for the fresh culture medium is seen, in the middle a pump station, the cell culture housing area with the heat element, and at the right side, a second cooling container for the waste culture medium. The top of the apparatus is covered by a plexiglass lid. In the front of the apparatus, the panel for pump, cooling, temperature, and culture atmosphere adjustment is seen.

tained by Peltier elements (2 × 70 Watt cooling power). This cooling method was chosen to obtain a refrigerated atmosphere with minimal vibrations. Because the Peltier cooling technique has a limited capacity compared to that of an ordinary compressor, we used only precooled media in the refrigerated stock containers.

The pump station consists of 4 single roller pumps with 2 channels each. The two-line pumping heads, drives, gear boxes, and the motors were obtained from Ismatec (Weinheim/FRG). It allows a variation of culture medium flow rate between 0.1 and 10 ml/h/channel. The orientation of the pumps into 8 single working lines gives the opportunity to superfuse each culture container separately and independently.

The base of the cell culture housing area is constructed from stainless steel which can be sterilized by ethanol (70%) or UV-irradiation. An aluminium holder for the different cell culture containers runs from the front to the rear of the compartment. The profile of the holder is machined, so that the culture chambers are inclined at an angle of 45°. This prevents any air-bubbles from being trapped within the chamber. Below the holder, an electronically regulated heater is installed to warm the culture containers to 37 °C. Under normal working conditions the whole area is covered by a plexiglass lid. Within this compartment all of the cell culture containers described below can be used.

All functions of the perfusion apparatus are set through controls on the front panel (Figs. 1, 2). Cell culture parameters such as roller pump rotations, (given as ml/h), cooling temperature, and the maintenance of constant temperature on the cell culture housing area are controlled by an on-board microcomputer. The electronic hard and software was devised in our faculty workshop. All construction details are available from our laboratory on request.

Cell holder sets for different culture containers

The constructed cell holder sets (MINUSHEET; [13, 14]) are suitable for mounting small or large diameter, flexible or inflexible support materials (Fig. 3). They provide the basis for our culture device. A wide range of support materials for cell attachment, for example, cellulose nitrate, polycarbonate or acetate filters, cellophane or biological membranes such as the renal capsule or chorion allantois membrane, can be used. The support materials can be trimmed to the standard coverslip diameter of 13 mm with a special punching tool. The support material is then fixed by set up rings made from Procan (Hoechst, Frankfurt a. Main/FRG). This is done by mounting the support material on a Procan-ring into which is then pressed a second tightly fitting ring. This manipulation completes the mounting procedure.

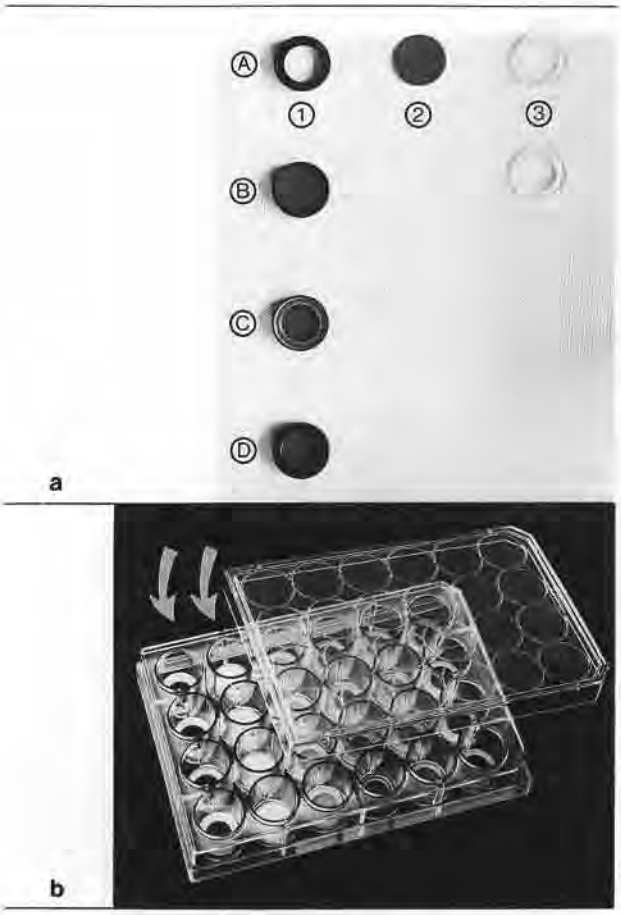


Fig. 3a. Photographic illustration of cell holder sets mounting. — (A) The MINUSHEET consists of 3 parts: the set up ring (1), the support material for cell attachment (2), and the small ring (3). — (B) For mounting, the support material (2) is placed into the set up ring (1). — (C) Then the tightly fitting ring (3) is pressed into the set up ring (1). — (D) The sheets can be used from both sides. After mounting, the sheets are used in conventional 24-well tissue culture plates for the preculture period (**b**).

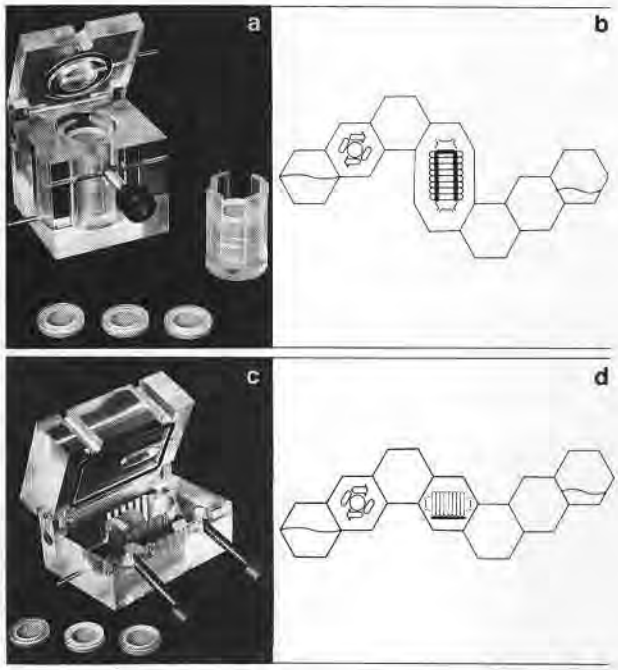


Fig. 4. Photographic (**a, c**) and schematic (**b, d**) illustrations of cell culture containers for permanent perfusion of media. — **a.** MINUSHEETs are stacked into a basket. The cell culture container is loaded by setting the basket into the opening and closing the lid. — **c.** Illustration of a cell culture container, where the MINUSHEETs are orientated in a vertical manner like the teeth of a comb. It enables quick access to exchange single sheets from the container. — **b, d.** Schematic illustration of the perfusion system. It consists of a medium bottle at the left side, a peristaltic pump for column chromatography, the perfusion cell culture container for horizontal (**b**) and vertical (**d**) stacking of sheets and a medium bottle at the right side.

Cell culture containers

Various cell culture containers for different purposes are available.

1) The flat dimensions of the MINUSHEETs allow them to be stacked like a pile of coins in a horizontal (Fig. 4a) and a vertical manner (Fig. 4c): The MINUSHEETs can be stacked in a basket (Fig. 4a). After inserting the basket in the cell culture container, an immediate perfusion with media is possible (Fig. 4b).

2) The MINUSHEETs can be orientated within a different cell culture container in a vertical manner (Fig. 4c). It enables quick access to the cultures, enabling individual rapid exchange or removal of single sheets from the container during perfusion with media (Fig. 4d).

3) The gradient perfusion chamber allows a new kind of cell culture (Fig. 5). It was specifically designed for epithelia. MINUSHEETs can easily be transferred by forceps to the perfusion chamber, which allows the separate superfusion of monolayers from their apical and basal sides (Fig. 5a). Using for example hypotonic or hypertonic media (*black*) against isotonic fluid (*white*) (Fig. 5b), the epithelia can thus be exposed to gradients over various periods of time. With this bilateral superfusion technique the culture conditions can be kept as close as possible to the physiological situation for most epithelia, which are commonly exposed to apicobasal gradients in their natural environment.

Protocol for a representative cell culture experiment

Sterilization of MINUSHEETs. The cell holder sets (Fig. 3) [14] suitable for renal capsula fibrosa material was sterilized by soaking in 70% ethanol for 10-min periods each. The sheets were then rinsed twice in sterile, isotonic phosphate buffered saline (PBS), 10 min each and transferred to standard 24-well tissue culture plates (Falcon, Becton Dickinson, Heidelberg/FRG).

Cell preculture. Thin cortical explants from the kidney of newborn New Zealand rabbits [11, 12] were mounted into the cell holder sets (Fig.3) [14]. The explants consisted of a piece of capsula fibrosa with adherent nephrogenic blastema containing S-shaped bodies and collecting duct anlagen. 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 anlagen [11]. The collecting duct cells spread over the outer surface of the explants. Within 24 h of culture the entire surface of the explant was completely covered by a

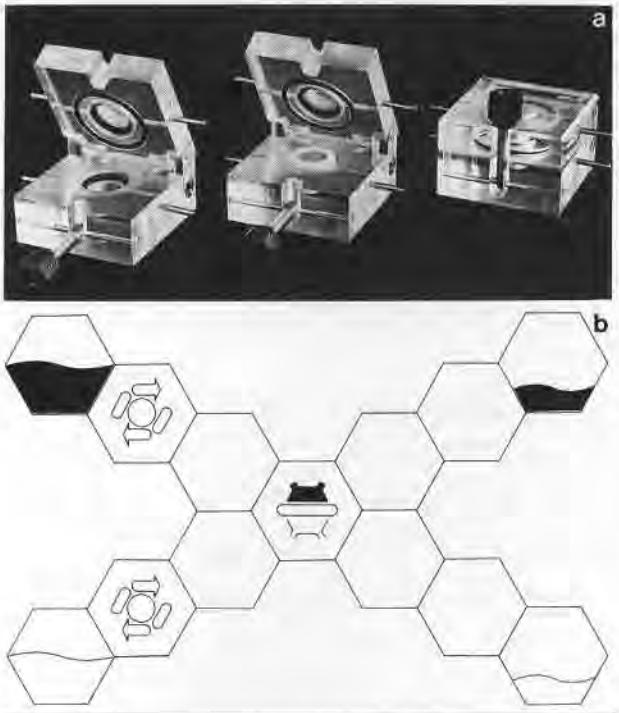


Fig. 5. Photographic (**a**) and schematic (**b**) illustration of mounting a MINUSHEET in the gradient perfusion chamber. — **a.** The chamber is opened by undoing the frontal lever screw (*left side*), then the MINUSHEET is placed. The chamber is closed by fastening the frontal lever screw. — **b.** The specific construction makes it possible to culture cells under permanent perfusion with different media at the luminal and basal side. The illustration demonstrates the perfusion of one medium (*black*) at the luminal and one medium (*white*) at the basal side of a sheet in a gradient perfusion chamber.

single-layered epithelium. Preculture was carried out in a Heraeus tissue incubator (Hanau/FRG) at 37 °C in a humidified atmosphere containing 5% CO_2/95% air.

Perfusion culture. 24 hours after starting the preculture, the MINUSHEETs were removed from the culture plate with a pair of fine forceps and transferred to the culture container as described above (Fig. 4a). The sheets were stacked and superfused with medium as seen in Figure 4b. Between 6 and 10 sheets were placed into a basket which was inserted into the cell culture container. Through a basal perfusion inlet cannule and an apical outlet cannule medium can be superfused for at least 4 weeks. For the described experiment the cells were superfused with the same medium from the apical and basal side. The cultures were superfused with IMDM/25 mM HEPES and a 1% mixture of antibiotic-antimycotic solution (Gibco-BRL Life Technologies, Eggenstein/FRG) for 13 days. The medium was contained in Schott glass bottles (500 ml), which were closed by screw caps (Nr. 47622, TECNOMARA, Fernwald/FRG). 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 in all experiments was 1 ml/h.

Culture medium. IMDM/HEPES containing 1 ml/100 ml antibiotic-antimycotic mixture was used as the basal control medium. Aldosterone (1×10^{-7} M), and arginine-vasopressin (1×10^{-6} M), and/or insulin (1×10^{-6} M) were added to the culture medium in the experimental series. The superfusion of cultures with medium and the application of the different hormones was started 24 h after preparing the cultures. Total culture time was 14 days (1 day preculture, 13 days perfusion culture).

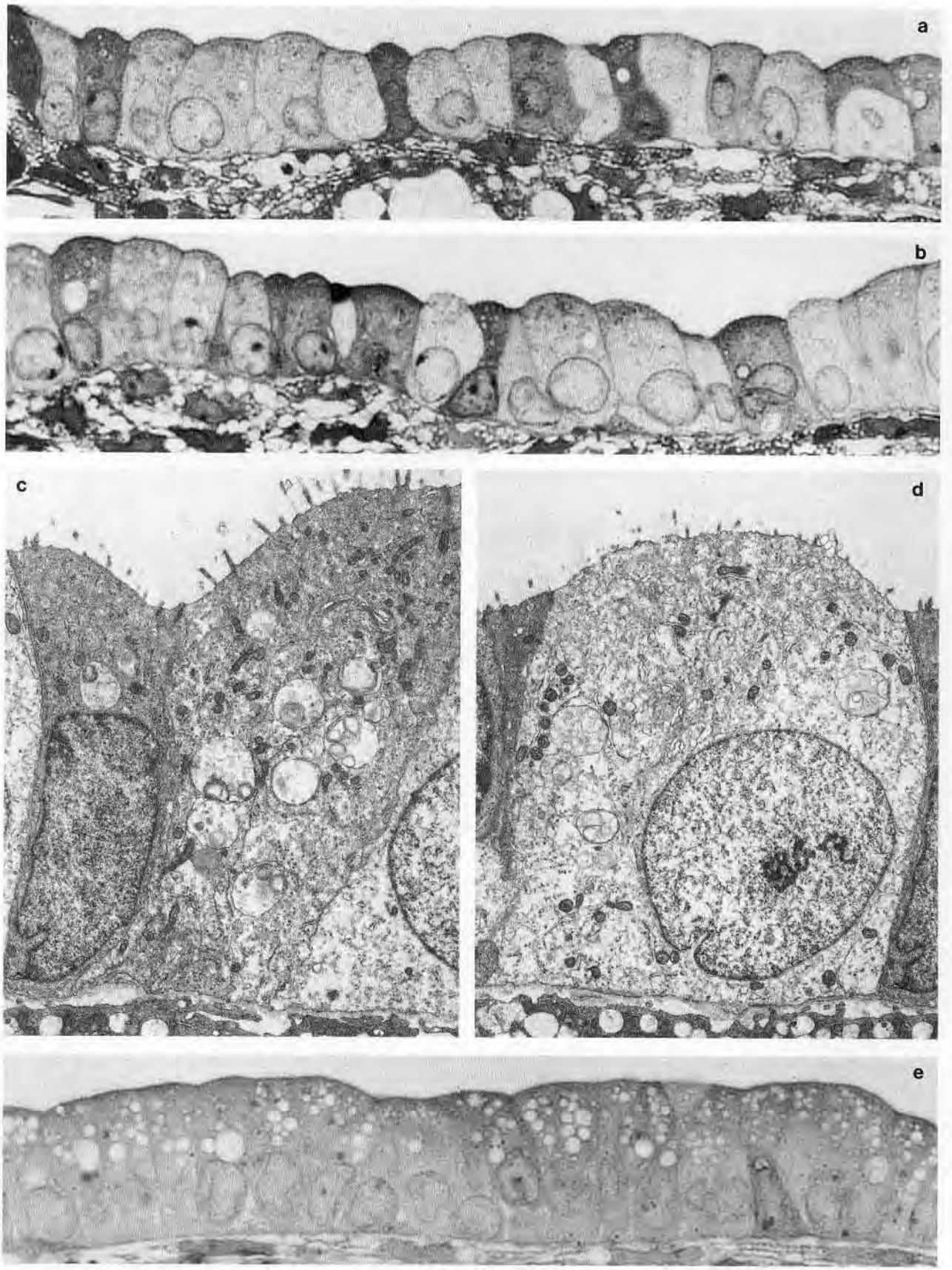
Culture medium and additives were obtained from Gibco-BRL Life Technologies. Aldosterone (Aldocorten) was donated by Ciba-Geigy (Wehr-Baden/FRG). Arginine-vasopressin (AVP) and insulin were obtained from Sigma (Deisenhofen/FRG).

Histology

For histological examination, mounted collecting duct epithelia were fixed after 14 days long-term perfusion for 20 min in IMDM containing 3% glutaraldehyde. Following the initial fixation step, the epithelia were removed from the cell holder sets by lifting the fixation ring with forceps. Then the epithelia were postfixed with 1% osmium tetroxide in PBS (*p*H 7.4) for 15 min. 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, ultrathin sections were stained with uranyl acetate and lead citrate, and then examined with a Zeiss electron microscope (EM 902). In total, more than 180 epithelia from perfusion cell culture experiments were analyzed by morphological methods.

Results and discussion

At first glance our culture system appears to be less convenient than the classical techniques [1, 9, 10]. Consequently, it has to be questioned whether the effort of our new technique justifies the outcome. Many different cell cultures exist, such as continuous cell lines, which grow excellently under ordinary culture conditions making the development of a new system unnecessary [3, 4, 6]. However, primary cultures are often more difficult to grow and have been shown to dedifferentiate very rapidly under conventional culture conditions [12]. Even when cultured over a prolonged period of time, the dedifferentiated cells do not achieve the completely differentiated characteristics observed in intact organs [4, 6, 7]. One of the major reasons for the arrest of primary cell cultures in this dedifferentiated state is thought to be the unphysiological conditions prevailing under ordinary culture environments. The development of the perfusion system described herein was intended to approximate as closely as possible the conditions in which anchorage-dependent cells are exposed in vivo. Major improvements are the extended choice of adequate culture conditions for different cell types and the easy handling of cultured cells for further manipulations. During our initial use of our new system we found an improvement of the differentiation of renal primary cultures (Fig. 6). In these experiments, we used small renal cortex explants from newborn New Zealand rabbits with the renal capsula fibrosa isolated as described previously [11]. A thin cortical tissue layer containing collecting duct anlagen and S-shaped bodies, was placed on to a MINUSHEET [13, 14]. Twenty-four hours after isolation, the surface of the explant was covered with proliferating collecting duct cells spreading out of the collecting duct anlagen. Until the 14th day, the cells were perfused with IMDM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medium containing aldosterone, arginine-vasopressin, and insulin. Light microscopic observations of semithin sections revealed a monolayer of polar-differentiated epithelial cells (Figs. 6a, b). When observed with transmission electron microscopy, the cells exhibited an excellent morphological appearance showing typical features of collecting duct intercalated (Fig. 6c) and principal (Fig. 6d) cells. No differences in the structural morphology were discernible when the in vitro samples were compared with samples obtained directly from the kidney [5, 8]. In contrast, Figure 6e shows cells grown with the same medium but under conservative conditions. The monolayer appears to be composed of a single cell type. The dark (intercalated) cell type does not differentiate.

The perfusion cell culture system requires basic components which are: two glass bottles with screw caps, silicone-tubing, a roller pump (0.5–10 ml/h), a perfusion cell culture container and a heat exchanger such as a circulating water bath or a thermo bloc. Most of these pieces of equipment can be found in an ordinary cell biological laboratory. For laboratory scale use we developed the above integrated apparatus, which renders the work with the superfusion cell cultures more convenient (Figs. 1, 2). Evaluating the pros and contras of our new system, the following features make our technique preferable to ordinary culture methods: (*i*) the option of constant medium perfusion, (*ii*) the choice of an optimal cell support, and (*iii*) the option to expose monolayers to an organospecific fluid environment, including that of a transepithelial gradient with different media at the apical and basal side.

In particular, the technique allows a continuous administration of fresh media and constant levels of hormones, it also allows removal of metabolites by the drainage of outflowing medium. Thus, we have a situation which resembles the organotypic conditions much more closely than conventional techniques. Furthermore, compared to a perfusion culture apparatus described previously [15], the cells can be kept on organospecific supports. All kinds of suitable support materials for cell attachment can be chosen to improve growth and differentiation of cultured cells.

In summary, the new and relatively simple technique may help to make many cell culture experiments more comparable to the situation which applies to cells under the physiological conditions in vivo. We are convinced that this is an important and necessary aspect which will have a major impact in cell culture technology for the future.

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Fig. 6. Photographic illustration of renal collecting duct epithelia cultured for 14 days with 1MDM containing aldosterone, arginine-vasopressin, and insulin under perfusion conditions (**a–d**) and under conservative conditions (**e**). – **a, b, e.** Light microscopic view. – **c, d.** Electron microscopic view. The perfusion technique produces an excellent appearance of the cultured renal collecting duct epithelium (**a, b**). By the new perfusion technique it is possible to differentiate embryonic cells into lightly (**d,** principal) and darkly (**c,** intercalated) labeled epithelial cells of the renal collecting duct. No differences in the structural phenotype were discernible when the cultured cells were compared with samples obtained directly from the kidney. – **e.** The micrograph shows a collecting duct epithelium grown under conservative conditions. In contrast to (**a**) and (**b**), the polarized epithelium appears to be composed of a single cell type derived from neonatal kidney. – 500× (**a, b, e**), 6000× (**c, d**).

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