Continuous Growth of Proximal Tubular Kidney Epithelial Cells in Hormone-supplemented Serum-free Medium

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ABSTRACT An epithelial cell line from pig kidney (LLC-PK₁) with properties of proximal tubular cells can be maintained indefinitely in hormone-supplemented serum-free medium. Continuous growth requires the presence of seven factors: transferrin, insulin, selenium, hydrocortisone, triiodothyronine, vasopressin, and cholesterol. The hormone-defined medium (a) supports growth of LLC-PK₁ cells at a rate approaching that observed in serum-supplemented medium; (b) allows vectorial transepithelial salt and fluid transport as measured by hemicyst formation; and (c) influences cell morphology. The vasopressin dependency for growth and morphology can be partially replaced by isobutylmethylxanthine or dibutyryl cyclic AMP. The medium has been used to isolate rabbit proximal tubular kidney epithelial cells free of fibroblasts.

Little is known concerning the in vivo conditions and factors controlling growth and expression of the differentiated state in kidney epithelial cells. Nor are the developmental conditions that give rise to distinct types of epithelial cells in the different segments of the kidney tubule understood. In order to provide information about these important biological problems, we have focused our studies on established cell lines derived from kidney tissue (1, 2). Of these kidney epithelial lines, the best characterized is the Madin-Darby canine kidney (MDCK) line (3–6). Our investigations have defined the in vivo (7) and in vitro (8, 9) growth properties of these cells. Differentiated characteristics of these cells have also been studied (10–12), and the polar nature of an MDCK cell epithelium has been established by functional (13, 14) as well as structural (15–17) studies.

Whereas the MDCK cell line appears to derive from the collecting duct or distal tubule, recent evidence suggests that another established cell line, designated LLC-PK₁ (18), may have been isolated from the proximal tubule of the porcine kidney. These cells form continuous epithelial sheets in vitro and retain Na⁺-dependent glucose uptake activity (19).

Although an increasing number of laboratories are initiating studies with the LLC-PK₁ cells, little is known yet concerning the growth properties of these cells or of primary epithelial cells derived from the proximal tubule. We therefore initiated comparative growth studies with this line, the first of which are reported here. It is shown that while the LLC-PK₁ cells will not grow for long periods of time in the serum-free medium

which supports rapid MDCK cell growth, a few qualitative and quantitative modifications of the medium allow continuous growth of these proximal tubular cells. The qualitative changes include substitution of vasopressin for prostaglandin E₁ and the inclusion of cholesterol in the growth medium. Some of the growth agents influence cell morphology and expression of specific differentiated characteristics of the cells. On the basis of the results reported, we tentatively conclude that the factors and conditions supporting growth of proximal tubular epithelial cells are basically similar to, but differ slightly from those supporting growth of epithelia from the distal segment or collecting duct.

MATERIALS AND METHODS

Cells and Growth Media

LLC-PK₁ cells (19) were received from Dr. D. S. Misfeldt (Veteran's Administration Hospital, Palo Alto, CA). Stock cultures were maintained at 37°C in a humidified 5% CO₂/95% air mixture. The growth medium, Dulbecco's modified Eagle's medium (DME, Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) was supplemented with 15 mM HEPES (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA), pH 8.2, 14 mM sodium bicarbonate, antibiotics (penicillin, potassium salt, (Sigma Chemical Co., St. Louis, MO) at 2 × 10⁵ IU/l; streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) at 2 × 10⁻⁴ M and ampicillin (Bristol Laboratories, Syracuse, NY) at 25 mg/liter), 7.5% horse serum (HS, Gibco Laboratories) and 2.5% fetal bovine serum (FCS, Gibco Laboratories) (DME-HSFCS). Serum-free stocks were passaged in a 50:50 mixture of DME and Ham's nutrient mixture (Gibco Laboratories) (DME/F12-SF) having the same supplementations as DME-HSFCS with the exceptions that HEPES was buffered to pH 7.4 and serum was lacking. All stocks were grown in 100-mm tissue culture dishes (Lux Scientific Corp., Newbury Park, CA).

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Cell Growth Conditions

Exponentially growing LLC-PK$_1$ cells were trypsinized by washing once with 3–4 ml of DME/F12-SF, adding 3 ml of a 0.03% EDTA/0.1% trypsin (Gibco Laboratories) solution, and incubating at 37°C for 3 min. The trypsin solution was then removed, and the cells were incubated at 37°C for 15 min. The cells were suspended in 3 ml of a 0.1% chick egg white trypsin inhibitor (Sigma Chemical Co.) solution, centrifuged, and washed twice in 10 ml of DME/F12-SF. A 0.5-ml aliquot of this cell suspension was counted with a Coulter counter (Coulter Electronics Inc., Hialeah, FL). Cells were plated at 3×10^4 cells/2 ml/35-mm Lux tissue culture dish (Lux Scientific Corp.) with the addition of the appropriate growth factors. All cell counts were performed in quadruplicate.

The initial growth studies were performed with cells grown initially in DME-HSFCS. Subsequent growth studies, performed to optimize final hormone concentrations, used cells that had been passaged a minimum of 10 times in hormone supplemented DME/F12-SF.

Isolation of Primary Cultures of Rabbit Proximal Tubular Kidney Epithelial Cells

A white New Zealand rabbit was killed by cervical dislocation, the left kidney rapidly removed, and a 1-mm slice of superficial cortex transferred to a dish of chilled medium (see below). Segments of proximal convoluted tubule were microdissected from the slice and transferred in a droplet of medium onto Millipore filters (Millipore Corp., Bedford, MA; THWP 02500), which allow visualization of the tubules with an inverted microscope. The tubules were incubated at 37°C in the following serum-free medium: DME/F12 containing transferrin 5 μg/ml, insulin 5 μg/ml, selenium 5×10^{-8} M, hydrocortisone 2×10^{-7} M, epidermal growth factor 25 ng/ml, and lysine vasopressin 100 μU/ml. Medium was changed every 4 d.

After 10 d, the filters with well-defined monolayers were removed and fixed initially in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, postfixed in 1% osmium tetroxide, and processed further as previously described (20). The specimen was oriented so that sections were taken perpendicular to the plane of the spreading cell culture; toluidine-blue stained, 1-μm thick sections were studied with the light microscope, while selected portions were prepared for and examined by electron microscopy.

Materials

Most hormones and growth factors (human transferrin, bovine pancreas insulin, 3,3,5-triiodo-L-thyronine (sodium salt) (T$_3$), hydrocortisone, and cholesterol from porcine liver) were from the Sigma Chemical Co. Selenium (Na$_2$SeO$_3$ 5H$_2$O) was from R. Ham, University of Colorado. Lysine-8-vasopressin, arginine-8-vasopressin and calcitonin (human) were products of Calbiochem-Behring Corp.

RESULTS

Table I summarizes the composition of the hormone-supplemented serum-free medium for the LLC-PK$_1$ cells and compares this medium with that for the MDCK cells (9). Both cell lines require transferrin, insulin, and selenium for growth as do numerous other cell lines (21, 22). Additionally, the two lines are growth stimulated by hydrocortisone and triiodothyronine both of which presumably act at the nuclear level. An

TABLE I

Hormone-defined Media for Two Kidney Epithelial Cell Lines

	LLC-PK$_1$	MDCK
Transferrin	25 μg/ml	5 μg/ml
Insulin	10 μg/ml	5 μg/ml
Selenium	5×10^{-8} M	5×10^{-8} M
Hydrocortisone	2×10^{-7} M	5×10^{-8} M
Triiodothyronine	10^{-9} M	10^{-9} M
Vasopressin	10 μU/ml	
Prostaglandin E$_1$		25 ng/ml
Cholesterol	10^{-8} M	

Optimal concentrations of the growth factors are indicated. The hormone-free basal medium for both cell lines was DME/F12, (see Materials and Methods). The medium for the MDCK cells was reported in reference 9.

adenylate cyclase stimulating hormone, (vasopressin in the case of the LLC-PK$_1$ line; prostaglandin E$_1$ in the case of the MDCK cells) is also required. Only the LLC-PK$_1$ cells showed growth stimulation with cholesterol. Epidermal growth factor was also noticeably stimulatory in the presence of transferrin and insulin, but had little effect in the complete defined medium (data not shown). The quantitative differences in concentration of those agents that are required by both cell lines may not be of particular significance since the two studies were not conducted under strictly parallel conditions.

The growth dependencies of the LLC-PK$_1$ cells on individual hormones as a function of concentration is reproduced in Fig. 1. None of the agents tested inhibited growth at concentrations in excess of those that gave nearly optimal growth. However, in several cases, concentrations much lower than those included in Table I were markedly growth-stimulatory. The results summarized in Fig. 2 show the effects of hormone deletion on growth, measured 4 and 6 d after transfer to the deficient medium.

The LLC-PK$_1$ cells have been grown continuously for over 6 mo with weekly passages at a 1–10-fold dilution in this serum-free medium (Table I). They grow slightly slower in the defined medium, with a doubling time of 26.4 h, compared to a doubling time in serum (7.5% HS, 2.5% FCS) of 21.6 h (Fig. 3). The plating efficiency of the serum-free cells is 70% while the serum-grown cells routinely plate with 97% efficiency. Cells grown in serum-containing and serum-free, hormone-supplemented media form hemicysts with approximately equal frequency. The hemicysts were nearly identical with respect to number, size, and morphology (data not shown).

The growth requirement for vasopressin could not be replaced by prostaglandins or other adenylate cyclase stimulating hormones. Arg-vasopressin stimulated cell growth to the same extent as did lys-vasopressin, and human calcitonin, reported to be active in LLC-PK$_1$ cells (23), had little or no effect. Neither did other hormones tested enhance cyclic AMP production to the extent observed with vasopressin (data not shown). However, the vasopressin growth dependency could be partially replaced by isobutylmethylxanthine (a potent inhibitor of cyclic AMP phosphodiesterase) or dibutyryl cyclic AMP (Fig. 4). These two agents caused cellular morphological changes analogous to those caused by vasopressin. The cells appeared more flattened and assumed a less ragged, more healthy appearance (data not shown).

The LLC-PK$_1$ cells were examined by electron microscopy after growth in either serum-containing medium or in the complete serum-free hormone-defined medium (Figs. 5 and 6). As shown, the cells grown under these two conditions appeared similar. Normal junctional complexes were present in both cases. The only striking difference noted was the appearance of the brush border. Cells grown in the hormone-supplemented medium (Fig. 6) possessed more dense but shorter microvilli than were observed for the serum grown cells (Fig. 5).

MDCK cells were found to grow in the medium defined for the LLC-PK$_1$ cells, but no hemicyst formation was observed. This observation correlates with the fact that vasopressin stimulates cyclic AMP production in MDCK cells much less efficiently than do the E prostaglandins. The LLC-PK$_1$ cells did not grow continuously in the serum-free medium which supports MDCK cell growth.

The hormone-supplemented serum-free medium for the LLC-PK$_1$ cells was used to isolate primary cultures of epithelial cells, free of fibroblasts, from microdissected segments of prox-
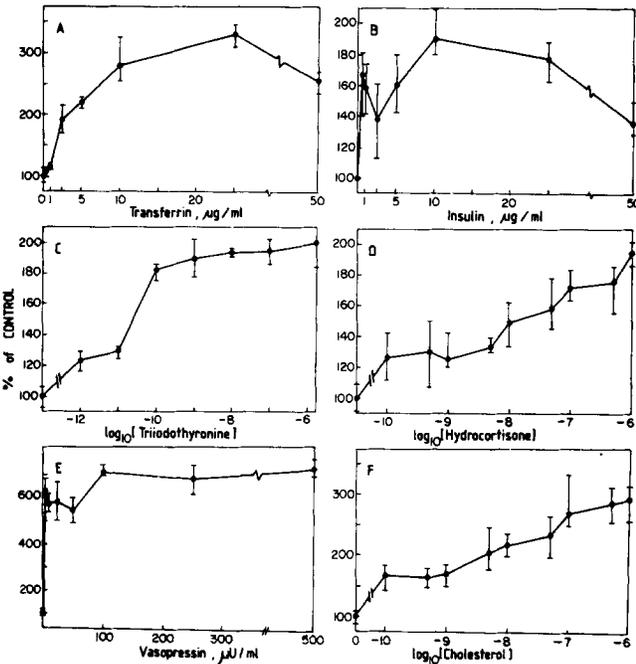
FIGURE 1 Growth dependencies of LLC-PK₁ cells as a function of hormone concentration. The concentrations of the hormones other than the one being varied were as reported in Table I. Experimental conditions were as described in Materials and Methods. Dose response curves were determined for (A) transferrin, (B) insulin, (C) triiodothyronine, (D) hydrocortisone, (E) vasopressin, and (F) cholesterol.
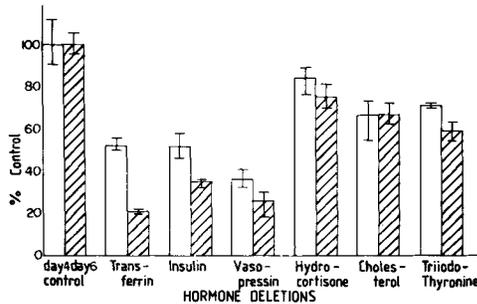

FIGURE 2 Effect of single-factor deletion from the defined medium (DME/F12-SF) on LLC-PK₁ cell growth. Conditions were as described in Materials and Methods. The growth supplement which was deleted is indicated on the abscissa. Solid bars indicate relative growth after 4 d in the single-hormone deficient medium. Cross-hatched bars indicate relative cell number after 6 d in the deficient medium. In complete medium (DME/F12-SF) (standard) the number of cells per 35-mm plate was 5.2×10^4 on day 4 and 1.5×10^5 on day 6.

imal kidney tubules. Proximal tubules were identified with certainty and care was taken to insure that no transfer of floating cells occurred. Cells were seen to proliferate from the broken ends of the proximal tubular segment and to spread out concentrically over the Millipore filter within 48 h. In 10 d the area covered by the monolayer was more than 100 times greater than that covered by the initial explant, indicating that proliferation rather than migration of cells had occurred. Light microscopy examination indicated the cells to be somewhat flattened and elongated cuboidal epithelial cells. Although they grew on the membrane filter as a monolayer, they were often present in collections of two or more cells in thickness, and occasionally were arranged as very small papillary growths of

four to six cells deep. Scattered cells had large clear cytoplasmic vacuoles. No fibroblasts were seen by light or electron microscopy. These results establish that the epithelial cells which proliferated were of proximal tubular origin.

To test whether the serum-free medium defined for the LLC-PK₁ cells was specific in supporting the growth of proximal tubular cells, explants of dissected cortical collecting tubules were also studied. Cells from these tubule segments also proliferated in this medium. Thus, while the medium does support the growth of proximal tubular cells, it cannot be regarded as being selective for this cell type.

Ultrastructural study revealed the free surfaces of the cells to possess widely and irregularly separated microvilli (Fig. 7, arrows). The cells were attached to one another by tight junctions at the apical surface; they interdigitated with one
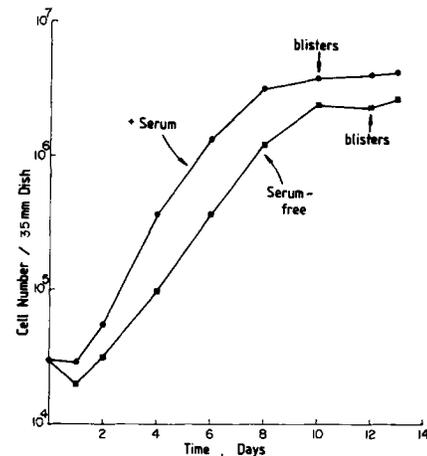

FIGURE 3 Comparison of LLC-PK₁ growth in serum-containing medium, DME/F12-HSFCS (●) versus complete serum-free medium, DME/F12-SF (■). Serum-containing medium was DME/F12 supplemented with 7.5% HS and 2.5% FCS. Cells were plated at 3×10^4 cells per 35-mm dish in their respective media. Maximal doubling times were 21.6 h and 26.4 h, respectively for serum and serum-free media. Corresponding plating efficiencies were 97% and 70%, respectively.
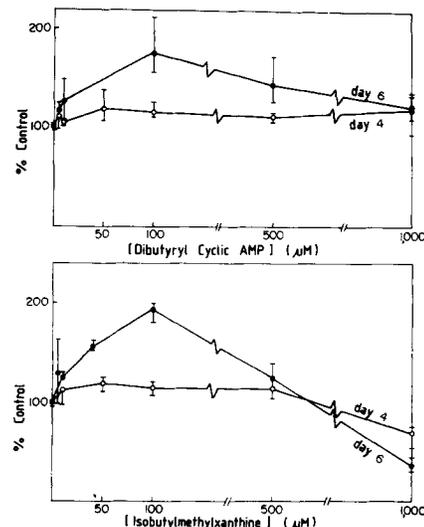

FIGURE 4 Effects of dibutyryl cyclic AMP or isobutylmethylxanthine (IBMX) on growth of LLC-PK₁ cells in DME/F12-SF lacking vasopressin. Conditions were as delineated in Materials and Methods. Cells were counted after 4 (○) and 6 d (●) respectively.
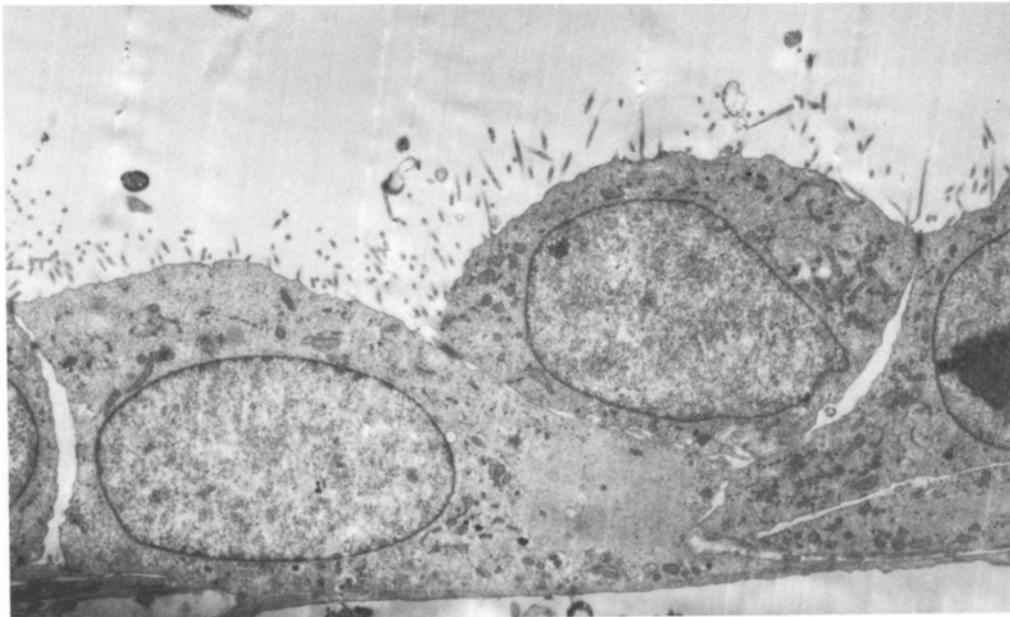
FIGURE 5 LLC-PK₁ cells grown on plastic in serum-containing medium. Tight junctions and villous projections into the intercellular spaces are evident × 4,825.
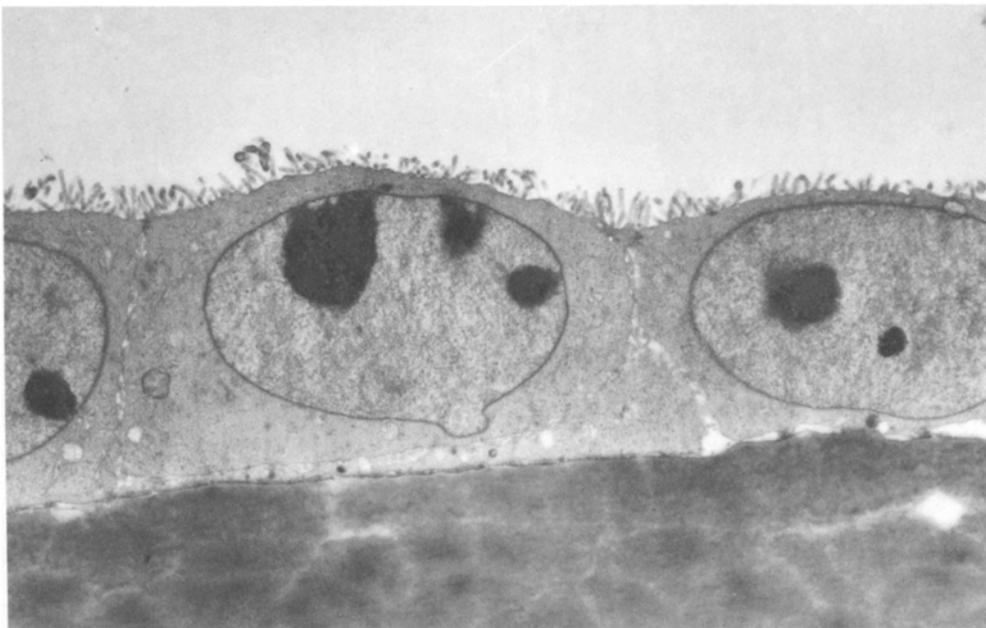

FIGURE 6 LLC-PK₁ cells grown on plastic in hormone-supplemented, serum-free medium. Compared with cells grown in serum, the brush border is denser but microvilli are shorter in length (See Fig. 5). × 4,825.

another on the lateral surfaces by complex infoldings. The nuclei were located in central or basal portions of the cells. Organelles, consisting mainly of elongated mitochondria and cisternae of endoplasmic reticulum were randomly distributed throughout the cytoplasm; numerous free ribosomes constituted the bulk of the cytoplasm. A basement membrane underlying the basal portion of the cells was not evident.

DISCUSSION

We have shown that the LLC-PK₁ cell line, a kidney epithelial line of presumed proximal tubular origin, can be maintained in continuous growth in the absence of serum. Growth depends on several agents as listed in Table I. The growth requirements of the LLC-PK₁ cells are similar to those of the MDCK cells studied previously, except that vasopressin substitutes for prostaglandin as an adenylate cyclase stimulatory hormone, and cholesterol is required. The specificity of this medium is indi-

cated by the facts that (a) the medium supports rapid growth of the LLC-PK₁ cells in the absence of serum; (b) the medium supports hemicyst formation with a frequency similar to that observed in serum-containing medium; and (c) the medium has been successfully used to isolate epithelial cells, free from fibroblasts, from dissected proximal tubules of rabbit kidney. Because the growth rate of LLC-PK₁ cells in serum exceeded that in defined medium, it can be anticipated that an additional factor will be found that stimulates cell division. This suggestion is substantiated by the fact that the primary cultures of proximal tubular epithelial cells, although polarized with apical membrane microvilli and tight junctions, did not fully differentiate in vitro into cells with a dense, microvillous apical brush boarder, characteristic of proximal tubular epithelia (Fig. 5). The medium should nevertheless prove useful in defining (a) the cell surface receptor properties of the LLC-PK₁ cells, (b) the growth requirements of these cells in molecular terms,
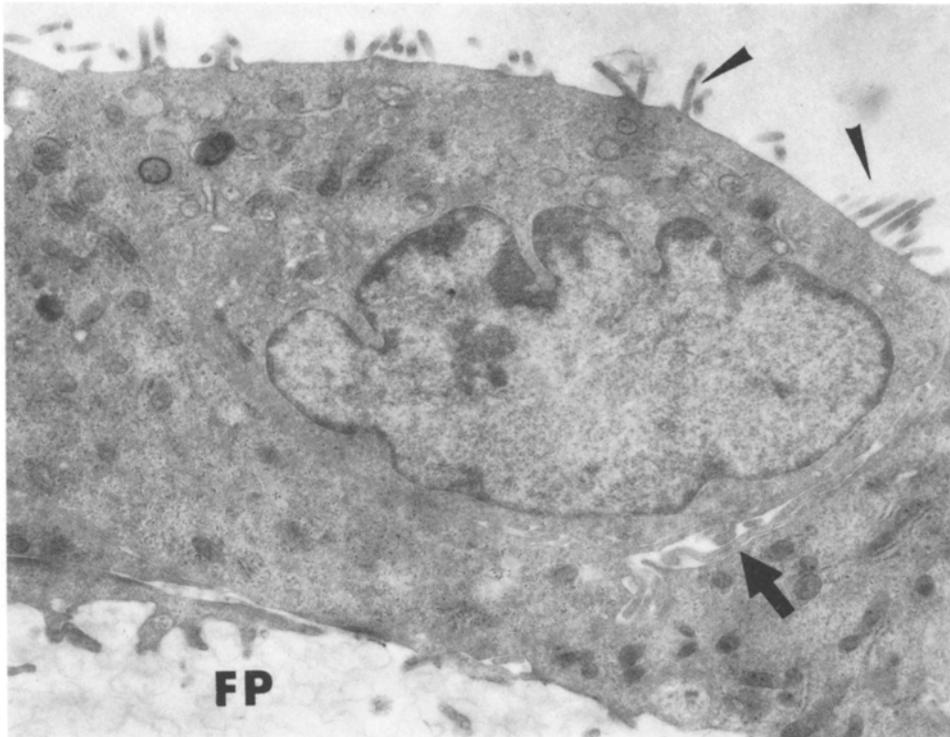
FIGURE 7 Electron micrograph showing group of proximal tubular epithelial cells with scattered microvilli (arrow heads) on free surface. There are villous interdigitations between cells (arrow); tight junctions are not visible in this photograph. Methods of isolation and fixation were as described in Materials and Methods. FP, filter paper. × 14,000.

and (c) the hormonal dependencies for expression of differentiated biochemical traits. It should also be of use in the isolation of epithelial cells from the proximal tubule of a variety of mammalian species.

After completion of this manuscript, an abstract describing a defined medium for the growth of primary rabbit kidney epithelial cells appeared (24). The two media are qualitatively similar although quantitative differences were observed.

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