Characterization of Primary Rabbit Kidney Cultures that Express Proximal Tubule Functions in a Hormonally Defined Medium

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ABSTRACT Primary cultures of rabbit-kidney epithelial cells derived from purified proximal tubules were maintained without fibroblast overgrowth in a hormone-supplemented serum-free medium (Medium RK-1). A hormone-deletion study indicated that the primary cultures derived from purified rabbit proximal tubules required all of the three supplements in Medium RK-1 (insulin, transferrin, and hydrocortisone) for optimal growth but did not grow in response to EGF and T₃. In contrast, the epithelial cells in primary cultures derived from an unpurified preparation of rabbit kidney tubules and glomeruli grew in response to EGF and T₃, as well as insulin, transferrin, and hydrocortisone. These observations suggest that kidney epithelial cells derived from different segments of the nephron grow differently in response to hormones and growth factors.

Differentiated functions of the primary cultures derived from proximal tubules were examined. Multicellular domes were observed, indicative of transepithelial solute transport by the monolayers. The proximal tubule cultures also accumulated α-methylglucoside (α-MG) against a concentration gradient. However, little or no α-MG accumulation was observed in the absence of Na⁺. Metabolic inhibitor studies also indicated that α-MG uptake by the primaries is an energy-dependent process, and depends upon the activity of the Na⁺/K⁺ ATPase. Phlorizin at 0.1 mM significantly inhibited 1 mM α-MG uptake whereas 0.1 mM phloretin did not have a significant inhibitory effect. Similar observations have been made concerning the Na⁺-dependent sugar-transport system located on the luminal side of the proximal tubule, whereas the Na⁺-independent sugar transporter on the peritubular side is more sensitive to inhibition by phloretin than phlorizin. The cultures also exhibited PTH-sensitive cyclic AMP synthesis and brush-border enzymes typical of proximal cells. However, the activities of the enzymes leucine aminopeptidase, alkaline phosphatase, and γ-glutamyl-transpeptidase were lower in the cultures than in purified proximal-tubule preparations from which they are derived.

Primary kidney-cell culture is valuable for studying renal functions in vitro (1). Although several established kidney-cell lines have been used to study the mechanism of transepithelial solute transport by the kidney (2, 3, 4, 5, 6), investigations using such cell lines have several limitations. First, the available cell lines may only resemble particular kidney-cell types to a limited extent. Secondly, only a limited number of established kidney-cell lines are available for study. Consequently, culture systems are also needed that closely resemble cells in a number of nephron segments (7). Primary kidney-cell culture provides an excellent means to resolve these problems (8).

Previous studies with primary-kidney cultures have been, however, impeded by the presence of serum in the tissue-culture medium (9). The transporting epithelial cells in primary-kidney cultures maintained in serum-supplemented medium, have been overgrown by fibroblasts. The fibroblast...
overgrowth can be attributed to the serum (10). Furthermore, serum may be cytotoxic to the differentiated cells of interest (10, 11).

The culture conditions for primary kidney epithelial cell cultures may be improved significantly by using hormonally defined, serum-free medium (12, 13). For example, a hormone-supplemented serum-free medium (Medium K-1) developed for the MDCK cell line (14) has permitted the establishment of primary cultures of baby-mouse kidney epithelial cells from a heterogeneous population of kidney cells, without fibroblast overgrowth. The baby-mouse kidney primaries expressed differentiated functions observed in MDCK cell cultures but lacked Na⁺-dependent sugar uptake, which is typical of proximal-tubule cultures (10).

This paper illustrates the use of hormonally defined medium as a means to maintain primary rabbit kidney cultures enriched for proximal-tubule cells. The proximal-tubule cells increased in number and maintained an epithelial morphology when cultured in serum-free medium supplemented with insulin, transferrin, and hydrocortisone. Each of these three supplements was required for a maximal number of cells to be observed. However, the proximal tubule cultures did not increase significantly in number in response to factors such as EGF and T3. These characteristics differ from those of primary cell cultures derived from unpurified rabbit-kidney preparations. The proximal-tubule cell cultures possess a number of characteristics typical of proximal tubules, including Na⁺-dependent α-methylglucoside uptake, PTH-sensitive cyclic AMP synthesis, and the brush-border enzymes leucine aminopeptidase, γ-glutamyl transpeptidase, and alkaline phosphatase.

MATERIALS AND METHODS

Culture Medium

The basal-culture medium used, SFFD, was a 50:50 mixture of DME and Ham's F12 Medium supplemented with 15 mM HEPES buffer, 1.2 mg/ml sodium bicarbonate, 192 IU/ml penicillin, and 200 μg/ml streptomycin. Water for medium preparation was first distilled, then purified using a Milli-Q reagent grade water system (Millipore Corp., Bedford, Mass.), and finally redistilled. Growth supplements were added to SFFD immediately before use. Rabbit proximal-tubule cultures were grown in SFFD supplemented with bovine insulin (5 μg/ml), human transferrin (5 μg/ml), and hydrocortisone (5 × 10⁻⁷ M) (Medium RK-I). Cultures derived from unpurified kidney preparations were grown in Medium RK-2, which consisted of Medium RK-1 further supplemented with epidermal growth factor (EGF, 10 ng/ml) and triiodothyronine (T₃, 5 × 10⁻⁷ M). MDCK cells (obtained from Dr. J. Holland, University of California at San Diego) were cultured in Medium K-I, which consisted of Medium RK-1 further supplemented with T₃ (5 × 10⁻⁷ M) and progaglandin E₂ (25 ng/ml). Primary cultures of baby-mouse kidney epithelial cells, prepared from BALB/c mice as described by Taub and Sato (10), were also cultured in Medium K-I.

Initiation of Primary Rabbit Kidney Cultures and Cell Growth Studies

Adult New Zealand white rabbits (male, 4-6 lb) were killed by cervical dislocation. The kidneys, with the renal artery and vein intact, were immediately removed and washed with sterile SFFD. The renal artery was cannulated with a 20-gauge stainless-steel needle (sterile with blunted end) and sutured. Each kidney was then perfused with PBS until clear of blood.

To obtain purified proximal tubules, the kidneys were then perfused with 0.5% solution (wt/vol) of iron oxide (which becomes associated with glomeruli) until the kidney was grey black. The kidney was washed with SFFD and decapacitated; the cortex was removed, minced into 1-cm pieces, and homogenized with four strokes in a sterile Dounce homogenizer (loose pestle). The resulting homogenate was poured first into a 253-μm-mesh nylon screen, washed with PBS, and then poured onto an 83-μm-mesh nylon screen, repeating the PBS wash. The tubules and glomeruli remained on top of the 83-μm-mesh screen were removed and resuspended in a tube containing SFFD and a magnetic stir-bar. During the 30-min incubation period at 37°C that followed, glomeruli associated with iron oxide were attracted to the stir bar. The stir bar was then removed from solution. The procedure described above is a modification of the method of Brendel and Meenan (15, 16).

To make a preparation of unpurified tubules and glomeruli (a whole kidney preparation), the kidney was perfused with PBS and decapacitated. Both the cortex and medulla were minced into 1-cm pieces and homogenized in a sterile Dounce homogenizer (four strokes using a loose pestle). The resulting suspension of tubules and glomeruli was then washed by centrifugation to remove debris.

Purified proximal-tubule and whole-kidney preparations were incubated at 37°C in SFFD containing 0.125 mg/ml collagenase (Class IV, Worthington Biochemical Corp., Freehold, NJ) and 0.0025% soybean trypsin inhibitor (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) for two minutes, washed twice by centrifugation, and resuspended into SFFD for inoculation into 35-mm dishes. The concentration of the inoculum was measured using either the Bradford protein assay (17) (generally 0.5 mg protein was added per 35-mm dish) or a hemocytometer. The cultures were maintained in a humidified 5% CO₂/95% air incubator at 37°C. Medium was regularly exchanged every 5 d.

Cultures for cell-growth studies were incubated for appropriate time intervals. The cells were then removed from the dishes by trypsinization, and were counted with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Unless otherwise mentioned, all determinations were made in triplicate.

Determination of Cyclic AMP Production in Primary Cultures

The effects of hormones on cyclic AMP synthesis by 2-wk-old primary cultures were determined as follows. The culture medium was removed, the cells were washed once with SFFD, and then SFFD with appropriate supplements was added to each dish. The cultures were then incubated for 2 h in a humidified 37°C incubator in a 5% CO₂/95% air environment. To determine extracellular cyclic AMP, the culture medium was collected at the end of the incubation period. Each sample of medium was acidified with trichloroacetic acid (TCA) (the final TCA concentration was 5%). To determine total cyclic AMP (intracellular and extracellular), the medium was collected and acidified as described for extracellular cyclic AMP. The intracellular cyclic AMP was obtained by incubating the cells in each dish at 37°C for 2 h with 5% TCA. The extracellular and intracellular cyclic AMP from each sample were then pooled. The cyclic AMP in the samples was purified using a Dowex 50 column (18). The purified samples were dried by lyophilization and resuspended in 0.05 M sodium acetate buffer, pH 6.2. Aliquots were analyzed for their cyclic AMP content by a cyclic AMP radioimmunoassay (19), and representative dishes were assayed for protein by means of a modified Lowry procedure (20).

Membrane Transport Studies

α-Methylglucoside and monoglucoside transport was measured using 12-28-d-old primary cultures of rabbit-kidney epithelial cells on 35-mm dishes. Before the uptake period, the monolayers were washed twice with a modified Kreb's Ringer solution (NaHCO₃ was omitted, and the pH was adjusted to 7.3 with Na₂HPO₄). The monolayers were then incubated at 37°C in the modified Kreb's Ringer with [³²P]α-MG and unlabeled α-MG at the appropriate concentration. At the end of the uptake period, the monolayers were again washed three times with the modified Kreb's Ringer buffer. After the final wash, the cells were solubilized using solution C of the Lowry protein-assay (20). After 20 min, the Lowry solution was neutralized with HCl. To determine the [³²P]α-MG incorporated intracellularly, half of each sample was removed, and the label was determined in a scintillation counter, using Liquisint scintillation fluid (National Diagnostics, Inc., Advanced Applications Institute Inc., Somerville, NJ). The remainder of each sample was used for a Bradford protein determination (17). The radioactive counts in each sample were then normalized with respect to protein and were corrected for zero-time uptake per mg protein (i.e., label not removed by the washing procedure). All such uptake determinations were made in triplicate.

The intracellular water space was estimated from measurements of cell diameter using an ocular micrometer (21-23). The diameter of 50 cells in a triplicated cell suspension was determined and then averaged. The total volume per cell that was calculated was similar to a volume determination made by Coulter Counter. The intracellular-water space was estimated as being 70% of the total volume. The number of cells per mg protein was determined by Coulter Counter, and the Bradford assay, respectively (see Fig. 2). An intracellular-fluid volume of 17.5 μl/mg protein was calculated for rabbit-tubule cells. The intracellular-fluid space of the LLC-PK₁-cell line was estimated as being 25 μl/mg protein by this method, and as 3.5 μl/mg protein by the procedure of Mills et al. (24).

Enzyme Assays

Leucine aminopeptidase was assayed using l-leucine-p-nitroanilide as sub-
p-nitroanilide. The substrate of the alkaline phosphatase reaction, 6.6 mM p-
strate (25). The culture medium was removed and the cultures were washed two
times with PBS. The cultures were then incubated at 23°C with 1 ml of PBS
containing 1 mM L-leucine-p-nitroanilide. The release of p-nitroanilide was
assayed periodically over a 20-min period by reading the OD at 405 nm. Each
determination was made using duplicate dishes, and was standardized with
respect to protein. Bradford protein-determinations (14) were made in triplicate,
similar procedures. The substrate for the γ-glutamyl transpeptidase assay, 0.20
mM γ-glutamyl-p-nitroanilide, was in 150 mM NaCl/Tris buffer, pH 9.5. The
reaction product was
similar procedures. The substrate for the γ-glutamyl transpeptidase assay, 0.20
mM γ-glutamyl-p-nitroanilide, and the acceptor molecule, 0.3 mM glycylgly-
cine, were in 150 mM NaCl/Tris buffer, pH 9.5 (26). The reaction product was
p-uitrophenol, was determined at 420 nm.

Materials

EGF was purchased from Collaborative Research Inc. (Walsham, MA), and
parathyroid hormone (bovine, 1-34 peptide) from Beckman Instruments Inc.
(Fullerton, CA). Other hormones, transferrin, and chemicals were purchased
from Sigma Chemical Co. (St. Louis, MO). [14C] a-methyl glucopyranoside and
cyclic AMP RIA kits were obtained from New England Nuclear (Boston, MA).

RESULTS

Maintenance of Primary Cultures Derived from
Purified Rabbit Kidney Proximal Tubules
in Defined Medium

Purified rabbit-kidney proximal tubules were inoculated di-
rectly into tissue culture dishes containing SFFD supplemented
with insulin, transferrin, and hydrocortisone (Medium RK-1).
Within several days the outgrowth of epithelial cells from the
kidney tubules was observed microscopically. After several
weeks, the kidney epithelial cells in Medium RK-1 formed
confluent monolayers without fibroblast overgrowth (Fig. 1 a).
However, fibroblasts were observed in parallel cultures main-
tained in SFFD supplemented with 10% fetal calf serum (Fig.
1 b). Viable cultures were maintained in RK-1 for over 6 wk
and could survive subculturing.

Fig. 2 illustrates the cell number and protein content during
a 3-wk culture period. A significant increase in protein content
was not observed for the first 4 d. However, between days four
and nine the proximal tubule cultures almost doubled with
regard to both cell number and protein content. After day nine,
the amount of cellular material remained fairly constant. These
studies suggest that the cells observed in culture during the first
4 d migrated from the attached kidney-tubules, although sub-
sequently cell proliferation may have occurred. Confluent
monolayers were obtained after a 2-wk culture period.

Effect of Individual Supplements in Defined
Medium on Cell Number

The effects of the individual supplements in Medium RK-1
on the number of cells in the proximal tubule cultures were
compared by means of a hormone deletion study. The cultures
were maintained for either 10 or 15 d in Medium RK-1, or in
Medium RK-1 individually deleting each of the three supple-
ments. Cells with an epithelial morphology were observed
under all of these culture conditions. Fig. 3 a illustrates that
significantly fewer cells were observed following growth in
Medium RK-1 individually lacking either insulin, transferrin,
or hydrocortisone, than in Medium RK-1 with all three supple-
ments. The addition of EGF and/or T3 to Medium RK-1
did not significantly increase the number of cells observed in
the proximal-tubule cultures after either 10 or 15 d of growth
(Fig. 3 a). PGE1, a component in Medium K-1 for MDCK
cells, also did not significantly increase the number of cells
observed in the proximal-tubule cultures after these time inter-
vals (data not included).

Presumably, the primary cultures established from purified
rabbit-kidney proximal tubules differ from primary rabbit-
kidney cultures prepared by other means. To examine this
possibility, primary rabbit-kidney cultures were initiated using
a preparation of unpurified tubules and glomeruli, and the
effects of medium supplements on the number of cells in such
primary cultures were examined. Toward these ends, primary
cultures derived from an un purified kidney preparation were
initiated in (a) Medium RK-1, (b) Medium RK-1 individually
deleting each of the three supplements, and (c) Medium RK-1
adding EGF and/or T3. Under all of these growth conditions
the cultured cells were epithelial in morphology rather than
fibroblastic.

Fig. 3 b compares the cell number in these cultures under
different growth conditions. The cell number observed
after the cultures were grown in Medium RK-1 individually
deleting either insulin, transferrin, or hydrocortisone was at
most 20% lower than in the control condition, Medium RK-1.
However, when these cultures were grown in Medium RK-1
containing either EGF, or EGF and T3, 50% more cells were
observed, as compared with the control condition (Fig. 3 b). An
equivalent effect of EGF on primary cultures derived from
purified proximal-tubules was not observed (Fig. 3 a). This
study suggests that the cells in primary cultures derived from
purified proximal-tubule preparations have growth properties
distinct from those of cultures derived from an un purified
population of tubules and glomeruli.

Sodium Dependent α-Methylglucoside
Transport

An important function of the proximal tubule is the reab-
sorption of sugars from the lumen of the tubule back into the
blood (29). This reabsorption is mediated by a sodium-depend-
ent sugar-transport system, which is located on the apical
surface of proximal-tubule cells, as well as a sodium-indepen-
dent transport system, located on the basolateral surface of the
cells (30, 31). The Na"-dependent sugar-transport system is not
observed in other segments of the nephron and consequently
is a good proximal tubule marker.

To determine whether the proximal tubule cultures possess
this hexose-transport system, the transport of α-methylgluco-
side (α-MG), a nonmetabolizable sugar, was studied. Fig. 4
illustrates the accumulation of 1 mM α-MG by 2-wk-old rabbit
proximal tubule cultures. In the presence of Na", α-MG uptake
occurred at a constant rate, 0.14 nmol/min/mg protein, during
the first 1–2 h. Previous studies by Mullin et al. (32) suggest
that α-MG uptake has no effect on cell volume during the first
hour in culture. An α-MG concentration of 26 nmol α-MG/
mg protein was achieved in Na"-containing buffer, and <1
nmol α-MG/mg protein was accumulated in the absence of
Na". The intracellular-fluid volume was estimated to be 0.75
± 0.02 µl per mg protein. Using this value, the intracellular α-
MG concentration achieved in this experiment was determined
as being 33 mM in the presence of Na" and 1.3 mM in the
absence of Na". In contrast, the rabbit renal cortex, which
contains primarily proximal tubules, concentrates 1 mM α-MG
only five-fold at 25°C (33).
FIGURE 1  Primary rabbit-kidney cultures. Purified rabbit proximal tubules were distributed at 0.5 mg protein per 35-mm dish containing either (a) Medium RK-1 or (b) SFFD supplemented with 10% fetal calf serum. 3 wk later, representative microscope fields of the cultures were photographed.
FIGURE 2 Growth of primary rabbit-kidney cultures derived from purified proximal tubules. Purified rabbit-kidney proximal tubules were plated at 0.5 mg protein/35-mm dish containing Medium RK-1. Medium was changed every 5 d. Both cell number (O) and protein (C) were determined in quadruplicate over a 3-wk growth period.

FIGURE 3 Hormone-variation study. The effects of medium supplements on the growth of rabbit-kidney epithelial cells derived from (A) purified proximal tubules and (B) unpurified tubules and glomeruli (a whole-kidney preparation) were examined. Purified rabbit proximal tubules (a proximal preparation) and unpurified rabbit tubules and glomeruli (a whole-kidney preparation) were inoculated at 104 nephron fragments per 35-mm dish into dishes containing either (i) SFFD supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and hydrocortisone (5 × 10⁻⁸ M) (control medium); (ii) control medium individually deleting either insulin, transferrin, or hydrocortisone; or (iii) control medium supplemented with EGF (10 ng/ml) and/or 5 × 10⁻¹² M T₃. The medium was changed every 5 d and the cells were counted in triplicate dishes on days 10 (A) and 15 (B). The average cell number in each condition was expressed as the percent of the average cell number in control medium.

FIGURE 4 Time course of α-methylglucoside uptake. The uptake of 1 mM α-methylglucoside into primary cultures derived from purified proximal tubules was examined over a 210-min time interval at 23°C in either modified Kreb's Ringer (●) or Na⁺-free modified Kreb's Ringer (○). Na⁺-free Kreb's Ringer was made isotonic using sucrose. [¹⁴C] α-methylglucoside was present at 0.5 mCi/mM. The incorporation of [¹⁴C] α-methylglucoside in the cultures was determined periodically during the uptake period, as described in Materials and Methods. All uptake determinations were in triplicate and were corrected for zero time uptake.

Effects of Inhibitors on α-MG Uptake

Sodium and α-MG are cotransported across the apical membrane of proximal cells by a secondary active-transport system (30). The driving force for Na⁺/sugar cotransport by this system is the electrochemical gradient for Na⁺ across the plasma membrane (30, 34, 35). The Na⁺/K⁺ ATPase in the proximal tubule maintains a low intracellular Na⁺ concentration and, consequently, an electrochemical gradient for Na⁺ across the plasma membrane. A decrease in the activity of the Na⁺/K⁺ ATPase in proximal tubule cells, then, would result in an increase in intracellular Na⁺ and, as a consequence, a decrease in the rate of α-MG uptake by the Na⁺-dependent sugar-transport system (36).

If a similar α-MG-transport system exists in the primary cultures, then metabolic inhibitors and ouabain would similarly be expected to decrease the rate of α-MG uptake as well as the activity of the Na⁺/K⁺ ATPase. Indeed the respiratory inhibitors cyanide and 2,4-dinitrophenol inhibited α-MG uptake by 34% and 23%, respectively (Table I). Inhibition of α-MG uptake by the glycolytic inhibitor iodoacetate was also observed, both in the presence and in the absence of cyanide (Table I). The inhibitory effects of all these agents were observed most dramatically in acetate-free Kreb's Ringer (unpublished observation). Presumably, this carbon source had to be deleted, so as to reduce intracellular-ATP levels enough to inhibit α-MG uptake.

The uptake of 1 mM α-MG was similarly inhibited by 10⁻⁴ M ouabain (53% inhibition was observed; Table I). However, α-MG uptake was inhibited to a greater extent when Na⁺ was deleted from the uptake buffer (90% inhibition was observed; Fig. 4). The differences between these two sets of observations may be explained if the treatment with ouabain (and the other metabolic inhibitors) did not permit the intracellular Na⁺ concentration to increase to extracellular levels. That is, ouabain
treatment may not completely eliminate the electrochemical gradient for Na⁺ across the plasma membrane. Potassium-free Kreb's Ringer was used in examining the effect of ouabain on α-MG uptake (K⁺ inhibits ouabain binding to the Na⁺/K⁺ ATPase). The α-MG uptake observed in K⁺-free buffer lacking ouabain was significantly higher than in the control buffer. This observation may be explained by an increase in membrane potential that can be expected to result from the reduced intracellular K⁺. So, these results are consistent with the hypothesis that α-MG is transported into the primary cultures by a single secondary active-transport system, which depends upon an electrochemical gradient for Na⁺ (34, 35).

The similarity between the α-MG-transport system in the primary cultures and the Na⁺-dependent sugar-transport system in the rabbit proximal-tubule was tested further, using the drugs phlorizin and phloretin. Phlorizin and its derivative phloretin have been shown to block the Na⁺-dependent glucose-transport system localized on the apical surface and the Na⁺-independent glucose-transport system localized on the basolateral surface of proximal cells, respectively (30). Table II shows that, in the proximal tubule cultures, phlorizin at 0.01 and 0.1 mM inhibited 0.1 mM α-MG uptake by >50% and even inhibited 1 mM α-MG uptake, although to a smaller extent. However, phloretin at equivalent concentrations either did not significantly affect α-MG uptake or inhibited 0.1 mM α-MG uptake by <25%. The decreased α-MG uptake at 0.1 mM phloretin may be explained by an effect of this dosage of phloretin on the Na⁺/K⁺ ATPase rather than on the Na⁺-dependent sugar transport system (37). Consequently, these observations are consistent with the existence of a sugar-transport system in the proximal tubule cultures that resembles the Na⁺-dependent sugar-transport system localized on the brush border of proximal tubule cells. The observations with phlorizin are consistent with studies by Kimmich and Randles, which suggest that α-MG is not transported by the Na⁺-independent glucose-transport system at all (38).

Transport as a Function of Culture Age

The usefulness of these primary cultures for transport studies depends upon constant expression of the transport functions over an extended culture period. Fig. 5 illustrates that the initial rate of 1 mM α-MG uptake is indeed maintained at a constant rate between 12 and 28 d in culture. The studies described above suggest that the α-MG uptake rate depends upon the maintenance of a Na⁺ gradient across the plasma membrane by the Na⁺/K⁺ ATPase. Consistent with these studies, the initial rate of ouabain-sensitive Rb⁺ uptake was also constant over this culture period (data not presented), indicating that Na⁺/K⁺ ATPase activity was stable over this time period.

Effects of Hormones on cAMP Production

Hormone-dependent cAMP production by the primary cultures derived from proximal tubules was studied. The primaries were treated with parathyroid hormone (PTH), calcitonin, and arginine vasopressin, hormones that act on different segments

| Table I | Effects of Metabolic Inhibitors on α-Methylglucoside Uptake |
|----------------|----------------------------------------------------------|
| Uptake condition | Inhibitor concentration | [14C] α-MG | % Control uptake |
| Acetate-free Kreb's Ringer | — | 100 ± 5 |
| with no additions (control) | 2,4-Dinitrophenol 0.5 mM | 77 ± 10 |
| | KCN 1 mM | 66 ± 11 |
| | Iodoacetate 0.5 mM | 69 ± 5 |
| | Iodoacetate + KCN 0.5 mM, 2 mM | 53 ± 15 |
| | K⁺-free/acetate-free Kreb's Ringer | — | 129 ± 4 |
| | Ouabain in K⁺-free/acetate-free Kreb's Ringer | 0.1 mM | 47 ± 10 |

The effects of metabolic inhibitors on the uptake of α-methylglucoside (α-MG) into primary cultures derived from purified proximal tubules were studied. Before the uptake period, cells were preincubated for 20 min at 23°C in acetate-free Kreb's Ringer, in the presence of the appropriate inhibitor. Uptake assays were then conducted in the same buffer, with 1 mM α-MG (0.5 mCi/mmol) and the same inhibitors, over a 20-min period at 23°C. Each determination was made in triplicate and was corrected for zero-time uptake. The average of each set of uptake determinations was compared percentagewise to control uptake (uptake in acetate-free Kreb's Ringer). The standard deviation for each set of data is included.

| Table II | Effects of Phloretin and Phlorizin on α-Methylglucose Uptake |
|----------------|----------------------------------------------------------|
| Agent | [14C] α-MG Uptake (0.1 mM) | % Control |
| | α-MG | % Control |
| None (control) | 100 ± 28 | 100 ± 11 |
| Phloretin 0.01 mM | 134 ± 14 | 98 ± 15 |
| | 0.10 mM | 77 ± 15 | 93 ± 13 |
| Phlorizin 0.01 mM | 34 ± 4 | 65 ± 19 |
| | 0.10 mM | 15 ± 8 | 62 ± 12 |

Primary cultures derived from purified rabbit proximal-tubules were used for α-methylglucoside (α-MG) uptake studies as described in Materials and Methods. During the uptake period, the cultures were incubated in Modified Kreb's Ringer containing either 0.1 mM α-MG and [14C] α-MG at 0.5 mCi/mmol or 10 mM α-MG and [14C] α-MG at 0.5 mCi/mmol, as well as the appropriate inhibitor. The incubation period was 20 min at 23°C. The α-MG uptake under conditions was averaged from determinations made with three dishes, and was corrected for zero-time uptake. Control uptake with 1 mM α-MG was 4.6 nmol α-MG/mg protein and with 0.1 mM α-MG was 0.64 nmol α-MG/mg protein. The standard deviation for each data set was calculated.
of the nephron (39). PTH at a physiological concentration increased extracellular cAMP levels by 0.77 ± 0.10 pmol cAMP/mg protein, which could account for the total increase in AMP production caused by PTH (0.9 ± 0.4 pmol CAMP). Thus, PTH treatment apparently stimulated CAMP efflux as well as net CAMP synthesis. Calcitonin and arginine vasopres- sin did not significantly affect CAMP production (Table III), although a slight stimulation of CAMP synthesis by arginine vasopressin was possible. In the presence of arginine vasopres- sin 1.2 ± 0.09 ng/ml CAMP was produced as compared with 0.92 ± 0.01 ng/ml CAMP produced by control cells. Similarly, in the rabbit-kidney proximal-tubule a response to PTH, but not to arginine vasopressin or calcitonin, was observed (39).

**Enzyme Markers of Primary Cultures**

A number of other differentiated functions were also observed in the primary cultures derived from purified proximal tubules. 2-wk-old proximal tubule cultures maintained in Medium RK-1 were epithelial in morphology, exhibited multiciel- lular domes as well as leucine aminopeptidase, alkaline phosphatase, and γ-glutamyltranspeptidase activities (Table IV). However, the activities of all three enzymes were higher in rabbit-kidney primaries cultured for 2 wk in Medium RK-1 rather than in SFFD supplemented with 10% fetal calf serum (Table IV). The rabbit-kidney primaries in serum-supple- mented medium were overgrown with fibroblasts, which generally exhibit very low activities of these enzymes. The activities of these functions were also determined in primary baby-mouse kidney epithelial cells and in MDCK cells, an established kidney epithelial cell line with distal tubule properties (Table IV). Dome formation by the proximal tubule cultures in RK-1 medium occurred at a lower frequency than in either primary baby-mouse or MDCK cell cultures (Table IV). In contrast, leucine aminopeptidase activity was even higher in these primary cultures than in either baby-mouse kidney or MDCK cell cultures (Table IV). Alkaline phosphatase was also observed at slightly higher levels in the primaries than in MDCK, whereas γ-glutamyltranspeptidase activity was equivalent in both cell types (Table IV).

### Table III

Effect of hormones on Cyclic AMP Production by Primary Cultures Derived from Proximal Tubules

| Assay condition | Extracellular cyclic AMP pmol/mg protein | Total cyclic AMP pmol/mg protein |
|-----------------|----------------------------------------|---------------------------------|
| No supplement   | 0.59 ± 0.13                            | 0.76 ± 0.10                     |
| 0.5 mM Isobutyl (IBMX) meth- | 0.15 ± 0.03                            | 0.92 ± 0.01                     |
| y-xanthine      |                                        |                                 |
| 10−4 M arginine vasopressin + | 0.05 ± 0.01                            | 1.16 ± 0.09                     |
| 0.5 mM IBMX     |                                        |                                 |
| 10−4 M salmon calcitonin + 0.5 | 0.11 ± 0.02                            | 0.59 ± 0.21                     |
| mM IBMX         |                                        |                                 |
| 5 ng/ml parathyroid hormone + | 0.92 ± 0.07                            | 1.8 ± 0.38                      |
| 0.5 mM IBMX     |                                        |                                 |

The effects of hormones on the production of cyclic AMP by primary cultures derived from purified proximal tubules were studied as follows. The cultures were washed twice with SFFD, and then incubated 2 h with SFFD containing the appropriate supplements. At the end of 2 h, (a) extracellular cyclic AMP and (b) extracellular and intracellular cyclic AMP was determined as described in Materials and Methods. All determinations were made in triplicate. The averages of the determinations and the standard deviations are illustrated.

### Table IV

Expression of Differentiated Renal Functions in Kidney Cell Cultures

| Cell type                  | Primary rabbit kidney cultures derived from purified proximal tubules | Purified proximal tubules | MDCK | Primary baby mouse kidney |
|----------------------------|-----------------------------------------------------------------------|---------------------------|------|---------------------------|
| Growth medium              | Medium RK-1 (epithelial (see Fig. 1))                                 | SFFD + 10% FCS epithelial cells with extensive fibroblast overgrowth (see Fig. 1) | —    | Medium K-1 (epithelial)   |
| Cell morphology in culture | —                                                                      | —                         | Medium K-1 (epithelial) | —    |
| Dome formation              | +                                                                     | —                         | +++  | ++                         |
| Initial rate of Na⁺ depen- | 0.16 nmoles α-MG per min per mg protein                              | 0.29                      | 17.8 (r = 0.89)          | 2.0  |
| dendent α-MG uptake         | —                                                                     | —                         | none detected            | ++   |
| γ-glutamyl transpeptidase   | 2.7 (r = 0.99)                                                       | 0.29                      | 17.8 (r = 0.89)          | 2.0  |
| activity (nmoles p-ni-    | —                                                                     | —                         | none detected            | ++   |
| trophilon released/ min/mg protein) |                  |                            |                          |      |
| Leucine aminopeptidase     | 12 (r = 0.98)                                                        | 2.1                       | 35.2 (r = 0.93)          | 0.5  |
| activity (nmoles p-nitro-  | —                                                                     | —                         | none detected            | 1.7  |
| philin released/ min/mg protein) |                  |                            |                          |      |
| Alkaline phosphatase ac-   | 0.75 (r = 0.996)                                                     | 0.16                      | 7.8 (r = 0.99)           | 0.2  |
| tivity (nmoles p-nitro-     | —                                                                     | —                         | —                          |      |
| philin released/min/mg protein) |                  |                            |                          |      |

Primary kidney cultures derived from purified proximal tubules were grown in Medium RK-1 or in SFFD + 10% FCS for 2 wk. MDCK and primary baby mouse kidney cultures were grown to confluence in Medium K-1 (7). Alkaline phosphatase activity was determined from the slope of the linear region of the curve. The correlation coefficients for the fit of the data points to the curve were calculated.
DISCUSSION

This paper has investigated the possibility that hormone-supplemented serum-free medium may be used to selectively grow particular types of kidney epithelial cells in primary culture. The results of these studies suggest that at least two populations of epithelial cells exist in the rabbit kidney that differ with respect to their growth responses to hormones and growth factors. Presumably, the addition of different sets of factors to the culture medium may cause an enrichment in particular cell types in primary cultures derived from an unpurified preparation of kidney tubules and glomeruli. For example, Medium RK-1 may permit increased growth of proximal cells as compared to other cell types in these cultures (particularly those that require EGF for optimal growth).

The primary cultures derived from purified proximal tubules have retained many properties typical of rabbit proximal-tubule cells. Sodium-dependent α-methyl-glucoside (α-MG) transport is a distinctive proximal-tubule function. The studies presented here indicate that α-MG is accumulated against a concentration gradient and that little or no α-MG uptake occurs in buffer lacking sodium. The uptake of α-MG was inhibited by ouabain, as well as the metabolic inhibitors cyanide and iodoacetate. Similar observations have been made with the proximal tubule of the kidney (33, 37) and have supported the hypothesis that α-MG transport is a secondary active process that depends upon an electrochemical gradient for Na⁺ (33, 35). The possibility that an α-MG-transport system is present in the primary cultures, which closely resembles the sugar transport systems in the proximal tubule, was further examined using the drugs phlorizin and phloretin. Alpha-MG uptake by the primary cultures was inhibited by 0.01 mM phlorizin, when 1 mM α-MG was present. However, 0.01 mM phloretin had no significant effect. Similar observations have been made in studies concerning the Na⁺-dependent sugar-transport system in the brush border of the proximal tubule (30). In contrast, the Na⁺ independent sugar-transport system present on the basolateral membrane of proximal cells is more sensitive to inhibition by phloretin than phlorizin (31).

The primary cultures derived from purified proximal tubules exhibited other functions observed in the proximal tubule. Like the cells in the proximal tubule, the cell cultures were particularly responsive to physiologic concentrations of PTH, whereas calcitonin and ADH had no significant effect. Admittedly, other segments of the rabbit nephron are also responsive to PTH (the thick ascending limb and the distal convoluted tubule) (39). However, the cells in these other segments possess hormone responses that are not observed in the proximal tubule. For example, the thick ascending limb is also responsive to calcitonin, a response that was not observed in the rabbit primary cultures.

The activities of enzymes present in the brush border of the proximal tubule were also studied in the primary rabbit cultures. The primary kidney epithelial cell cultures that originated from purified proximal tubules had much higher levels of alkaline phosphatase, γ-glutamyltranspeptidase, and leucine aminopeptidase when grown in defined medium than in serum-supplemented medium. The low levels of activity in cultures in serum may be explained by the fibroblast overgrowth of these cultures, or by the effects of unidentified agents in the serum. Kidney-tubule epithelial cells characteristically have much higher levels of these enzymes than do other cell types in the kidney (33, 40, 41).

The primaries derived from purified proximal tubules also exhibited, in defined medium, higher levels of leucine aminopeptidase and alkaline phosphatase than the established cell line MDCK. MDCK resembles distal tubule cells in many respects. In fact, the three brush-border enzymes studied do exhibit higher activities in the proximal tubule than in other segments of the nephron (41-43). The existence of high levels of these enzymes in the proximal tubule has been demonstrated by studies in which the proximal tubule of the kidney has been specifically stained by histochemical reagents for these enzymes (40-42, 44).

In the primary cultures the activities of these brush-border enzymes were lower than in the purified proximal tubules from which the cultures were derived. The reduced enzymatic activity may simply be due to the cultures' lacking a highly developed brush border typical of the proximal tubule (Taub, M., and C. Bentzel, unpublished observation). Such observations may also result from a decrease in the expression of these functions during the culture period. Such a decrease may be explained by the selective growth of proximal straight tubules rather than proximal convoluted tubules, for example. The decrease in the specific activity of these enzymes may also be a result of the absence of a three-dimensional structure and/or matrix interactions, as indicated by the studies of Lola Reid (45).

We cannot exclude the possibility that other cell types are present in these cultures. However, these cultures appear to be highly enriched for proximal cells. First, accumulation of α-MG by these cultures is comparable to that observed in rabbit kidney cortical slices (46). Such slices are enriched fourfold with respect to proximal cells. Second, the cultures also have much higher activities of leucine aminopeptidase and γ-glutamyltranspeptidase than would be expected if the cells were endothelial or fibroblastic. Third, as observed in the proximal tubule, the primary cultures are responsive to PTH, whereas calcitonin and arginine vasopressin did not significantly affect cAMP production. Our data do not exclude the possibility that vasopressin has a small effect on cyclic AMP synthesis by the cultures. Nevertheless, these studies do indicate that the primaries are more highly enriched with respect to proximal tubule cells than the renal cortex. The renal cortex possesses adenylyl cyclase activity that is equally responsive to PTH and calcitonin. An affect of arginine vasopressin on adenylyl cyclase is also observed in the cortex, although to only one-third the magnitude of the PTH effect. The responsiveness of the renal cortex to calcitonin and arginine vasopressin is probably due to the presence of a relatively small number of cells from the loop of Henle and the distal tubule (39). Notably, the proximal tubule cultures are not similarly responsive to these hormones. In yet greater contrast, the renal medulla, which is not enriched with proximal tubules, exhibits dramatic responsiveness to arginine vasopressin (47).

In vitro studies using "proximal" cultures in hormonally defined medium have distinctive advantages over previous studies that used tissue taken directly from the animal. The uptake of α-MG is stable over a considerable culture period, and the results obtained are more highly reproducible than with isolated tubules for example. The cell's long-term viability facilitates studies concerning the effects of hormones on membrane transport systems, and the interrelationships between metabolism and transport. Furthermore, the use of cell monolayers avoids the diffusion limitation problem of slices.

Although the tissue culture technique has been used previously to study renal functions, the primary cultures described here may prove to possess a closer resemblance to proximal

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useful for examining hormonal regulation of transport.

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