Preparation and study of fragments of single rabbit nephrons

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BURG, M., J. GRANTHAM, M. ABRAMOW, AND J. ORLOFF. Preparation and study of fragments of single rabbit nephrons. Am. J. Physiol. 210(6): 1293-1298. 1966.—A method has been developed for the dissection and in vitro study of fragments of single rabbit nephrons. Fragments of different tubule segments 5-4 mm long were dissected from sections of kidney immersed in oxygenated Ringer solution. Electrolyte and water composition of proximal tubule fragments were determined after incubation in appropriate solutions. It was found that isolated proximal tubules maintain large transcellular concentration gradients for Na, K, and Cl similar to kidney slices and mixed suspensions of kidney tubules. In order to measure transcellular transport, individual tubule segments were perfused and changes in the volume and composition of the effluent perfusion fluid measured. The viability of perfused proximal tubules was demonstrated by the presence of both active PAH transport and net fluid absorption.

renal tubule; PAH transport; tubule electrolyte content; tubule perfusion

Previous studies of transport by mammalian kidney tissue in vitro have generally been performed using kidney slices or tubule suspensions. Interpretation has been complicated by uncertainty as to the locus of the observed transport; that is, whether transport occurs at the luminal or peritubular border of the cells and whether, in the absence of glomerular filtration, transcellular transport persists. In addition, it has not been possible to specify with certainty whether there are differences in function of the many segments of the nephron present in these preparations.

In the present studies methods have been developed to dissect, incubate, perfuse, and analyze fragments of specific segments of single rabbit nephrons. Evidence is presented that tubules prepared in this manner remain viable and are suitable for the study of transport processes.

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METHODS

Dissection. In the initial studies the kidneys were dissected following treatment with collagenase. It was found that tubules pretreated with collagenase were not satisfactory for perfusion studies (see RESULTS), and subsequent to this observation only fresh tissue, with no preliminary enzyme treatment, was used for dissection. The rabbit was killed by exsanguination and the kidney was immediately removed and perfused through the renal artery with 15-30 ml modified Ringer solution (2) (to flush out the remaining blood). A slice, approximately 2 mm thick, was removed from a vertical cut through the center of the kidney, and placed in the modified Ringer solution. The solution was stirred constantly and oxygenated by bubbling with a mixture of 95% O₂ and 5% CO₂. Using steel needles and fine forceps (Dumont no. 5) the tubules were dissected by hand at room temperature under a stereoscopic microscope at 15-40X magnification.

Transfer of the tubules. In order to avoid pulling the tubules through an air-water interface, they were transferred from one solution to another in a small volume of medium (ca. 10 μl) in a siliconized pipet with a wide tip (ca. 1 mm diam.).

Incubation of the tubules. Tubules were incubated in approximately 0.3 ml medium in 2-ml beakers contained in a constant-temperature (25 C) water bath. The incubation fluid was bubbled with 5% CO₂ 95% O₂ through a fine hypodermic needle. A stereoscopic microscope (26X magnification) was used to observe the tubules in the incubation beakers.

Preparation for analysis. After incubation the tubules were transferred in a small drop of medium onto a siliconized glass cover slip on a cooling stage (designed and constructed by Dennis Prager). The purpose of cooling was to minimize evaporation of adherent extracellular fluid during blotting in order that extracellular solute not dry on the tubules. The temperature of the stage was adjusted to maintain a fine film of moisture on the cover slip without visible droplets of water. The tubule was pulled out of the small drop of bathing solu-
I. Apparatus for perfusing isolated tubule fragments. Concentric perfusion pipets (on right) are supported in a holder which provides access to the lumen of the individual pipets and permits finely controlled axial movement of the inner pipet with relation to the outer. The holder for the collecting pipets (on left) differs in that the lumen of the outer pipet is left open and a rack and pinion drive permits rapid insertion and removal of the inner (volumetric) pipet. The tissue chamber (center) is mounted in the mechanical stage of an inverted microscope. The tubule is held between the pipet tips in the slot at the bottom of the chamber and is separated from the microscope objective only by a thin cover glass. The slot contains approximately 0.1 ml fluid and remains filled throughout the experiment. The chamber above the slot holds approximately 2.0 ml additional fluid which may be drained and replenished through the fittings shown without disturbing the tubule. Not shown are 1) two small stainless steel tubes for bubbling gas through the fluid in the chamber in order to oxygenate and mix it. 2) the clear plastic cover for the chamber. 3) A thermistor in one end of the slot and resistance wire in the bottom of the chamber for temperature control. (The pipet holders were designed and constructed with the assistance of Mr. James White and Mr. Kenneth Bolen and include design features from similar apparatuses demonstrated to us by Dr. Walter Freygang and Dr. Philip W. Davies. The chamber was designed and constructed with the assistance of Mr. Dennis Prager.)

FIG. 2. Procedure for tubule perfusion. Concentric perfusing pipets are on the left. The end of the tubule is drawn by suction into the tip of the outer pipet, which supports it and seals the inner pipet within the tubule lumen. Collecting pipets are on the right. The tubule is drawn into the tip of the outer collecting pipet by suction and remains lodged there when the suction is stopped. The inner pipet is introduced periodically to remove aliquots of fluid and along the surface of the cover slip, using a steel needle, and then blotted with no. 50 Whatman filter paper which had been cut to a fine point. After blotting, the tubule was transferred with a clean steel needle to an uncontaminated section of the cover slip and dried in a stream of N₂ gas. The dried tubules were weighed using Bonting's modification of the fishpole quartz fiber balance (1) in a special room in which the relative humidity was maintained at less than 40%.

Determination of radioactivity. For C₁⁴ and P₃¹ determinations the dried tubules or aliquots of perfusion fluid were placed in the center of a planchet and counted using a low-background (<1 count/min) Geiger system. The medium was counted by drying 20-100 µl of suitable dilutions in the center of other planchets. For H₂ in the presence of C₁⁴ 10-µl aliquots of 75 N nitric acid extracts of tubules or medium (see below) were counted in 5 ml scintillation fluid (PPO 5 g, dimethyl-POPOP 0.3 g, ethanol 100 ml, toluene to 1 liter).

Measurement of Na and K. The dried tubules were extracted under mineral oil for 4 hr in 1 µl (measured with a calibrated Misco MCA 1 pipet) of 75 HNO₃ containing 3 mM CsNO₃ and 3 mM (NH₄)₂HPO₄. Na and K were determined simultaneously on approximately 3-µl aliquots of the extract using the helium-glow discharge photometer of Vurek and Bowman (8). Blanks and standards were handled in exactly the same manner as the tubule extracts. Na and K in the medium were determined using a Baird DB-5 flame photometer. In order to evaluate the micromethod, 75 N HNO₃ extracts of large quantities of rabbit cortex were also prepared and analyzed. In 14 measurements made from 3 extracts the ratio of the results using the helium-glow discharge photometer compared to those using the conventional flame photometer were Na, 1.016 ± 0.06 (50) and K, 1.015 ± 0.06.

Measurement of Cl. Dried tubules were extracted for 1 hr under mineral oil in 100 nl 75 HNO₃ (delivered with a calibrated quartz self-filling pipet (6)). The electrometric titration method of Ramsay (7) was employed with the following modifications: No H₂SO₄ was added since an acid extract was used. Aliquots of the extract were pipetted with a calibrated 10- to 15-µl self-filling pipet. An end point of 280 mv was used. References electrodes were prepared daily. The electrical apparatus (designed and constructed by Dennis Prager) included a vibrating-
reed electrometer (Cary) to measure voltage, and condensers of polystyrene with very high leakage resistance (Donner). The coefficient of variation with NaCl standards was ±1.6%. The Cl concentration in a 0.75 HNO₃ tissue extract was essentially the same with the micro-method (1.06 mEq/liter⁻¹) as with macroelectrometric titration (1.81 mEq/liter⁻¹ (5)). In preliminary experiments it had been found that kidney tubules in a suspension lose virtually all their Cl when incubated in a Cl-free solution (NO₃ replacing Cl). Single dissected proximal tubules were incubated under identical conditions and tissue Cl was measured. Mean tissue Cl content was 2 mEq/kg⁻¹ dry wt (4 tubules) which is less than 2% of the Cl measured in the tissue under normal conditions and indicates that there is no significant contamination during the extraction and analysis.

Measurement of water content. Tubules which had been incubated for 1 hr in a bathing solution containing THO were incubated for an additional 30 sec in a medium containing inulin carboxyl-C¹⁴ in addition to the THO. Then, a drop containing the tubule was placed under mineral oil. The tubule was pulled from this drop, through the mineral oil and into 10 µl 0.75 N HNO₃. After 1 hr of extraction the tubule was removed, washed with chloroform, dried, and weighed. (In control experiments it was found that the extraction and washing with chloroform results in a loss of dry weight of 5% and the calculations have been appropriately corrected.) Radioactivity in the extract was determined by liquid-scintillation counting (see above).

Tubule perfusion. Tubules were placed in a specially designed chamber (Fig. 1) above an inverted biological microscope. Concentric glass pipets mounted in a specially designed holder (Fig. 1) were used to position and perfuse the tubules. These pipets were prepared using a vertical pipet puller (constructed by National Institutes of Health Instrument Section) and a de fonbrune microforge. Fluid was collected from the distal end of the tubule using a single pipet. The procedure for perfusion is illustrated in Figs. 2 and 3. A Sage variable-speed syringe pump with a Hamilton Teflon-tipped, 50-µl gas-tight syringe was used to deliver the perfusion fluid. Pump rate, as calculated from timing the displacement of the piston in the syringe was in good agreement with measurements of delivery of albumin ¹²⁵I into a small drop of water in the center of a planchet.
FIG. 5. Freshly dissected rabbit tubules. The segments present (in order of decreasing maximum diameter) are proximal straight tubule, collecting tubule, and thick ascending limb. Note the thinning of the proximal straight tubule (bottom) at the transition to the thin descending limb.

TABLE 1. Electrolyte content of rabbit tubules

<table>
<thead>
<tr>
<th>Tubule Type</th>
<th>Suspension†</th>
<th>Suspension‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal Convoluted</td>
<td>mEq kg⁻¹ Dry Wt</td>
<td>mEq liter⁻¹ Tissue Water</td>
</tr>
<tr>
<td>Na</td>
<td>155.4 ± 13.3 (33)</td>
<td>69.7</td>
</tr>
<tr>
<td>K</td>
<td>261.4 ± 9.4 (46)</td>
<td>110.</td>
</tr>
<tr>
<td>Cl</td>
<td>132.4 ± 6.4 (24)</td>
<td>55.7</td>
</tr>
</tbody>
</table>

Values are means ± SE of means. Number of tubules analyzed are in parentheses. *Ref. 3.

The coefficient of variation of pumping rate as measured by radioisotope delivery was ±4% for consecutive 10-min collections at the perfusion rates used (5-30 nl/min⁻¹).

The apparatus for collecting perfusion fluid is also illustrated in Figs. 2 and 3. Perfusion fluid was observed to rise smoothly in the collecting pipet. Collection periods were begun when the meniscus passed an arbitrary point on the outer collecting pipet as measured with an ocular micrometer. At the end of each period fluid was collected in a calibrated, uniform-bore capillary (i.d. 54 μ). The volume was calculated from the length of the fluid column in the capillary. Calibration of capillaries from measurement of diameter and length was in good agreement with calibration with albumin 131 and the former calibration was generally used. To determine radioactivity in perfusion fluid and collected samples the contents of the measuring capillary were washed into a small drop of fluid in the center of a planchet and were dried.

Solutions. Medium used for dissections and incubation contained (in mM): NaCl 115, KCl 5, NaHCO₃ 25, Na acetate 10, NaH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.0, and 5% v/v calf serum (Microbiological Associates). For perfusion 100 mg/100 ml glucose was added to both the outside bath and perfusion fluid and calf serum was omitted from the perfusion fluid. Collecting tubules were perfused with an identical solution diluted 1:5 or with a solution containing NaCl 30 mM, CaCl₂ 1.0 mM, MgSO₄ 1.2 mM, and K phosphate buffer pH 7.35, 2.5 mM.

RESULTS

Microdissection. In the initial experiments collagenase was used prior to dissection and only proximal convoluted tubules and collecting tubules were obtained. Later, when fresh tissue without collagenase was used, it was possible to identify and dissect all of the portions of the nephron contained in cortex and outer medulla.
SINGLE RABBIT NEPHRONS IN VITRO

FIG. 6. Attempted perfusion of an isolated proximal straight tubule which had been dissected after collagenase treatment. The tubule distended and ruptured near the tip of the perfusion pipets.

TABLE 2. Inulin carboxyl-C\textsuperscript{14} recovery in isolated perfused proximal straight tubules

<table>
<thead>
<tr>
<th>Tube Length, mm</th>
<th>Perf. Rate, nl min\textsuperscript{-1}</th>
<th>Mean Coll. Rate, nl min\textsuperscript{-1}</th>
<th>Mean Inulin-C\textsuperscript{14} Conc. Ratio\textsuperscript{*}</th>
<th>Mean Inulin-C\textsuperscript{14} Recovery</th>
<th>No. of Coll.</th>
<th>Total Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
<td>13.3</td>
<td>12.1</td>
<td>1.13</td>
<td>109</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>1.76</td>
<td>13.3</td>
<td>11.1</td>
<td>1.13</td>
<td>94</td>
<td>7</td>
<td>80</td>
</tr>
</tbody>
</table>

\* Recovered fluid/perfused fluid.

The fresh tissue was easier to handle since the tubules are less friable than collagenase-treated specimens and do not adhere to one another as readily as do the latter.

Intact fragments were more easily obtained from straight segments (straight proximal tubule, collecting tubule, and ascending limb) than from proximal and distal convoluted tubules. In order to avoid injury to the tubules, an attempt was made to manipulate only the cut ends with the instruments during dissection and to avoid stretching the tubules. Dissected tubules are shown in Figs. 4 and 5.

In contrast to results with New Zealand White Rabbit kidney, all attempts to dissect tubules from rat, dog, necturus, and toad were unsuccessful.

*Extracellular contamination.* The amount of bathing solution which blotting failed to remove was estimated by dipping the tubules into an otherwise identical medium containing inulin carboxyl-C\textsuperscript{14} or albumin-I\textsuperscript{131} and calculating extracellular contamination from the radioactivity remaining on the tubules after blotting and drying. The results using inulin carboxyl-C\textsuperscript{14} and albumin-I\textsuperscript{131} did not differ significantly. Contamination of proximal convoluted tubules was .29 ± .02 SEM (41 tubules analyzed) liter kg\textsuperscript{-1} dry wt.

Measurements of tubule sodium and chloride have been corrected by subtracting the electrolyte content of this volume of medium. Measurements of tubule water content with THO were individually corrected (by inulin carboxyl-C\textsuperscript{14} determinations) for extracellular water.

*Water and electrolyte content.* The water content of proximal convoluted tubules was 2.37 ± .10 (17) liters kg\textsuperscript{-1} dry wt. This is not significantly different from the value of 2.24 ± 0.4 previously found under the same conditions in tubule suspensions (2). The electrolyte content of proximal convoluted tubules incubated for one or more hours and of tissue suspensions is compared in Table 1. The Na and Cl concentrations are similar, but the K concentration is approximately 20% lower in the proximal tubule than in the tissue in the suspension. Large electrolyte concentration gradients were maintained between the proximal tubule tissue and the medium, evidence that active electrolyte transport continues in these tubule cells. The similarity of Na, K, and Cl concentrations and water content in the proximal tubule cells to those previously measured in rabbit tubule suspensions and cortical kidney slices (if correction is made for the extracellular space in the latter) is not unexpected since proximal tubules comprise approximately 80% of the mass of rabbit kidney cortex (4).

*Tubule perfusion.* Initially, tubes were perfused which
had been dissected after treatment with collagenase. The results as noted earlier were unsatisfactory. Proximal tubules are collapsed in vitro. When fluid was injected into the lumen, the collagenase-treated tubules discarded at this point and ruptured, whereas the remainder of the tubule remained collapsed (Fig. 6). This is presumably owing to removal of the basement membrane by collagenase. The epithelial cells in the absence of a basement membrane lack rigidity and cannot support the pressure necessary for perfusion.

Tubules dissected without collagenase were readily perfused (Fig. 3). In order to assess the value of the perfused proximal tubules for physiological experiments, inulin carboxyl-Cl4 was added to the perfusion fluid, and both the final inulin concentration and inulin recovery were measured. It was possible to obtain essentially complete recovery of injected inulin and to demonstrate net absorption of fluid, as indicated by loss of volume and increase of inulin concentration during perfusion (Table 2).

Collecting tubules were also tested similarly and it was found that albumin-I131 recoveries were complete. In 40 control collections in 6 collecting tubules the mean recovery was 99.2 ± 2.6%. In general the collecting tubules were easier to perfuse and could be maintained for longer periods of time (up to 6 hr in some experiments).

**PAH transport.** In order to demonstrate PAH secretion, proximal straight tubules were placed in a bathing solution containing PAH carboxyl-Cl4 (10⁻⁴ m) and were perfused with a PAH-free solution. Cl4 concentration was measured in the collected perfusion fluid and in the outside bath. The results for two tubules which were successfully perfused are shown in Table 3. The mean PAH concentration in the collected perfusion fluid (which initially contained no PAH) was approximately three times as high as in the outside bath, indicating active PAH transport. This concentration ratio is lower than that between tissue and medium in kidney slices and tubule suspensions (2). However, the results are not directly comparable. The tissue-to-medium ratio in slices and suspensions probably represents the maximal concentration gradient which the cells can achieve in the absence of net transtubular transport. When the tubules are perfused, however, PAH is lost continuously from the cells into the lumen, and it is not unreasonable to expect the resulting concentrations in tubule cells and luminal fluid to be considerably less than in the absence of net transport.

**DISCUSSION**

In the present experiments it has been shown that it is possible to perform physiological studies on isolated fragments of single mammalian nephrons. Since virtually all of the individual segments can be dissected from the kidney, more of the nephron is available for study than with conventional in vivo micropuncture techniques which are limited to those portions of the tubule which appear at the kidney surface. That the dissected nephrons are viable is attested to by the maintenance of concentration gradients for Na, K, and Cl in proximal convoluted tubule cells and by the demonstration of net fluid absorption and PAH secretion by perfused proximal straight tubules. The preparation is advantageous for in vitro studies of kidney function since it permits measurement of net transtubular transport, and allows comparison between different segments of the nephron. By combining tubule perfusion and tissue analysis it may also be possible to define with greater certainty the separate contributions of the peritubular and luminal membranes to transport in each segment.

**REFERENCES**