Preparation and study of fragments of single rabbit nephrons

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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.

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 Table).
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 15–40 x 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 (26 x 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 solution 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 (I) in a special room in which the relative humidity was maintained at less than 40%.

Determination of radioactivity. For C¹⁴ and I¹³¹ 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 Ca(NO₃)₂ and 3 mM (NH₄)₂HPO₄. Na and K were determined simultaneously on approximately 3-nl 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 medi-
um 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 ± .058 (SD) and K, 1.015 ± .069.

**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- nl 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 .75 HNO₃ tissue extract was essentially the same with the micromethod (1.86 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

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**FIG. 1.** 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.)
mineral oil. The tubule was pulled from this drop, through the mineral oil and into 10 μl .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 collect-

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**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.

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**FIG. 3.** Perfusion of a single collecting tubule. The direction of perfusion is from left to right.

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**GUEST COMMENTARY**

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I often look at the “tubule rig” shown in the photograph (the first one built in Alabama) and wonder how I ever ended up doing experiments involving an ultramicro technique. Although I had done my Ph.D. thesis at the University of Michigan in the area of transport, I had carefully avoided technically demanding procedures such as intracellular microelectrode recording. I had also declined a suggestion from Art Vander that, because of my interest in renal physiology, I might consider going to the Department of Physiology at Cornell to learn micropuncture. It took a real salesman to get me to approach isolated renal tubule perfusion. Tom Andreoli “made me an offer I couldn’t refuse” when, in 1969, I came to his lab as a postdoctoral fellow in the Department of Physiology at Duke. Actually, when I found out that virtually all of the other postdoctoral fellows in Tom’s and neighboring labs were trying to find something that would transport something across lipid bilayers or were measuring ion fluxes in red blood cells, I decided that doing something different might have its advantages.

Moe Burg was extremely generous with information about both the practical and theoretical aspects of isolated tubule perfusion, and it was this generosity that led to the wide acceptance of this technique and the many discoveries that resulted from it. Before my arrival, Tom Andreoli had already visited Moe Burg’s lab to learn what he could about the technique. While there, he listened to presentations and talked with Moe and the fellows in his lab who were doing...
ed 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 $^{131}$I into a small drop of water in the center of a planchet. 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 $^{131}$I 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, NaHCO$_3$ 25, Na acetate 10, Na$_2$HPO$_4$ 1.2, MgSO$_4$ 1.2, CaCl$_2$ 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 con-
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. 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 dip-

TABLE I. Electrolyte content of rabbit tubules

<table>
<thead>
<tr>
<th></th>
<th>Proximal Convoluted Tubules</th>
<th>Tubule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mEq kg⁻¹ Dry Wt</td>
<td>mEq liter⁻¹ Tissue Water</td>
</tr>
<tr>
<td>Na</td>
<td>165 ± 13 (33)</td>
<td>69.7</td>
</tr>
<tr>
<td>K</td>
<td>261 ± 9 (46)</td>
<td>110</td>
</tr>
<tr>
<td>Cl</td>
<td>132 ± 6 (22)</td>
<td>55.7</td>
</tr>
</tbody>
</table>

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

tubule perfusion, including Jared Grantham, Sandy Helman, Juha Kokko, and Bruce Tune. I particularly remember Tom’s admiration for Moe and his willingness to share the details of his methods. Moe had, to be sure, provided Tom with virtually every detail one needed to perfuse tubules, including suppliers’ names and telephone numbers. What struck Tom in particular was that, when he asked Moe Burg why he was so generous, Burg emphasized that good science should not be a monopoly and that the quicker more people did these experiments and, among other things, confirmed his work, the more all of us would learn. It struck Tom then, and both of us now, that Moe’s approach to science was singularly admirable.

From his visit to Moe’s lab, Tom took back to Duke voluminous notes and blueprints of the pipette advance mechanisms that were later invaluable to me. Tom also had the machine shop in the Duke Physiology Department construct the first set of pipette holders and V-tracks that were made by anyone other than Jim White at the National Institutes of Health. Jim had constructed the original tubule rig parts and continues to supply some tubule perfusers with his beautiful handiwork. When I arrived at Duke the pipette holders were waiting for me, and I began to order and assemble the rest of the rig, including the Unitron microscope, Boley slide rests, the aluminum base sheet, and all of the rest. I used Tom’s notes to begin making perfusion pipettes. All of this was done with frequent calls to Moe for help as I needed it, and I needed a lot. In August of 1969 I, too, visited Moe’s lab to get some firsthand answers to my questions, and he was a cordial and helpful host.

I thought that I had observed everything as carefully as I could and that I had made detailed notes. There was, however, one slight variation in the rig that rose on the bench at Duke. Of course the classic article by Burg et al. (1) was our Book of Genesis, but I may have followed it too closely when I assembled the rig parts. In Figure 3 of that article, it clearly states that: “The direction of perfusion is from left to
TABLE 2. Inulin carboxyl-C¹⁴ recovery in isolated perfused proximal straight tubules

<table>
<thead>
<tr>
<th>Tubule Length, mm</th>
<th>Perf. Rate, nl min⁻¹</th>
<th>Mean Coll. Rate, nl min⁻¹</th>
<th>Mean Inulin-C¹⁴ Conc Ratio</th>
<th>Mean Inulin-C¹⁴ 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>1.15</td>
<td>103</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>1.76</td>
<td>13.3</td>
<td>11.1</td>
<td>1.15</td>
<td>94</td>
<td>7</td>
<td>80</td>
</tr>
</tbody>
</table>

* Recovered fluid/perfused fluid.

TABLE 3. PAH transport by isolated proximal straight tubules

<table>
<thead>
<tr>
<th>Tubule Length, mm</th>
<th>Perf. Rate, nl min⁻¹</th>
<th>Mean PAH Conc Ratio</th>
<th>No. of Collections</th>
<th>Total Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>5.4</td>
<td>3.44</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>1.28</td>
<td>5.4</td>
<td>2.75</td>
<td>4</td>
<td>72</td>
</tr>
</tbody>
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* Collected perfusion fluid/outside bathing solution.

ping the tubules into an otherwise identical medium containing inulin carboxyl-C¹⁴ or albumin-I¹³ⁱ and calculating extracellular contamination from the radioactivity remaining on the tubules after blotting and drying. The results using inulin carboxyl-C¹⁴ and albumin-I¹³ⁱ did not differ significantly. Contamination of proximal convoluted tubules was 2.9 ± 0.2 SEM (41 tubules analyzed) liter kg⁻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-C¹⁴ determinations) for extracellular water.

**Water and electrolyte content.** The water content of proximal convoluted tubules was 2.37 ± 0.10 (17) liters kg⁻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, tubules 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 distended 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-C\(^4\) 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-\(^1\)\(^3\) recoveries were complete. In 40 control collections in 6 collecting tubules the mean recovery was 99.2 ± 2.0%. 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-C\(^4\) (10\(^{-4}\) m) and were perfused with a PAH-free solution. C\(^4\) 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.

The impact of the isolated perfused tubule technique has also been immense in the broader field of epithelial transport. The preparation provided an epithelial system that could be studied in the absence of the serosal cell layers and stroma that complicate experiments in other epithelia, such as the intestine, gallbladder, frog skin, and toad bladder. The isolated tubules consist of a single cell layer with its surrounding basement membrane, providing an excellent opportunity to study epithelial function without the complication of unstirred layers at either surface of the epithelium. Thus the cells responded rapidly to changes in solution composition and hormones or other agents. The absence of cells other than epithelial cells also provided easy access to the basolateral membrane for intracellular recording and reduced the complications of measuring intracellular solute concentrations. If only the isolated nephron segments had been as easy to dissect and handle as a toad bladder, they would have become a model for all epithelial systems.

Particular advantage was taken of the relative simplicity of the isolated nephron segments in studies of water transport in the cortical collecting duct and the proximal tubule. In the collecting duct, work on the biophysics of water permeation led to the concept that a water-filled pore, which excluded even the smallest solutes and ions and which constrained water molecules to single-file diffusion, was responsible for the water permeability induced by vasopressin (16,17). We now know this pore to be a member of the family of aquaporins that have recently been cloned and sequenced.

The fundamental problem of isosmotic volume reabsorption in the proximal tubule and other epithelia was also more easily addressed using the isolated perfused tubule preparation. The process was shown to depend both on the active reabsorption of solutes, primarily those cotransported with Na\(^+\) (18), as well as on the development of passive driving forces because of the preferential reabsorption of solutes with reflection coefficients higher than NaCl (19,20). Thus isosmotic volume reabsorption required the development of only a small absolute transepithelial osmolarity difference because of luminal dilution and interstitial fluid hyperosmolarity, and the presence of an additional “effective” osmolarity difference produced by solute reflection coefficient differences (21,22).

Work in the proximal tubule established not only how solute reabsorption was coupled to water reabsorption, but also that volume reabsorption could be diminished or reversed when active solute secretion was dominant (23). In fact, because most solute secretion occurs in the proximal straight tubule, the isolated perfused tubule technique was essential to the study of this process (24,25).

Obviously, in the very limited space of this article, I have been able to provide merely a glimpse of the knowledge that has been gained using the isolated perfused tubule technique. Moe has made landmark contributions to both renal and epithelial transport physiology, and he continues to...
REFERENCES


