A simplified method for isolation of large numbers of defined nephron segments

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Simplified method for isolation of large numbers of defined nephron segments. Am. J. Physiol. 273 (Renal Physiol. 42): F650–F657, 1997.—We describe a simplified method for isolation of large numbers of nephron segments from rat and rabbit kidneys. In contrast to most previous protocols, the kidneys are not perfused. After removal from the animal, the kidney is sliced and torn in pieces that are subsequently digested in culture medium containing 0.5 mg/ml of collagenase at 37°C. If the preparation is agitated only very gently and infrequently, then the tissue gradually falls apart into a suspension containing long nephron fragments, often consisting of multiple connected segments. These are easily sorted into homogeneous segment populations that can be used for enzyme assays, protein extraction for immunoblotting, and RNA extraction for reverse transcription-polymerase chain reaction, all of which have been done successfully in our laboratory. For comparison, we have also examined cortical collecting tubule segments and cells prepared by the more rigorous protocol described previously (E. Schlatter, U. Froébe, and R. Greger. Pfliegers Arch. 421: 381–387, 1992). Even after the isolation of single cells in a Ca²⁺-free medium, the cells maintain their normal architecture and a distinct separation of apical and basolateral membranes.

Burg et al. (5) subsequently developed the technique for microdissecting and perfusing isolated nephron segments without collagenase treatment. Work by this and other laboratories concentrated on this preparation because of the advantages of the in vitro technique for transepithelial transport processes in regions of the nephron that could not be accessed directly by micro puncture. However, the isolated nonperfused tubule preparation was found to be preferable for many studies of segmental metabolism as refined by Guder et al. (11) and of receptor localization and intracellular second messenger coupling as refined by Morel and colleagues (15, 16, 19, 20). For example, using collagenase treatment, the latter group was able to identify and isolate the multiple segments of the distal convoluted tubule from the rabbit (15) and rat (16) and measure their adenosine 3’,5’-cyclic monophosphate (cAMP) production in response to arginine vasopressin (AVP), parathyroid hormone, isoproterenol, and calcitonin. In the studies in the rabbit, they were able to dissect entire complexes of multiple distal convoluted tubules, with arcades and cortical collecting duct (CCD) intact (see, for example, the micrograph in figure 4 of Ref. 20). The primary shortcoming of this approach is the skill and labor that is required to dissect the nephron segments, which results in a practical limitation to quantities that can be obtained.

Schafer et al. (25) have recently modified the enzyme incubation procedure to prepare isolated CCD segments or cell clusters for patch clamping. In their preparation, the kidneys are perfused in situ with a medium containing both collagenase and another protease. Slices from the kidneys are then incubated with the same enzymes at 37°C, resulting in a more thorough dispersion of the nephron segments. When the digested slices are subsequently transferred to the dissection dish, it is found that many individual segments are completely dissociated from the tissue slices, so that little or no dissection is required. Thus numerous CCD segments could be gathered merely by sorting them from other segments. Cell-attached or excised membrane patch-clamp recordings could then be made from the basal side of the epithelium of the selected segments. Further treatment of these segments in a

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Ca$^{2+}$-free buffer produced clusters of highly uncoupled cells that could be used for fast or slow whole cell patch clamping (25). Despite the enzymatic treatment and disaggregation, these cells were subsequently shown to have normal electrophysiological responses to AVP (25, 28) and normal responses of intracellular Ca$^{2+}$ and Na$^+$/$H^+$ exchange to a variety of hormones (1–3, 24, 26).

We undertook the present study to examine the morphological integrity of the cells isolated by this procedure and to refine the method so that it would be suitable for the dissection of even larger amounts of individual nephron segments for RNA extraction for reverse transcription-polymerase chain reaction (RT-PCR), for multiple enzymatic assays, and for extraction of sufficient protein for immunoblotting. Although we (31) and others (e.g., 30) have conducted RT-PCR on RNA extracted from 1–2 mm of isolated nephron segments, it is desirable to have more tissue for the detection of low-abundance messages. McDonough et al. (18) have also microdissected sufficient amounts of CCD by the conventional collagenase treatment method for immunoblotting of Na-K-adenosinetriphosphatase isomur forms; however, on the order of 100 mm of CCD is required to obtain sufficient protein (~10 µg) for a single lane on the blot. The method we describe below allows a single investigator to sort, measure, and transfer samples of over 50 mm of CCD and other nephron segments within 1 h following digestion. Furthermore, the gentle enzymatic and mechanical treatment used in this method preserves long segments of the nephron consisting of multiple regions. This procedure allows for easier identification and isolation of the desired nephron segments and decreases the time that must be expended in collecting and transferring the segments. We have also examined the nephron segments obtained by previous procedure of Schlatter et al. (25) with scanning electron microscopy (SEM) and have shown that, even with the more vigorous enzymatic and Ca$^{2+}$-free treatment, the CCD segments and cells maintain their normal morphology and polarization of the apical and basolateral membranes.

**METHODS**

First, we briefly describe the previously reported method (25) for the preparation of CCD segments using perfusion of the kidneys and their further digestion with collagenase plus protease, followed by the methods used to examine these cells with SEM. Second, we describe our newly modified procedure, which involves only collagenase digestion without prior perfusion of the kidneys and which permits dissection of larger numbers of relatively long nephron segments.

Collagenase plus protease perfusion and digestion protocol. Isolation of CCD tubule segments and uncoupled cells according to this protocol was similar to the method described before (25). and we will refer to it subsequently as collagenase + protease digestion. For isolation of other tubular segments such as proximal tubules, thick ascending limbs, or inner medullary collecting ducts, the enzyme concentrations and incubation times may require minor modifications. Female Wistar rats (Charles River, Sulzdorf, Germany) weighing 60–150 g were anesthetized with intraperitoneal injections of 100 mg/kg body wt Trapanal (Byk-Gulden, Constance, Germany). The anesthetized rats were placed on a heated table, and the left kidney was exposed. The renal artery was cannulated with fine polyethylene tubing, and 3 ml sterile culture medium (Ham's F12; GIBCO, Berlin, Germany) containing 1 mg/ml collagenase (type IV; Sigma, Deisenhofen, Germany) and 1 mg/ml protease (type XXV, Pronase E, Sigma) were infused through the kidney within 1 min. The kidney was removed immediately thereafter, decapsulated, and rinsed in sterile Ham's F12 medium. Small pieces (~2 mm cubes) were cut from the cortex. These pieces were incubated at 37°C in 2 ml sterile Ham's F12 medium containing 0.5 mg/ml collagenase and 0.5 mg/ml protease and were gassed with 5% CO$_2$-95% air in a glass vial for ~10 min with mild shaking. At this point the medium contained isolated glomeruli and short tubule segments. After centrifugation at 3,000 rpm for 2 min, the enzyme-containing solution was removed, and tubules were resuspended in ice-cold Ham's F12 medium. Individual tubule segments could be identified easily in a dissection microscope at ×25–40 magnification by their appearance and dimensions. These tubule segments are suitable for patch-clamp studies (cell attached, excised, and slow or fast whole cell configuration) with seals obtained from the basal side. The cells of these tubular segments can also be electrically and physically separated by an incubation in ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA)-buffered (5 mM) Ca$^{2+}$-free medium on ice with gentle shaking for an additional 10–20 min, which produces cell clusters with rounded, electrically uncoupled cells (see Fig. 2). This Ca$^{2+}$-free incubation resulted in isolated cells (see Fig. 3) within 10–20 min.

Scanning electron microscopy. Tubules or cells prepared by the collagenase + protease protocol were seeded on Thermoporo plates (10.5 × 22 mm; Nunc, Naperville, IL) coated with poly-L-lysine as an adhesive. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 1 h and then dehydrated in ethanol solutions with ascending ethanol content. Thereafter the critical point dried cells were coated with a 10-nm thick layer of platinum and examined using a Hitachi S-800 scanning electron microscope.

Simple collagenase digestion protocol. The animals used in these studies were male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) and female New Zealand White rabbits (Mertle's Rabbits, Thomlinson, NJ). The rats and rabbits were obtained 5–14 days before use and were maintained ad libitum on tap water and standard pellet diet chow (16% rodent diet no. 8746; Teklad, Madison, WI) or standard pelleted rabbit chow (Teklad 15% rabbit diet 8630). At the time the rats were used for an experiment, they weighed 100–170 g, and the rabbits weighed 2–2.5 kg. To obtain a kidney for isolation of nephron segments, rats were decapitated, and rabbits were anesthetized with a combination of ketamine (40 mg/kg) and xylazine (10 mg/kg) and decapitated when surgical anesthesia had been achieved.

In both species the kidneys were rapidly harvested after decapitation, and they were not perfused with any solution before removal. The capsules were stripped away, and 0.5- to 1.0-mm thick slices of tissue were made using a Thomas-Stadie-Riggs tissue slicer (Thomas Scientific, Swedeboro, NJ). When we wished to dissect nephron segments from the cortex, the slices were cut tangential to the cortical surface opposite the hilum. For the rat kidney, only one cortical section was taken, because the thickness of the cortex in rats of this size is only slightly more than thickness of the slice. For the rabbit kidney, which has a cortical thickness of ~4 mm, at least three slices could be taken. The sections from both species were carefully examined to be sure they contained no medullary tissue. Coronal slices were used to dissect nephron segments from the medulla or from medul-
lary rays. In the first case, the cortical tissue was removed before the tissue was digested, and in the latter case, the medullary tissue was removed.

Slices of cortex or medulla were then torn into small chunks using forceps (two to four pieces in the case of the rat slices and twice as many for the rabbit slices). These pieces of tissue were incubated in 2 ml of warm Eagle's minimal essential medium (MEM) containing 0.5 mg/ml of type 2 collagenase, 5 mM glycine, 50 U/ml deoxyribonuclease (DNase), and 48 µg/ml of soybean trypsin inhibitor in a 20-ml scintillation vial. [All reagents were from Sigma Chemical, St. Louis, MO, with the exception of the collagenase (catalog no. 4176), which was obtained from Worthington Biochemical, Freehold, NJ.] We refer to this medium below as "MEM-collagenase mixture." The cortical pieces were briefly (<5 s) and very gently agitated by hand and incubated without shaking at 37°C while exposed to room air. (We have also used a standard tissue culture incubator gassed with air plus 4% CO2 for the procedure, but this does not seem to be necessary.) At ~15-min intervals, the supernatant was poured off the sedimented tissue into a 5-ml ice-cold test tube. Fresh MEM-collagenase medium was added to the remaining tissue pieces, and digestion could be continued with supernatant removal at 10- to 15-min intervals in the same manner until the large sections were exhausted. Cloudy, tubule-rich supernatants were evident after 30-40 min of digestion. The progress of the digestion was followed by examining droplets of the supernatant under a dissecting microscope.

It was found that tubule segments sedimented rapidly in the test tubes. The supernatant overlying the tubule segments was carefully removed with a Pasteur pipette and replaced with 2 ml of ice-cold, enzyme-free dissection solution containing 1% bovine serum albumin (BSA), and the tubules were stored on ice until sorting began. We expected that the BSA would bind residual collagenase and any contaminating proteases, and this would help to prevent further digestion. For tubule selection, aliquots of the tubule-rich suspension were gently pipetted, using a large bore transfer pipette, into a dissection dish containing MEM (we have also used Ham's F12 medium successfully) with 0.05% BSA at 4°C. Tubule segments were sorted from the central group of tubule segments by using a 30-gauge needle to move them to a clean area of the dish. Selection of tubule segments could be carried out over 3–4 h. The recovered segments were measured individually with an ocular micrometer, and groups of selected segments with a total length of 20 mm were transferred in 10 µl of medium with a P-20 Pipetman (Eppendorff Scientific, Madison, WI) as needed for further experimentation.

To examine the viability of the nephron segments, several "control" tubules were transferred in a 10-µl sample to a 10-µl drop of MEM containing 0.4 g/dl of trypan blue. If more than five stained cells were found in a 0.5-mm length, none of the samples was used. When the tubule segments were to be used for RNA or protein extraction, they were rinsed by transferring them to a 1.5-ml microcentrifuge tube containing ice-cold fresh medium. The tubules were briefly centrifuged at ~1,000 rpm, and the supernatant was removed by pipette. The RNA extraction medium (TRIzol; Life Technologies, Gaithersburg, MD) or the protein extraction buffer was immediately added to the pellet, which dissolved instantaneously.

For the purposes of the present methodological study, we pipetted a 50-µl droplet containing the tubules onto a microscope slide and covered the droplet with a large coverslip. The tubules were examined using a Nikon Diaphot inverted microscope equipped with ×10 wide-field eyepieces. Incident light was either bright-field diffused through a white frosted filter or passed through a Hoffman condenser (Modulation Optics, Greenvale, NY). The objectives were as follows: a Nikon ×2 Plan, a Nikon ×10 Plan modified by Modulation Optics for Hoffman modulation contrast microscopy, and a Nikon ×60/1.40 Plan Achromat oil-immersion lens. In the case of these light micrographs (Figs. 1-4), photographs were taken using 35-mm Kodak T-Max 100 black and white film. Images were digitized from the negatives using a Management Graphics Solitaire 8xp Film Recorder (Dept. of Photography and Instructional Graphics, Univ. of Alabama). The images were digitally cropped, sized, and labeled, the contrast was optimized by Photoshop software (Adobe Systems, Mountain View, CA), and the image files were prepared for electronic transmission directly to the publisher (via FTP). Intermediate hard copies were produced using an Epson Color Stylus II ink jet printer.

**RESULTS AND DISCUSSION**

Collagenase + protease perfusion and digestion protocol. The protocol was used only to prepare cells for examination by SEM and thereby to verify that even the more vigorous enzymatic and Ca2+-free treatments did not alter the cells structurally. Figure 1 shows a rat CCD segment near a bifurcation. Segments such as these were observed after collagenase + protease treatment but without Ca2+-free treatment. Figure 2 shows a cluster of rat CCD cells prepared according to the
collagenase + protease protocol followed by 15 min of Ca\textsuperscript{2+}-free incubation on ice. The isolated CCD cell shown in Fig. 3 was also prepared by the collagenase + protease protocol followed by 15 min of Ca\textsuperscript{2+}-free incubation. In all cases, it can be seen that there is excellent preservation of the fine structure of the cells. More importantly, even when the cells are completely isolated, as in Fig. 3, they maintain their polarity.

The CCD segments obtained by the collagenase + protease procedure have been used for several physiological experiments including microfluorometric determination of cellular Ca\textsuperscript{2+} activities (2, 23, 26), cellular Na\textsuperscript{+} activities (23), cellular pH (25, 27), and patch-clamp experiments to record membrane voltages (14, 25, 27, 28), whole cell conductances (25, 27), or single-channel currents (3, 12, 13, 14, 25, 27). The enzymatic isolation procedure including the Ca\textsuperscript{2+}-free incubation apparently does not remove or inactivate hormone receptors, because in several studies we could functionally demonstrate the existence of V\textsubscript{1} and V\textsubscript{2} receptors (2, 25, 26), of acetylcholine receptors (2), of purinoceptors and angiotensin II receptors (26), and of \(\beta\)-receptors (2).

The same methods have been used previously to obtain medullary and cortical thick ascending limbs (MTAL and CTAL) and inner medullary collecting duct segments. Viability of the segments was indicated by intracellular voltage and pH measurements made in the case of the ascending limb segments (4) and by hyperpolarization of the intracellular voltage upon amiloride addition in the case of the inner medullary collecting ducts (unpublished observations).

Simple collagenase digestion protocol. Figures 4 and 5 show samples of nephron segments isolated from, respectively, rat and rabbit kidneys treated by the collagenase digestion protocol. As shown in Fig. 4A, the supernatant drawn from the collagenase digestion vial and placed in the dissection dish consisted of numerous separated nephron segments. Using either a 30-gauge needle or a fine forceps (Dumont no. 5), we found it quite easy to separate like tubule segments into collections in a clean area of the dissection dish from which they were subsequently transferred for microscopic observation or for experiments. In the case of cortical tissue slices, complexes of connected CCD, connecting tubules (CNT), and distal tubules (DT) were observed frequently and could easily be trimmed to isolate individual segments. For example, we frequently observed preservation of the entire structure of the DT from the CTAL of the loop of Henle to the CCD (e.g., see Fig. 4G), which allowed easy identification of the
macula densa region, early (bright) DT, late (granular) DT, and the CNT segments. We could also obtain full lengths of all the medullary segments from the coronal kidney slices (Fig. 4, C–E).

We found the most critical aspect of the collagenase digestion procedure and the subsequent transfer of the nephron segments was to avoid mechanical stresses that would shear the tubule segments. When the suspensions were aerated by bubbling, when the tubules were resuspended by swirling, or when the tubules were centrifuged even briefly at low speed, the segments were uniformly found to be much shorter. Therefore, when the tubules were to be rinsed with fresh medium, they were allowed to settle to the bottom of a container such as a microcentrifuge tube, the old medium was pipetted from the top, and the new medium was slowly introduced down the side of the container. When tubule segments were later transferred from the dissection dish to a microscope slide or to another container for further processing, groups of 5–10 were drawn slowly into a 20-µl Eppendorf Pipetman tip and slowly ejected, with both procedures being
conducted under observation with the dissecting microscope so that a complete transfer of the measured length could be confirmed. In this way a single investigator could separate, measure, and transfer over 50 mm of a given nephron segment within 1 h.

The integrity of the isolated segments was examined by testing their ability to exclude the vital dye trypan blue, which has been a frequently used criterion for viability (9). Figure 6 shows an example of a CCD-DT complex from a rat kidney that was purposely damaged by incubation at room temperature. Several cells that stained with trypan blue can be seen with the ×10 objective, and with the ×60 objective it can be seen that even those cells that are not stained with trypan blue are swollen and granular. Using trypan blue and cell appearance at high power as indicators of damage, we found that the tubules remained viable for at least 3 h when kept either at 37°C in an incubator gassed with air plus 4% CO₂ or at 4°C (as in the dissection dish). On the other hand, even relatively short incubation (<30 min) at room temperature with or without gassing caused significant cell damage, as seen in Fig. 6. We also varied the concentration of collagenase in the MEM-collagenase mixture and found that we could reduce it to 0.5 mg/ml and maintain good tissue digestion. This concentration is considerably lower than the 2 mg/ml more typically used in previous procedures.

It is not clear why the tubules are more labile at room temperature, although we might speculate that, because metabolic and transport processes have differing temperature sensitivities, the persistence of some metabolism at room temperature causes greater cell damage than the cessation of all metabolism at 4°C. Also, it has long been standard procedure to use ice-cold solu-

Fig. 5. Rabbit cortical nephron segments prepared by collagenase protocol. A: collection of nephron segments sorted from the dissection dish and transferred to a microscope slide in a droplet of MEM. B: a CCD (right) with attached CNT and DT segments. C: proximal convoluted tubule. D: Short segment of CCD (pointing toward the bottom left) and attached connecting and DT segments. Micrograph in A was taken with bright-field illumination and the ×2 lens; all other micrographs were taken with the ×10 lens and Hoffman contrast modulation.

Fig. 6. Effect of incubation at room temperature on tubule integrity. A: complex of rat CCD, CNT, and DT segments (Hoffman contrast modulation ×10 objective) was incubated at room temperature for 40 min in MEM medium and then transferred in 10 µl of the medium to an equal volume of medium containing 0.4% trypan blue. Damaged cells that are permeable to the vital dye can be identified by the dark spots corresponding to nuclei. B: (×60 oil-immersion objective) higher magnification of the region of the leftmost confluence of two distal segments in A shows four cells with nuclei that are stained by trypan blue. Other cells are swollen and granular in appearance.
tions for tissue preservation, for example, in the preservation of organs for transplantation. In this regard, it might be possible to preserve the nephron segments from the digested kidney slices even longer if we were to use a hyperosmotic solution with a composition more like that of intracellular fluid (21, 22) as is common practice in organ transplantation. However, we did not find this necessary in these experiments.

We have done several series of experiments in which RNA was extracted from isolated proximal tubule and CCD segments for amplification by RT-PCR (29, 32), and the method provides more than enough RNA for the detection of even low-abundance messages. We have also used this method for analysis of adenyl cyclase activity in isolated, nonpermeabilized rat CCD segments. These studies confirmed markedly enhanced cAMP production with 20 or 200 nM AVP and that this stimulation could be inhibited by either 100 nM epinephrine or 10 µM dopamine (17). These observations demonstrated that receptors for all three hormones were intact and that metabolic ATP production and adenyl cyclase activity were normal. This was expected because, even with the more vigorous isolation using the collagenase + protease protocol followed by disaggregation of the cells in a Ca²⁺-free buffer, there was normal receptor and transport activity as discussed above.

The method is also valuable for extracting sufficient protein from a discrete nephron segment for immunoblotting. We have found that ≈10 µg of protein can be isolated from 100 mm of proximal and/or CCD segments and that this is sufficient for immunoblotting of all but very low-copy proteins. This can be compared with the observations of McDonough et al. (18), who found that 6.8 µg of protein could be extracted from 40 mm of proximal straight tubule but that only 0.12 µg of protein could be extracted from 40 mm of CTAL or MTAL. On the other hand, 100 mm of proximal or CCD segments provides only on the order of 10 ng of total RNA after standard preparation by guanidium isothiocyanate/acid phenol extraction with one propanol and one ethanol precipitation. This small amount of total RNA is, unfortunately, insufficient for the isolation of mRNA for either Northern blotting or ribonuclease protection assays. Thus other methods, such as immunodissection (10), will have to be perfected to obtain larger amounts of RNA from a pure segment population.

Nevertheless, this method provides a simple and quick method for the preparation of relatively large amounts of individual tubule segments that are very easily identified and thus homogeneous, and the method does provide sufficient material for immunoblotting of proteins and for RT-PCR of mRNA from individual nephron segments. If samples from multiple investigators were pooled, then it would be possible to obtain sufficient RNA to prepare cDNA libraries of specific nephron segments from rats and rabbits that have been maintained and monitored while on a variety of dietary protocols.

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