METHODS OF COLLECTING FLUID FROM KNOWN REGIONS OF THE RENAL TUBULES OF AMPHIBIA AND OF PERFUSING THE LUMEN OF A SINGLE TUBULE

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The development of technique for microscopic observation of the amphibian kidney during life, for collecting fluid from single renal corpuscles and for determining quantitatively various urinary constituents in exceedingly minute amounts of fluid has resulted in the acquisition of information, of a sort not previously available, concerning the glomerular circulation and the composition of glomerular urine (1, 2, 3). Subsequent work has made it possible to subject the renal tubules of amphibia to similar direct study with the result that quantitative data of comparable quality can now be presented concerning the more important changes which the glomerular filtrate undergoes during its passage through the several parts of the uriniferous tubule. Fluid from different levels of the tubule has been collected and analyzed; the parts of the tubule which it had traversed have been identified; and, in special experiments designed to reveal the passage of substances through the tubule wall, the lumina of portions of single tubules have been perfused with artificial solutions.

In the papers which follow this the data thus far obtained will be presented; in this paper are described the manipulative and experimental procedures by which they were obtained.

The animals employed were adult Necturus (N. maculosus) and frogs (R. pipiens). The tubules in Necturus are of larger size and less tortuous course than those of the frog kidney.2 Consequently the greater number of experiments has been made with Necturus.

NECTURUS. The induction and maintenance of anesthesia with urethane,

1 The expenses of this work have been defrayed in large part from a grant by the Commonwealth Fund of New York. Brief descriptions of the technique have been presented before the American Physiological Society (This Journal 109:87, 107, 1934) and before the Harvey Society (Am. J. Med. Sci. 190: 727, 1935).

2 The kidney of an adult Necturus is about 6 times as large as that of a large frog (pipiens). The number of nephrons in one such Necturus kidney was 784; the average number in that of the frog is about 2000. The average capacity of a proximal convoluted tubule of Necturus (7 measurements) was 0.31 cu. mm.; in frogs, it was estimated to be about 0.05 cu. mm.
the exposure of the kidneys and their illumination by reflected light have
been previously described (4, 5). Intravenous injections were made
through a cannula inserted either into the anterior abdominal vein or a
mesenteric vein. The ventral surface of the kidney was usually kept dry
with cotton during an experiment to avoid the possibility of entrance of
surface fluid into the tubule through its nephrostome. Urine from the
ureter was collected either from an incision or by means of a cannula.
Blood samples were taken from the posterior vena cava by puncture with
finely pointed glass capillary pipettes containing dry heparin or potassium
oxalate.

The anatomy of the kidney of Necturus has been well described by
Chase (6). It seems desirable, however, to give a brief description of the
disposition of the different parts of a nephron as they present themselves
when the ventral surface of the kidney is studied with the binocular mi-
croscope (fig. 1).

The visible glomeruli are arranged in an irregular row parallel with and
close to the mesial border of the kidney. The ciliated neck of the tubule
and the ciliated tube which connects with the nephrostome on the surface
of the kidney are rarely visible unless made so by an intracapsular injection
of some opaque or colored material. The beginning of the proximal tubule
is recognizable by reason of its large diameter, the pigmentation and appar-
ent thickness of its wall. The first section of the proximal tubule, approxi-
mately one-quarter its total length, takes a relatively direct course toward
the lateral border of the kidney. There it makes several convolutions,
only fractions of which are visible; it ends as a short straight segment
directed back toward the mesial border and is recognizable by the direct-
ness of its course, the transparency and relative lack of pigmentation of its
wall. As it approaches the region of the glomeruli it narrows abruptly to
form the intermediate tubule or narrow segment.

The narrow, ciliated intermediate tubule takes a relatively straight
course towards the mesial border of the kidney but is rarely visible unless
special procedures are adopted. At the level of or mesial to the glomeruli
it becomes the distal tubule which remains narrow in its first part, convol-
lutes several times in the immediate neighborhood of the glomeruli and
usually lies too deep to be seen. The second segment of the distal tubule is
wider and portions of it are often visible at the kidney surface; these are
readily recognizable as small, highly transparent lacunae in the interstices
of proximal tubules, blood vessels and connective tissue.

The collecting duct arises from the distal tubule about half way between
the mesial and lateral borders of the kidney, runs a straight course towards
the ureter, and is uniformly so deep within the kidney tissue as to be
invisible.

The following figures are the averages of measurements by Dr. R. T.
Kempton\textsuperscript{3} of the first four parts of the tubule in the kidneys, after dehydration, which have served in our experiments. In parentheses are given the number of measurements averaged. Neck, 0.9 mm. (135); proximal convoluted tubule, 13.9 mm. (133); intermediate tubule, 1.5 mm. (63); distal convoluted tubule, 8.1 mm. (65).

The precise site of puncture of the wall of a tubule with reference to the nephron as a whole can not usually be determined by simple inspection. Consequently in nearly all of the experiments, at the conclusion of a collection of tubule fluid, the pipette was filled with a 1:10 dilution of Higgins' india ink, reinserted into the puncture hole and the lumen of the entire nephron filled with ink. The kidney was then quickly excised and placed in absolute alcohol for dehydration, cleared in clove oil and an accurate scale drawing made of the injected nephron. The puncture hole was usually seen distinctly in the cleared preparation and measurements were made of the distances from it to the ends of the section of the tubule in which it occurred.

\textit{Collection of tubule fluid.} The apparatus used was the mercury-filled, quartz capillary pipette system of Wearn and Richards (2). The tip of the pipette was sharply bevelled and about 20\(\mu\) in diameter. The stand and micromanipulator were placed so that the pipette was in the same vertical plane as the section of tubule to be punctured and at an angle of about 30° with the surface of the kidney. The force required to push the point of the pipette through the peritoneum and tubule wall sometimes displaced the kidney to such an extent that when puncture suddenly occurred the wall of the tubule was torn or the tubule lying immediately beneath it was also punctured. Such an accident necessitated the selection of another tubule for the experiment.

For correct interpretation of analytical results it is essential that the

\textsuperscript{3}A more complete account of the dimensional relationships of the Necturus tubule will be published by Dr. Kempton in the Journal of Morphology.
collected fluid shall not be contaminated with fluid drawn back into the pipette from a part of the tubule distal to the site of puncture. One precaution against this source of error was the practice of keeping the levelling bulb of the collecting system at such a height that the surface of the mercury in it was slightly higher than the tip of the collecting pipette in the lumen of the tubule. Greater certainty was secured by blocking the lumen of the tubule at a point immediately distal to the site of puncture. This was easily done when collection was to be made from the very beginning of the proximal tubule by inserting the pipette at the desired point and injecting a globule of mercury into the lumen; prevented from moving proximally by the narrowness of the tubule neck, it took a position just distal to the point of the pipette. Mercury, similarly injected, was sometimes used to block the distal end of the proximal tubule before collecting from its terminal segment; we came to regard this as an unnecessary precaution because, in a number of trials, we were unable to draw fluid back from the distal tubule through the narrow, ciliated intermediate tubule.

When fluid was to be collected from other parts of the proximal tubule a relatively thin mineral oil was chosen as the obstructing fluid in preference to mercury because, owing to its lower surface tension, its injection through the capillary pipette could be controlled with far greater nicety. The injected column of oil moved with the current of tubule fluid to a position distal to the point of the pipette where it remained as long as all of the tubule fluid coming to the pipette was collected. It could be caused to move by slight change in the height of the levelling bulb of the collecting system: hence throughout a collection, it served as a sensitive indicator of the correctness of adjustment of the point of the pipette within the lumen of the tubule.

Collection of fluid from the intermediate tubule is difficult because its

Fig. 2. Illustrating the insertion of a micropipette for the collection of tubule fluid from the beginning, A; the middle, B, and the distal end, C, of a proximal convoluted tubule of Necturus. Stippling at A represents a globule of mercury; at B, a column of colored oil.

\textsuperscript{4} Atlantic Refining Company's "250 T" oil, saturated with Scharlach-R to increase its visibility.
lumen is narrow and it usually lies too deep to be easily visible. Its visibility can be increased by injecting a minute amount of diluted india ink into the distal end of the proximal tubule and allowing it to flow through the intermediate, or by injecting air and then mercury into the lumen of a distal tubule, thus forcing the air to move proximally through the intermediate tubule. The mercury blocks the distal end of the intermediate tubule, and the air in it is gradually absorbed. Then fluid can be collected in a pipette the tip of which has been thrust through the wall of the intermediate tubule.

Collection of fluid from the distal tubule is also difficult in that only a few small portions of its convolutions are visible at the ventral surface of the kidney. With practice they are easily distinguished by their location and appearance, but no estimate can be made of the site of puncture until the experiment has been finished, the tubule injected with ink and the map of the tubule drawn. For this reason it is largely a matter of chance whether or not the data accumulated in a number of experiments will show the progress of the changes which take place in fluid as it passes through the distal tubule.

Another difficulty arose because of the extreme ease with which fluid comes back into the pipette from the collecting duct and ureter. To avoid this the ureter was incised so that there was no impediment to escape of urine from it. The lumen of the tubule was blocked distally to the site of puncture by the injection of colored oil as described above.

Success in the collection of enough fluid for analysis from any part of the tubule obviously depends upon the delivery of an adequate amount of glomerular filtrate into the tubule. For this reason sluggish glomerular circulation, more frequently encountered in Necturi than in frogs, has, in our experience, required many animals to be discarded. For the same reason fluid may stagnate in the tubules and care must be taken that stagnant fluid be not included in tubule fluid collections. On occasion, also, tubule fluid from Necturi contains protein in concentrations from one-hundredth to one-tenth of that in blood plasma. The analyses however indicate that this circumstance is not associated with decrease in tubule function. Only rarely has either of these disadvantages been encountered in frogs.

Perfusion of the lumen of a proximal tubule. In order to answer questions concerning the passage of individual constituents of the urine through the wall of the tubule (reabsorption, secretion or diffusion) a procedure was developed in which, after the abolition of function of the glomerulus, an artificial solution, differing from normal glomerular urine in one or more particulars, was introduced into the lumen of the tubule at one point, collected for analysis at another. The procedure was as follows (fig. 3): Two capillary pipette systems were set up, one for perfusing, \( P \), the other
for collecting, C. A nephron was chosen of which both ends of the proximal tubule were visible and accessible to puncture. One pipette, P, was inserted through the capsule of Bowman and mercury injected until the capsule, the neck of the tubule and the lumen of a short stretch of the proximal tubule were filled with mercury. (Pressure of the mercury remaining within the capsule stops glomerular filtration.) This pipette was then withdrawn, charged with the perfusion solution and its point inserted into that portion of the lumen of the proximal tubule which was filled with mercury. Upon forcing some of the solution to flow in, the mercury column in the tubule was broken and the distal fragment driven through the lumen to the distal end of the proximal tubule where it was arrested by the narrowness of the intermediate tubule. Injection of fluid from pipette P was stopped and the point of pipette C inserted into the lumen of the tubule at a point proximal to the mercury which blocked its distal end. (It was often necessary to remove pipette P during puncture with C to avoid tear of the wall at P. When this was the case, reinsertion of P was made through the original puncture hole.) Perfusion from pipette P and collection in pipette C were then begun, the heights of the levelling bulbs of the two systems being so adjusted that the perfusion rate was similar to the normal rate of flow of glomerular fluid into the tubule. Both ends of the tubule being blocked, measurements of the amount of fluid which enters the tubule from pipette P and is collected in pipette C give information concerning the entrance or escape of fluid through the wall of the perfused tubule. By a slight modification of the technique the perfusion could be confined to any desired portion of the tubule.5

5 In about half of the experiments of this type the collected perfusates contained easily detectable traces of protein, despite the facts that the original perfusion fluid contained none and glomerular fluid did not have access to the tubule. When such a perfusate was mixed with serum from a rabbit which had been immunized against Necturus serum, a precipitate formed, showing that in Necturus, under the conditions of these experiments, serum protein can find entrance into the lumen of the tubule through its wall (experiment by Dr. Chas. H. Hudson (7)).
Perfusion of the lumen of a distal tubule. The procedure just described could not be applied successfully to perfusion of the distal tubule because so much of it is invisible and inaccessible to puncture. Consequently a plan of perfusion via the ureter was adopted. A cannula was tied into the ureter and perfusion fluid introduced into it in such a way as to leave the tip filled with air. After connecting the cannula by rubber tubing with a reservoir containing the perfusion fluid the air in the cannula was forced back through the collecting ducts and tubules until it could be seen in the renal corpuscles or emerging from the nephrostomes. A capillary collecting pipette was then inserted into the distal end of a proximal tubule, a globule of mercury injected to prevent the descent of fluid from the glomerulus to the level of the point, and the perfusion reservoir raised to such a height as was necessary to provide a slow, constant flow of fluid through the distal and intermediate tubule into the collecting pipette.

In some experiments both the distal and the proximal tubule were perfused; in these the collecting pipette was inserted into the proximal end of the proximal tubule. In both instances the initial portion of the collection was rejected.

 Frogs. Preparation. The brain was crushed with hemostatic forceps, the right kidney exposed and arranged for illumination with transmitted light in the manner described in previous papers from this laboratory. Urine samples were taken from the ureter; blood samples usually from the ventricle by puncture with capillary pipettes. The points of the quartz pipettes used in puncturing the tubules were commonly less than 10\mu in diameter.

Collection of fluid from tubules. The frog's kidney possesses two advantages over that of Necturus; there are no nephrostomes through which fluid can gain direct access to the lumen of the tubule; the glomerular circulation is more vigorous so that the chance of collecting fluid which has been stagnant in glomeruli or tubules is absent. With respect, however, to ease of carrying out such procedures as those described above the frog's kidney is highly disadvantageous because of the much smaller size of the tubules and because of their tortuosity (fig. 4). The classical description by Nussbaum (8) of the disposition of the different parts of the tubule in the frog's kidney applies to the thicker portions of the kidney and not to the thinner part near the lateral border in which the more accessible tubules lie. Here the proximal and distal convolutions are not localized at the dorsal and ventral surfaces respectively. Both sections of the tubule are spread out in a thin sheet of tissue and direct inspection furnishes no clue as to the part of the tubule in which any visible loop is situated or the glomerulus from which any particular tubule arises. Most frequently the entire proximal tubule and the distal two-thirds of the distal tubule are more or less intertwined in the region lateral to the glomerulus; part of the first portion of the distal
tubule usually loops about the glomerulus to form a convolution mesial to it. For these reasons collection of fluid from predetermined levels of the tubule is exceedingly difficult. It is frequently possible to inject a dye solution or graphite suspension into the capsular space, watch it pass through the successive parts of the tubule and so to make rough identification of the relations of a visible segment to the entire tubule. Puncture of the wall of this segment can then be made and fluid collected with fair assurance of the approximate level of the tubule from which it came. In the majority of experiments however the plan has been adopted of selecting a distended segment of tubule at random and identifying its relations after collection from it was finished. This was done by filling the pipette with dilute india ink, reinserting it into the tubule and injecting the ink so slowly that the number and extent of convolutions through which it passed before it appeared in the glomerulus, in the first portion of the distal tubule or in the ureter could be observed. The accuracy of this procedure is only approximate, but, after experience had been gained, it was usually possible to be certain in which chief section of the tubule the puncture had been made and also in which third of that section. In some instances in which this method failed the punctured tubule was filled with india ink, the kidney excised and embedded in paraffin, and serial sections made. These revealed the site of puncture and showed whether it was in the proximal or

Fig. 4. Single nephrons in the lateral border of the frog's kidney. From a scale drawing of ink-injected cleared specimens. Stippled portions are the capsules and proximal convoluted segments.
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distal segment. The method of clearing the kidney and mapping the injected tubule which was effective in work with Necturus kidney was unsuccessful because the convolutions are so confusingly superimposed.

While the design of collecting fluid from various levels of the renal tubule in the frog can be accomplished by this method if the number of experiments is sufficient, the distal regions of the distal tubule are least apt to be included in a series of experiments by this chance method of approach. Consequently a special technique for identifying and puncturing this part of the tubule was devised. Oil colored with Scharlach-R was injected into the ureter through a cannula. Under the microscope it could be seen to fill the collecting ducts and to enter the terminal convolutions of some of the distal tubules. The location of these was marked for later identification by reference to fixed points in the field (e.g., blood vessels, pigment cells, other tubules), the ureteral pressure released and the tubules, collecting ducts and ureter allowed to be cleared of oil by the current of tubule fluid. The pipette was then inserted into one of the marked tubules and a short column of Scharlach-R oil injected. As soon as this had moved distally from the point of the pipette collection of fluid was begun. While blockage of the tubule distal to the point of puncture to prevent contamination of collected fluid is not believed to be as uniformly necessary as in Necturus the frequency with which reversed flow has been seen in the frog's tubule makes it an advisable part of the technique. It has the additional advantage of indicating the direction of flow within the tubule.

Perfusion of the lumen of the frog's tubule. The procedure is the same in principle as that described on p. 115. Mercury, however, can not be used to arrest glomerular filtration and to block the tubule because of the difficulty of injecting a sufficiently small amount. Oil is more easily managed; hence in preparing to perfuse a frog's tubule the perfusing pipette, charged with colored oil, was inserted into the intracapsular space and enough oil injected to fill the space and the lumen of about 0.5 mm. of proximal tubule. The pipette was then withdrawn, filled with the perfusion fluid, inserted into the oil-filled lumen of the proximal tubule and enough fluid injected to break the oil column and drive the distal fraction through the tubule to a position immediately distal to that judged to be favorable for the insertion of the collecting pipette. The injection was stopped during insertion of the collecting pipette and when this had been accomplished the perfusion was begun.

Perfect insertion of the two pipettes is more difficult in the frog than in Necturus because of the more compact arrangement and smaller dimensions of the tubule. Great caution is necessary here as well as in collections from normal tubules to avoid penetrating the dorsal wall of the tubule and entering the tubule which lies beneath it.

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drawings which illustrate this paper and for his valuable help in identifying sites of tubule puncture in the experiments described in the papers which follow this.

SUMMARY

Procedures have been described by which fluid, in amounts sufficient for analysis, can be collected from various identified levels of the renal tubules of Necturi and frogs. Methods are also described for perfusing different parts of the lumen of a single tubule with artificial solutions.

REFERENCES

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