INVITED REVIEW

DILUTION OF URINE THROUGH RENAL FLUID SECRETION: ANATOMO-FUNCTIONAL CONVERGENCE IN MARINE ELASMOBRANCHS AND OLIGOCHAETES

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Abstract

Homer Smith's finding that urea concentration in shark urine is lower than in plasma suggested that marine elasmobranch nephrons actively reabsorb urea from the glomerular filtrate in the renal tubules. This suggestion has not been supported by experimental data. Attempts to locate a segment of the renal tubule responsible for the alleged active reabsorption have resulted in detailed descriptions of the unique and complex configuration of the renal nephrons of marine sharks and skates, without identifying a urea-reabsorbing segment. On basis of several findings of renal tubular secretion of fluid in fish nephrons, including sharks and skates, I suggest that the lowering of urea concentration in urine below that of plasma is due to tubular fluid secretion rather than to active urea reabsorption. The functional significance of the complex configuration of the elasmobranch nephron may be reevaluated by comparison with that of the nephridia of earthworms and leeches which also produce dilute urine. These animals exhibit complex configurations of their excretory tubules, surprisingly similar to those of the marine elasmobranchs. It appears that these configurations have evolved independently in order to produce a urine hypoosmotic to plasma through fluid secretion, and that the lowering of the urea concentration, useful in elasmobranchs, is incidental to this other function.

Introduction

Marine elasmobranch maintain plasma and tissue urea concentrations as high as 350 to 400 mmol/L. An accumulation of urea and trimethyl amine oxide (TMAO) in body fluids serves to raise the osmotic pressure to a value slightly above that of the surrounding seawater. Thus, marine elasmobranchs need to prevent urea loss through their gills and kidneys. There is now good functional evidence that retention of urea through the gills depends on active urea reabsorption in the gill epithelium (in additon to a specialized basolateral membrane with very low permeability to urea) [8]. By homology cloning with newly discovered facilitated urea transporters in mammals (see review in [2, 17]), two such transporters have been cloned from kidneys of the spiny dogfish shark and the Atlantic stringray [15, 30], but facilitated diffusion of urea alone could not prevent urea escape in the urine. It is thus not yet clear whether urea conservation in the kidney depends on active urea reabsorption, as first proposed by Homer Smith, but not yet clearly demonstrated in this organ, or could result from other yet unidentifed mechanisms.

In 1929, Homer W. Smith was the first to describe that the concentration of urea in the urine of the marine elasmobranch, *Squalus acanthias* is significantly lower than in the plasma (see review in [31]). From his finding that the urea urine-to-plasma concentration ratio (U/P) in marine elasmobranchs normally ranges from 0.5 to 0.1, Smith concluded that urea was actively reabsorbed from the glomerular filtrate in the renal tubules and this conclusion was based on the assumption that all of the fluid entering the renal tubules did so through glomerular filtration of plasma [31]. It was subsequently repeated in most subsequent papers dealing with elasmobranch renal function although my own experimental findings [21, 28, 29] did not support Smith's conclusion. That the concentration of urea in the tubules could be lowered not only by active reabsorption of urea, but also by tubular secretion of fluid was never considered at that time in spite of the fact that certain marine teleost fish, which have no glomeruli in their kidneys and yet produce urine [17], must secrete fluid into the tubules, and that many invertebrates are able to secrete fluid into excretory tubes or nephridial canals.

In the following, I shall discuss similarities in renal morphology and physiology between marine elasmobranchs, leeches and earthworms. Based on these similarities I have arrived at the following novel hypothesis that, in elasmobranchs, tubular fluid becomes diluted by fluid secretion into certain segments of the renal tubule rather than by active reabsorption of urea and other solutes. What do leeches and earthworms have in common with marine elasmobranchs? They have an internal milieu that is hyperosmotic to their environment, so like the elasmobranchs they gain water by diffusion that they

must bail out through their respective excretory systems. Leeches live in fresh water and earthworms in moist soil that at times becomes saturated with fresh water. Marine elasmobranchs live in seawater or brackish water, but because the osmolality of their body fluids is always slightly higher than that of seawater, they are at all times hyperosmotic to their surroundings, just as leeches and earthworms are.

All freshwater invertebrates are hyper-osmoregulators, i.e., their body fluids are hyperosmotic to the pond water in which they live, and they gain water by diffusion through the body surface. In all classes, excess water is excreted through the excretory organs, which transport a filtered and/or secreted fluid, and have a "diluting segment", i.e., a tubular segment in which the fluid is diluted relative to the plasma. No freshwater animals are without kidneys of some sort, which can produce urine hypoosmotic to their body fluids [23]. Even the unicellular amoeba has its contractile vacuoles to bail out fluid with a lower osmolality than its cytoplasm [27].

Renal Fluid Secretion

The existence of fluid secretion into the nephron is now a well established concept. In 1982, Klaus Beyenbach demonstrated fluid secretion by direct visual observation in isolated flounder tubules [3]. Subsequent studies, mostly from his group, have firmly established that net fluid secretion takes place in the proximal tubule of aglomerular as well as glomerular fishes and also in sharks [3-5, 26]. This fluid secretion is secondary to secretion of ions, such as Cl, Na or in some cases Mg [7]. Solute and fluid secretion has also been shown to occur in the mammalian nephron under certain circonstances. Addition of *para*-amino-hippuric acid (an organic acid known to be actively secreted by the proximal tubule epithelium) to the bath of isolated rabbit pars recta was shown to induce the formation of a widely open lumen in tubules which had been previously collapsed by spontaneous solute and water reabsorption [10]. Analysis of the newly formed luminal fluid revealed that fluid secretion was coupled to PAH transport and was inhibited by probenecid, ouabain and hypothermia. More recently, fluid secretion has also been documented in the isolated perfused mammalian collecting duct [33].

However, as explained above, forty years ago, neither Homer Smith nor other scientits (including myself) could imagine that secretion of fluid could take place in elasmobranchs. When in 1972 Patel and I found evidence for fluid secretion in skate tubules [22, 25], it suddenly struck me that Homer Smith's proof for active urea reabsorption was no longer valid. It was only then that I opened my mind sufficiently to accept another explanation. In 1975, we showed that net tubular fluid secretion takes place in the American eel when it is acclimated to fresh water (FW) and produces dilute urine [26]. The urine-to-plasma (U/P) osmolality ratio in such eels was 1/4 that in sea water (SW) acclimated eels (0.15 vs 0.59), and the urine flow rate was two to ten times higher [26]. It was clearly a mechanism for excreting excess water. The increased urine flow rate in FW- compared to SW- adapted eels was due to a greater volume of fluid secreted and a lower fractional reabsorption of fluid. Prior to our findings, Hickman and Trump had demonstrated fluid secretion in flounder kidneys [14], but it was still difficult to convince the reviewers that the phenomenon was real. Thirty years later, I have come to realize that the

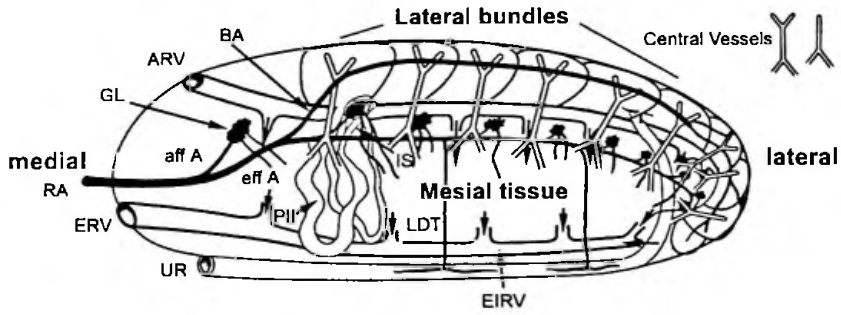

Figure 1. Schematic drawing of a cross section through the kidney of the skate *Rana erinacea*. The lateral bundle zone is located dorso-laterally on the mesial tissue. The oldest nephrons and their bundles are situated near the medial border, whereas young and developing nephrons occupy the lateral margin of the kidney. Central vessels, leading from the interior of the bundles (details not shown) to the mesial tissue, are indicated by open tubes with Y shape (see symbols at top right). The arterial system, shown in black, consists in renal, intrarenal, and bundle arteries (RA, IRA, and BA, respectively), and afferent and efferent arterioles (affA and effA, respectively) to the glomeruli (GL). The renal venous portal system, shown in white, consists in afferent renal vein (ARV), leading to the venous sinusoid capillaries (not shown) in the mesial tissue via the afferent intrarenal vein (AIRV). The mesial tissue is drained by efferent intrarenal veins (EIRV) to the efferent renal vein (ERV). PII = proximal tubule, IS = intermediate segment, UR = ureter. Reproduced from [13].

anatomical and functional convergence between the elasmobranch kidney and that of leeches and earthworms suggests that they operate in a similar way and that elasmobranchs dilute urea in urine by fluid secretion.

The Marine Elasmobranch Kidney

A. Configuration of the marine elasmobranch kidney

In search of a urea-reabsorbing segment, a number of investigators studied the rather peculiar configuration of the renal tubules of the marine elasmobranch kidney. This configuration is unique among fishes. It is not found in freshwater teleost, although they are also hyper-osmoregulators. Nor is it found in fresh water elasmobranchs. Hentschel et al. have presented a clear and detailed description of the nephron in skates and sharks as shown in Fig. 1 and 2 [13]. The oblong kidney comprises a mesial and a bundle zone. The glomeruli form a row between these zones. Each renal tubule is separate from the neighboring tubules. The loosely arranged proximal and distal convoluted tubules are located in the mesial zone, while the bundle zone contains one separate bundle from each tubule. Within the bundle, five tubular segments lie in parallel to one another wrapped within a flat sheath which holds the tubular segments tightly together (Fig. 2). Note that the term "bundle" used here is not equivalent to that found in "vascular bundles" of the mammalian kidney. Here, it represents a group of tubular structures belonging to a single nephron or nephridium and wrapped in a poorly distensible sheat. In mammals, it refers to groups of vessels, the arterial (descending) and venous (ascending) vasa recta, running in parallel and countercurrent in the renal medulla. These vascular bundles in mammals are never wrapped in a sheat. In a few rodents (mouse and species adapted to arid environment), thin descending limbs of short looped nephrons are also running within the vascular bundles [1, 16].

From each glomerulus a neck segment enters the bundle where it makes a hairpin loop (1 and 2 in Fig. 2). The proximal tubule leaves the bundle and forms several loose convolutions within the mesial zone. It then turns into the intermediate segment which re-enters the bundle zone, gradually changing into a distal tubule that forms a second hairpin loop (3 and 4 in Fig. 2). This loop, which is considerably longer than the first, forms a number of coilings at the tip of the bundle. The tubule then exits the bundle. The distal tubule forms a series of convolutions within the mesial tissue changing into a connecting tubule that re-enters the bundle as the fifth parallel tubular segment (5 in Fig. 2).

Within the bundle, the connecting tubule is in close proximity to the distal coilings near the tip of the bundle. Hentschel et al. specifically noted the following characteristic features [13]: "The limbs of the first hairpin loop adhere closely to each other. Because of its coiled organization, the second hairpin loop, consisting of diluting segment cells, is much longer than the first hairpin loop. The merging point of the collecting tubule with the collecting duct is located inside the bundle at a distance from the tip of the bundle." A single vessel is present in the center of each of the bundles. Dead end capillaries originate at the tip of the bundle in the vicinity of the coilings of the early distal tubule. As they join, they form a single central vessel, which eventually leaves the bundle at its proximal end. The side branches and central vessel carry no blood, only fluid probably generated by reabsorption from the structures in the bundle [13].

This organization suggests countercurrent exchange between the limbs of the proximal tubule and also between the coilings of the distal and connecting tubules. The connecting tubule from each of the bundles joins the collecting duct at the lateral edge of the bundle zone, just before it exits the tightly wrapped bundle. The tubules within the bundle are probably kept under pressure by the tight sheath that surrounds them.

B. Function of the marine elasmobranch kidney

In the shark *Squalus acanthias*, a diluting segment has been identified morphologically and electrophysiologically within the bundle [9, 12]. Marine skates as well as sharks produce urine hypoosmotic to blood, particularly when acclimated to dilute seawater. In *Squalus acanthias* adapted to pure seawater (SW), glomerular filtration rate (GFR) and urine output were both much lower than in sharks adapted to dilute SW (75%), and the inulin U/P ratio was 6.15 in the former vs 2.26 in the latter. Thus, it appeared that the increased urine flow after adaptation to dilute SW was established through an increase in GFR, a decrease in reabsorption of tubular fluid, and possibly an increase in fluid secretion, as later suggested by our data [25] and firmly established by Beyenbach and Frömter [5]. Initial experiments in *Squalus acanthias* suggested that urea reabsorption could be coupled to Na reabsorption, and thus be secondary active, because Na and urea were reabsorbed in a fixed ratio of 1.6 moles urea per mole Na over a very wide range of urea reabsorption rates (from 0.05 to 7.0 $mmol.h^{-1}.kg\ BW^{-1}$) (Fig. 3a) [28]. However, this relationship was not confirmed in later studies. In skates (*Raja erinacea*), the ratio of the amount of urea reabsorbed to that of Na

reabsorbed was not constant as in the shark but decreased with decreasing Na reabsorption (Fig. 3b) [25]. Furthermore, data by Murdaugh and Myers showed that when Na reabsorption was blocked in sharks by furosemide or ethacrynic acid, urea reabsorption was not diminished (cited in [21]). The data showed that very large amounts of urea can be reabsorbed passively in the proximal tubule (at least, 7 $mmol.h^{-1}.kg\ BW^{-1}$), indicating passive facilitated urea diffusion of filtered urea out of the tubules. Osmolality of final urine was lower than that of plasma in both skates and sharks, and far more so after adaptation to dilute SW [25, 28].

Micropuncture studies on elasmobranch kidneys would be necessary to understand where along the tubule the urine becomes hypoosmotic and where especially the urea concentration becomes lower than in the blood. Unfortunately, such studies do not seem possible due to the complex anatomical arrangement of the tubules in the bundle and their wrapping within a relatively tight sheath. Some deductions can however be made from existing studies. In skates (*Raja erinacea*) adapted to 75 % SW, bladder urine and fluid sampled from collecting duct outside the bundle were strongly hypoosmotic, while samples taken from the initial collecting duct just as it exits

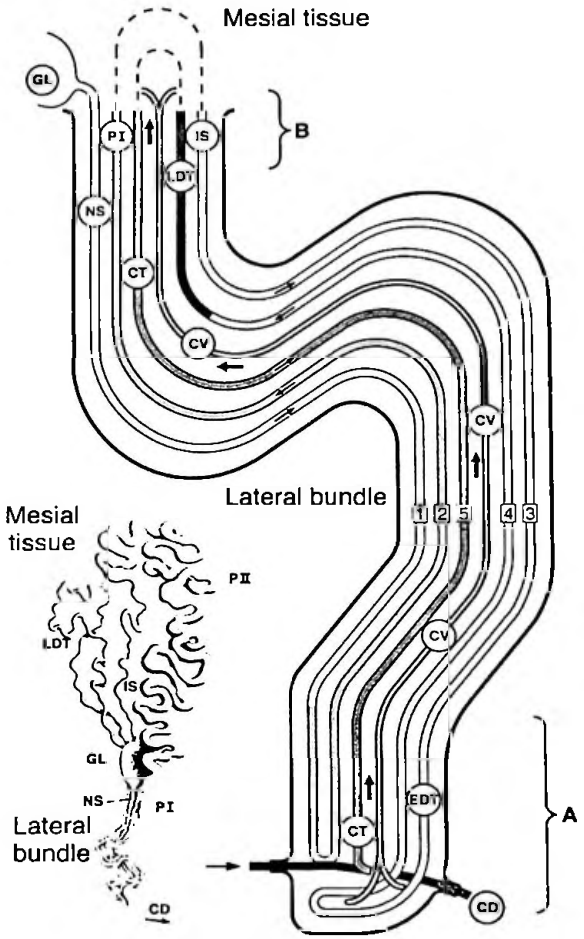

Figure 2. Schematic drawing of a "lateral bundle" of an elasmobranch kidney showing the arrangement of the nephron and portions of the connecting tubule-collecting-duct system enclosed in this bundle. Five tubular profiles (including two successive loops) and a central vessel are enclosed in the bundle sheath (thick line). The course of the nephron in the mesial zone (not shown, extending above the bundle zone) is indicated by broken lines (see text for more details and see inset for presentation of nephron segments in the mesial tissue zone). Thick arrows show the direction of supposed flow in the central vessel (CV) and thin arrows, the direction of urine flow. For simplicity, the actual thickness of the different nephron segments is not drawn to scale. GL = glomerulus, NS = neck segment, PI and PII = proximal tubule (initial and late parts, respectively), IS = intermediate segment, EDT = early distal tubule, LDT = late distal tubule, CT = collecting tubule, CD = collecting duct, CV = central vessel. Brace "A" refers to the distal end of the bundle and brace "B" to the region where the bundle merges with the mesial tissue. Arabic numerals indicate the order of successive limbs of the two loops and the collecting tubule. Inset on bottom left shows a young nephron of Scyliorhynus stellaris according to microdissections (bundle sheath and the central vessel are not drawn). Reproduced from [13].

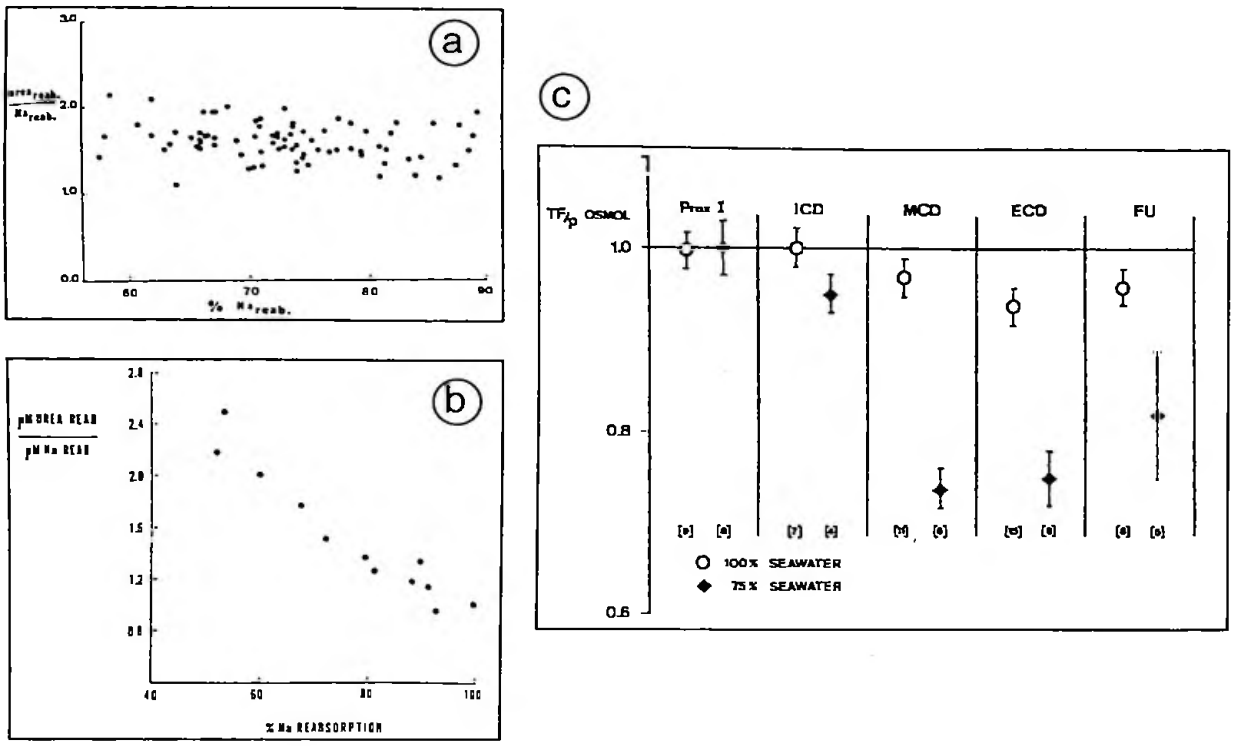

Figure 3. Experimental findings in elasmobranch kidneys. (a and b) Relationship between Na and urea reabsorption in renal tubules of the shark *Squalus acanthias* (a) and the little skate *Raja erinacea* (b). (c) Site of urinary dilution in the little skate *Raja erinacea*. The ordinate shows the calculated tubular fluid over plasma osmolality ratio (TF/P) in tubular fluid and urine samples collected from 100 % (open circles) or 75 % seawater- adapted skates (closed circles). Prox I = early proximal tubule; ICD, MCD, and ECD = initial, middle, and end collecting duct, respectively; FU = final urine. (a) reproduced from [28], (b) from [21] and (c) from [31].

the bundle were less hypoosmotic to the blood [32] (Fig. 3c). In fluid samples collected in dogfish sharks along the tubules in the mesial zone, urea concentration was always approximately 350-360 mmol/L, i.e., identical to that in plasma [29]. These results did not indicate active urea reabsorption but rather suggested that urea leaves the convoluted tubules through passive urea transport, as water is reabsorbed along the tubule. Actually, a facilitated urea transporter (SchUT) which can be responsible for this passive reabsorption has now been isolated from *Squalus acanthias* kidney [30].

In contrast, in the ureter and bladder of dogfish sharks, the urea concentration was as low as 40 to 100 mmol/L and the fall in urea concentration occurred at the same site where the urine became dilute. Micropuncture studies on the skate *Raja erinacea* have shown that tubular fluid in the collecting duct that exits the bundle is hypoosmotic to plasma [32]. The dilution begins already in the initial CD and increases abruptly at more distal sites in the same segment in which the urea concentration becomes lower than in the blood (Fig. 3c). Thus, it is quite possible that the dilution is caused by secretion of fluid with a lower concentration of urea than that of surrounding fluids. Could dilution of urea in urine be permitted by the elaborate arrangement of the renal tubules? Some insight may be gained through a comparison with the structure and function of the excretory organs of two invertebrates which are also able to produce dilute urine and secrete fluid, and show a configuration of their renal tubules very similar to that of the elasmobranchs described above.

The Nephridium of the Earthworm

A. Configuration of the earthworm nephridium

A number of investigators have described the nephridia of the fresh water annelid, the earthworm *Lumbricus terrestris*. The following description is adapted from that of Ramsay [18]. A row of nephridia is located on each side of the body, one pair for each body segment. Fig. 4 shows the loops of a nephridium. The parts of the nephridium may be functionally similar to parts of the mammalian renal tubule, the nephridiostome corresponding to the glomerulus and a tube with loops and convolutions corresponding to the renal tubule. The invertebrates do not have tubules but intracellular tubes or canals.

Ramsay described two structures that he called "loops". The first resembles the bundle and the second the tubular loops in the mesial zone of the elasmobranch kidney.

A narrow ciliated tube (proximal tubule), 30 μm in diameter, leads from the nephridiostome and forms a hairpin loop within the bundle (first loop). After leaving the bundle, the narrow tube forms a second loop, closely wound around the first loop, and corresponding to the proximal tubular loop in the mesial zone of elasmobranchs. The tube then re-enters the bundle to form a second hairpin loop in the opposite direction of the first loop. As the tube leaves the bundle, it suddenly widens into what Ramsay named the middle tube or middle segment. It forms a large loose loop as the middle tube widens and forms the ampulla which forms a third hairpin loop in the bundle, ending in a wide "distal segment" that connects to the bladder (Fig. 4a). Thus, the configuration of the earthworm nephridium resembles that of the elasmobranch nephron except that it has three hairpin loops instead of two and one half.

B. Function of the earthworm nephridium

In an elegant micropuncture study [18], Ramsay observed that the drop in osmolality of the nephridium fluid clearly begins in the wide middle segment which runs through the bundle (Fig. 4b). He stated "urine collected from the ampulla and beyond was always hypotonic to the medium surrounding the nephridium, and the average osmotic pressure was lower in the bladder than in the ampulla. A large part of the osmotic work producing hypotonic urine was thus carried out in the wide tube". Thus, in the earthworm, the dilution of the fluid begins in the bundle and becomes more pronounced in the following part of the nephridium [20].

The Nephridium of the Leech

A. Configuration of the leech nephridium

In leeches like *Hirudo medicinalis* or *Haemopsis sanguisuga*, excess fluid, gained by feeding or by diffusion through the body wall, is excreted through 17 pairs of nephridia located in the middle third of the body, one pair per body segment [24]. Each nephridium opens into a bladder measuring 2 to 3 mm in diameter when full. The bladder opens to the outside through the body wall. Fluid may enter the nephridium either directly through a nephridiostome [6] or by secretion through the tubular wall [24]. From injections of dye into the nephridia, we determined the direction of flow in the canals (see Fig. 5a) [24]. The nephridium begins in a tight bundle of several canals, and then proceeds in a broad loop laterally where it makes a few minor convolutions and a bigger loop prior to returning to the bundle. There, it makes a hairpin loop before proceeding laterally again to make a larger loop and finally returning to the bundle. The final segment of the tube makes a short loop in the bundle and then runs into the bladder.

Thus, in the leech as in the shark, five tubular segments traverse a tight bundle, whereas there are six in the earthworm. In all three animals, the final segment that carries urine to the bladder runs through the bundle, and in all three the bundle consists of hairpin loops of both proximal and distal tubules. The

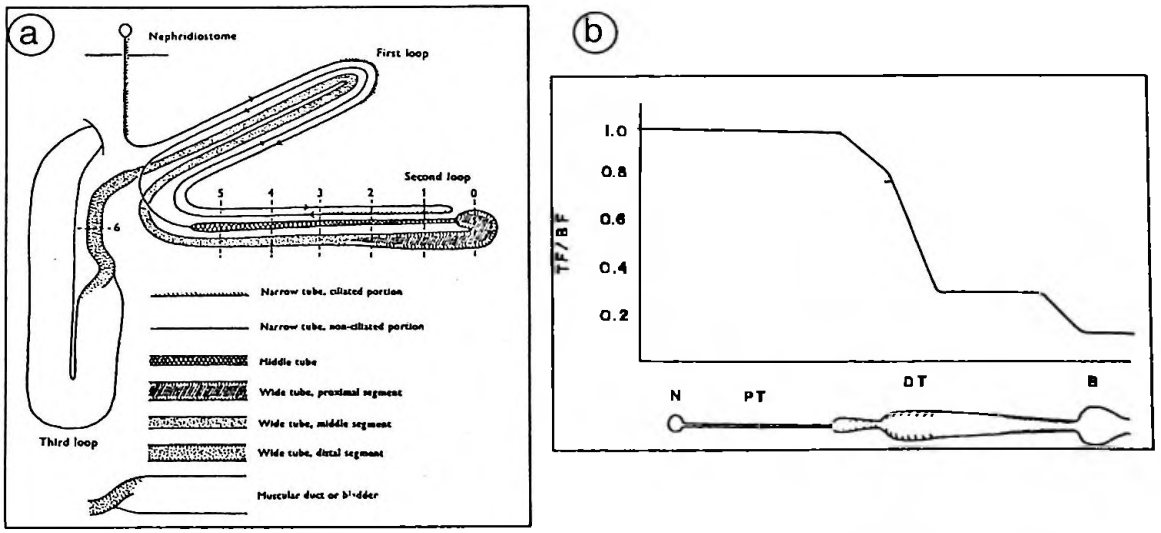

Figure 4. Anatomy of the earthworm nephridium and experimental findings. (a) Reconstruction of a nephridium. For reason of clarity, the three loops are shown displaced from their normal position. (b) Micropuncture data showing the tubular fluid over body fluid osmolality ratio (TF/BF) from the different regions of the earthworm nephridium. N = nephridiostome, PT = proximal tubule, DT = distal tubule, B = bladder. (a) and (b) reproduced from [17].

similarity in configuration suggests a similarity in function.

B. Function of the leech nephridium

In starved leeches, a gradual fall in osmolality along the nephridium was observed whereas in fed leeches, the osmolality dropped more precipitously between puncture site # III and bladder (Fig. 5b) [24]. In another set of experiments (Schmidt-Nielsen, unpublished observations), Na, and K were measured in the samples in addition to osmolality (Fig. 5c). Unexpectedly, osmolality, as well as Na and K concentrations in bladder fluid were all one third the concentration in the nephridial samples from the last puncture site. This could imply that fluid was added to the nephridium. It was shortly after this discovery in leeches that I found evidence for fluid secretion in the skate renal tubules [22, 25] and that fluid secretion then appeared as a possible explanation for the elusive "active urea transport" in elasmobranch renal tubules.

Hypothesis for the Function of the Bundle Region

Obviously, more data are needed to reach a valid conclusion for how the bundle region of the elasmobranch, earth worm and leech kidneys produces urine with lower osmolality and/or lower urea concentration than the blood. In all three, dilution already appears to take place in tubule segments within the bundle (Fig. 3c, 4b, and 5b). However, more pronounced dilution occurs in the tube leading to the bladder after it leaves the bundle.

Amphibians and mammals can also produce hypoosmotic urine, although their nephrons are not coiled individually in "bundles". Dilute urine is produced by a "diluting segment" which exhibits an extremely low permeability to water and in which sodium chloride is actively reaborbed. Earlier in evolution, in invertebrates and elasmobranchs, dilution is achieved in a different way. Each nephron seems to operate in a more independent fashion and this is in part due to the special architecture of the "bundles". The function of the bundles could be to create an environment for transfer of fluid into the final tubular segment traversing them. What complex interactions take place between the tubular segments within a bundle and its central vessel are not known. However, the spatial arrangement between these tubules suggests that the most important dilution and addition of fluid takes place after the collecting duct (shark) or excretory tube (leech and earthworm) leaves the bundle. The elasticity of the tubule may cause it to expand as it leaves the tightly wrapped bundle and thereby cause an inflow of dilute fluid, as described by Hammel and Scholander [11]. Elastic forces act as osmotic forces in drawing fluid from one compartment to another. The data from Stolte et al. [32] (Fig. 3) can be interpreted in this way. Similarly, the data from leeches and earthworms could mean that fluid is added after the last segment has left the bundle and is no longer under pressure within the bundle sheat.

In mammals, active urea reabsorption has been unequivocally characterized in the collecting duct, especially for reclaiming urea nitrogen during low protein intake (see review in [2, 19]). However, such active reabsorption might not be involved in urea conservation in elasmobranchs. The convergence of the anatomical arrangement of the nephron or nephridia in three widely different groups of animals, from invertebrates to the earliest vertebrates, suggests that other ways to dilute urine have developed during evolution.

In conclusion, I would like to propose that renal tubular fluid secretion, which is well demonstrated to occur in the proximal tubule of the nephron, could also take place in distal tubules in some species and play an important role in the production of a dilute urine. In particular, I propose that the bundle regions of some invertebrate and elasmobranch kidneys could serve the specific purpose of creating a hypo-osmotic environment for the tubules so that a hypo-osmotic fluid can be secreted. Finally, I suggest that, in elasmobranchs, urea is not actively reabsorbed in the kidney but is rather diluted through fluid secretion. Further studies are awaited to document this possibility.

Acknowledgements

Bodil Schmidt-Nielsen wants to thank Peter Cala and Larry Renfro for their suggestions and comments during the preparation of this review, as well as Emma Finn and Gayle Butler for their help with the bibliography.

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