ION ABSORPTION BY THE DISTAL TUBULES OF ONYCHOPHORAN Nephridia

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SUMMARY

The distal tubules of the 4th and 5th pairs of nephridia of Peripatus acacicoi (Onychophora) absorb ions from the fluid passing through them. Since water follows this movement only to a limited extent, the fluid in the lumen becomes hypotonic.

Absorption of ions stops when sodium is replaced by choline in the perfusion fluid and is much reduced by treatment with ouabain, particularly when this is applied to the basolateral faces of the cells. These results suggest that ion absorption may be driven by an Na/K exchange pump.

Cyclic AMP at 10^-2 mol l^-1 increases the rate of salt uptake by about four times; it seems likely from this that the tubules are hormonally controlled.

INTRODUCTION

Onychophorans, though a small group of animals, are of great phylogenetic and evolutionary interest. They have been described as the 'missing link' between the Annelida and Arthropoda (Burton, 1954). Indeed, it is probable that Annelida, Onychophora and Arthropoda had their origin in some simple wormlike common ancestor (Snodgrass, 1958). Very little is yet known of their physiology.

The first reference to the possible function of onychophoran nephridia was made by Picken (1936), who suggested that urine formation by these organs might involve filtration, with subsequent reabsorption and secretion, by mechanisms similar to those known in vertebrates. Manton & Heatley (1937) detected sulphate, phosphate and large quantities of ammonia in the urine of onychophorans, suggesting that nephridia may be able to eliminate the sulphate and phosphate arising from the protein-rich diet by combining them with ammonium ions derived from urea. Campiglia & Lavallard (1983a) analysed the urine in the segmental bladders and measured the rate of urine production. However, these data refer only to the small so-called 'néphridies typiques' (Gabe, 1957). These nephridia occur in all the segments of the body except the 4th and 5th segments. They have prominent bladders but only short tubules running into them.

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The nephridia belonging to the 4th and 5th pairs of lobopods are much larger than the 'nephridies typiques' and differ as well in their morphological, cytological and ultrastructural features (Gabe, 1957; Storch, Ruhberg & Alberti, 1978). The absence of bladders and the position of the excretory pore at the base of the feet makes it almost impossible to collect urine from them. Because of the size of the long tubular elements, which are about 6 mm in length, however, they can relatively easily be dissected under the microscope and are suitable for in vitro studies. The experiments described in this paper have been carried out using the longest of the segments in the nephridia, the 'segment à batonnets' (Gabe, 1957) or distal tubule (Lavallard, 1981). We have examined the specific ion absorbing abilities of the cells bordering on the distal segment of the 4th and 5th pairs of nephridia.

MATERIALS AND METHODS

Peripatus acacioi, Marcus & Marcus (1955), from a laboratory culture (Lavallard & Campiglia, 1975) were used in all the experiments. The animals were anaesthetized with carbon dioxide (Campiglia & Lavallard, 1983b), and opened dorsally so that the gut, glue glands, genital tracts and the ventral circular and dorsoventral muscles could be cut away. The 4th and 5th pairs of nephridia were then carefully dissected out, and the distal tubules, after being unravelled, were transferred to a drop of saline solution under liquid paraffin so that they could be cannulated and perfused following the technique described by Maddrell, Gardiner, Pilcher & Reynolds (1974). The coelomic vesicle and early nephrostome of the nephridium are inserted into the lobopod; the tubules form a loop and return to the same lobopod (Fig. 1). This arrangement results in the different segments at the level of the leg being attached in such a way that it is impossible to separate them without damage. Because of this, the end of the distal tubule held by the glass rod, where the samples were collected, contained lengths of the proximal tubule and nephrostome (Fig. 2). An advantage is that the beating of the flagella inside the nephrostomes can easily be seen and provides a crude check on the condition of the nephridia.

The volume of the drop of fluid emerging from the tubule was calculated from its diameter as measured with an ocular micrometer (Maddrell, 1963). For conductivity measurements, samples of known volume (at least 50 nl) of fluid were each diluted in 1 cm³ of glass distilled water in small polythene vials. From measurements of the conductivity of the water before and after addition of a sample of fluid and from the volume of the sample added, the conductivity change per microlitre was ascertained. Conductivity was measured with a Radiometer conductivity meter, type CDM 2e, fitted with a CDC 114 probe. Since the fluids sampled were composed almost entirely of ions, conductivity measurements provide a reasonable measure of osmotic as well as ionic concentration. By taking into account that K-rich fluids give slightly higher increases in conductivity than do Na-rich samples, the ionic concentrations of the samples could be calculated from calibration curves based on the values of conductivity of drops of different dilutions of the saline solutions used in the experiments.
The osmolarities of the bulk solutions were measured with a Knauer Osmometer, type M. The osmotic concentration of samples of fluid perfused through the tubules was measured using the melting-point depression method of Ramsay & Brown (1955). Measurements of the chloride concentration in samples of fluid were made using the electrometric method of Ramsay, Brown & Croghan (1955).

Possible water movement across the tubule walls was investigated by addition of $^{14}$C-labelled inulin (supplied by the Radiochemical Centre, Amersham) to the bathing medium and perfused solution. Inulin does not penetrate the walls of the

Fig. 1. (A) A drawing of the anterior half of the body cavity of an onychoporan showing the position of the 4th and 5th pairs of nephridia. (B) Schematic view of a nephridium.
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Fig. 2. Schematic view of the experimental arrangement used to perfuse the distal tubule.

Nephridia were perfused with standard saline solution and the conductivity of the emerging fluid was measured. The results of eight experiments, expressed as the ratio between the conductivities of the sample and of the saline before perfusion (S/R), showed a value of 0.82 ± 0.02, that is, on its way through the tubule, the
conductivity of the perfused fluid falls by about 18%. This lowering of the ionic concentration of the fluid passing through the tubule could be achieved either by ion absorption or by inward movement of water or both. To distinguish between these two possibilities, $^{14}$C-labelled inulin was added, both to the saline solution used as perfusion fluid and to the bathing medium, at a concentration ranging from 1000 to 2000 c.p.m. $\mu l^{-1}$. At this concentration there is a negligible effect on the osmotic concentration of the solution. If water were to move into the lumen, then this would reduce the concentration of inulin in the perfusing fluid relative to that in the bathing fluid – provided that inulin does not penetrate the wall of the tubule. The permeability of the wall of the distal tubules to inulin was checked in separate experiments by including inulin only in the bathing medium. No significant amount of the tracer was found in the samples of emerging fluid; clearly inulin does not cross the tubular wall at appreciable rates.

Table 1 displays the results obtained from ten experiments in which both the conductivity and the radioactivity of fluid emerging from tubules perfused with saline solution were measured. All the samples when compared with the saline solution exhibited lower conductivity, the mean S/R ratio being $0.83 \pm 0.01$ ($P < 0.01$). On average, the emerging fluid contained a slightly higher concentration of inulin than the saline solution, the mean S/R ratio being $1.07 \pm 0.03$ ($P < 0.1$). It is clear, then, that the fall in the concentration of the emergent fluid is caused by removal of ions from the lumen and not by water passing into the lumen. Since the luminal inulin concentration rose somewhat, some water must in fact have accompanied the movement of ions from the lumen.

In each experiment the volume of fluid emerging from the tubule per minute was calculated from the diameter of the drops, and the diameter and length of the tubule was measured. From all the data the analysis was extended as follows in order to get quantitative information about water and ion movements across the epithelium. For water absorption,

$$J_w = \frac{VI - VO}{A},$$

where $J_w$ is the rate of transepithelial water movement (nl min$^{-1}$ mm$^{-2}$), VI is the perfusion rate (nl min$^{-1}$), VO is the rate of collection of emergent fluid (nl min$^{-1}$) and A is the surface area of the tubule (mm$^2$). VI was calculated as,

$$VI = VO \frac{IC}{IP},$$

where IC and IP are the inulin concentrations (c.p.m. $\mu l^{-1}$) of the collected fluid and perfusion fluid, respectively. For ion absorption,

$$J_s = \frac{(VI \cdot COF) - (VO \cdot COS)}{A},$$

where $J_s$ is the net salt absorption (nmol min$^{-1}$ mm$^{-2}$), and COF and COS are the molar concentrations of the perfusion fluid and collected fluid, respectively. As stated
before, for these calculations the unit of conductivity was transformed to concentration (mmol l\(^{-1}\)) from a calibration curve using the conductivity meter with different dilutions of saline solution. Table 2 shows the results of such calculations.

On average, the net rate of solute absorption was 0·80 ± 0·12 nmol min\(^{-1}\) mm\(^{-2}\). The data concerning water movement showed a greater variability, ranging from a slight inward movement to an absorption of 4·2 nl min\(^{-1}\) mm\(^{-2}\). However, on average there is absorption of a hyperosmotic fluid of osmotic concentration of about 755 mosmol l\(^{-1}\) at a rate of 1·06 ± 0·53 nl min\(^{-1}\) mm\(^{-2}\).

While measurements of conductivity provide a reasonable measure of osmotic and ionic concentration, the exchange of small mobile ions in the lumen for larger less mobile ions could lead to a lowering of conductivity without a fall in osmotic concentration. We therefore measured the osmotic concentration of samples of fluid perfused through the lumen of the tubules. The results showed that a significant reduction of osmotic concentration occurred, the mean S/R ratio being 0·86 ± 0·01 (N = 16). This is somewhat higher than the figure for the reduction in conductivity, suggesting that some exchange of smaller ions for larger ones might have occurred. Accordingly, we measured the chloride concentrations in samples of fluid perfused through the lumen. These showed a substantial reduction in chloride concentration, the mean S/R ratio being 0·66 ± 0·02 (N = 11).

It is clear, then, that the distal segments of the nephridia can reduce the osmotic concentration of fluid flowing through the lumen and that this is achieved partly by an uptake of chloride ions. The results also suggest that there is some transfer into the lumen of osmotically active substances that contribute little to electrical conductivity. This in turn means that the absorption of ions leading to the reductions in

<table>
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<tr>
<th>R (c.p.m. µl(^{-1}))</th>
<th>S (c.p.m. µl(^{-1}))</th>
<th>S/R</th>
<th>R (µS cm(^{-1}) µl(^{-1}))</th>
<th>S (µS cm(^{-1}) µl(^{-1}))</th>
<th>S/R</th>
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<tr>
<td>870 ± 88</td>
<td>914 ± 48</td>
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<td>9·36 ± 0·00</td>
<td>7·40 ± 0·00</td>
<td>0·79</td>
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<tr>
<td>870 ± 88</td>
<td>1055 ± 43</td>
<td>1·21</td>
<td>9·36 ± 0·00</td>
<td>7·78 ± 0·18</td>
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<tr>
<td>1304 ± 73</td>
<td>1578 ± 70</td>
<td>1·21</td>
<td>8·24 ± 0·61</td>
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<td>2171 ± 82</td>
<td>2210 ± 47</td>
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<td>1340 ± 20</td>
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<td>8·53 ± 0·41</td>
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<td>1402 ± 19</td>
<td>1488 ± 70</td>
<td>1·06</td>
<td>9·94 ± 0·66</td>
<td>8·11 ± 0·19</td>
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<td>1929 ± 40</td>
<td>1920 ± 62</td>
<td>1·00</td>
<td>8·38 ± 0·83</td>
<td>7·37 ± 0·19</td>
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<tr>
<td>1929 ± 40</td>
<td>1912 ± 49</td>
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<td>8·10 ± 0·39</td>
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<td>0·79</td>
</tr>
<tr>
<td>1437 ± 6</td>
<td>1464 ± 27</td>
<td>1·02</td>
<td>8·10 ± 0·39</td>
<td>6·92 ± 0·43</td>
<td>0·85</td>
</tr>
</tbody>
</table>

Mean ± s.e. 1·07 ± 0·03* 0·83 ± 0·01**

Values are means ± s.e. of seven samples.

* P < 0·1, ** P < 0·01 (Student’s t-test, two-tailed).

R = Ringer, S = sample, S/R = sample/Ringer ratio.
Nephridial ion absorption

Table 2. The net movement of solute \( J_s \) and water \( J_v \) across the wall of distal tubules calculated from the conductivity and \(^{14}\)C-inulin concentration of the fluid collected at the distal end

<table>
<thead>
<tr>
<th>( J_s ) (nmol min(^{-1}) mm(^{-2}))</th>
<th>( J_v ) (nl min(^{-1}) mm(^{-2}))</th>
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<tr>
<td>0.22 ± 0.02</td>
<td>0.26 ± 0.02</td>
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<td>0.57 ± 0.22</td>
<td>0.55 ± 0.24</td>
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<td>0.36 ± 0.09</td>
<td>0.42 ± 0.11</td>
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<td>0.56 ± 0.22</td>
<td>-0.19 ± 0.07</td>
</tr>
<tr>
<td>0.97 ± 0.22</td>
<td>-0.55 ± 0.03</td>
</tr>
<tr>
<td>0.82 ± 0.09</td>
<td>-0.26 ± 0.08</td>
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<tr>
<td>1.58 ± 0.19</td>
<td>1.52 ± 0.30</td>
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<tr>
<td>0.88 ± 0.10</td>
<td>0.71 ± 0.19</td>
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<tr>
<td>1.03 ± 0.13</td>
<td>3.96 ± 0.31</td>
</tr>
<tr>
<td>0.97 ± 0.16</td>
<td>4.19 ± 0.63</td>
</tr>
</tbody>
</table>

Mean ± s.e. 0.80 ± 0.12*  1.06 ± 0.53**

* \( P < 0.01 \);  ** \( P < 0.1 \) (Student's \( t \)-test, two-tailed).

osmotic concentration and chloride concentration may be faster than the net rate of solute absorption calculated above.

**Transepithelial potential difference**

The transepithelial potential in distal tubules was found to be positive in the lumen when the tubules were perfused with standard saline solution. The results of nine experiments showed on average a value of +3.13 mV.

**Effects of changes in the ionic composition of the luminal fluid**

It is well known that in many transporting epithelia, primary active Na\(^+\) transport provides the driving force that is ultimately responsible for the rest of the transport processes. To find out if this is true for distal segments of the nephridia, a Na-free solution was run through tubules bathed in standard saline solution. To maintain the osmotic concentration, NaCl was replaced by choline chloride. The results of eight experiments showed that, in the absence of sodium ions in the lumen, there is no net movement of salt or water across the tubule wall, as judged by the lack of change in conductivity (Fig. 3).

It seems likely that when standard saline is perfused through the tubule the major cation absorbed is Na\(^+\), presumably accompanied by Cl\(^-\) ions. Since the transepithelial potential is lumen positive, it is conceivable that ion uptake is driven by a chloride pump.

A chloride pump might be expected to have a high specificity for chloride, other anions hardly being accepted. With this in mind, eight distal tubules were perfused with a saline in which all Cl\(^-\) ions were replaced by nitrate ions. The conductivity of the collected fluid, expressed as S/R ratio, was no different from experiments where
Fig. 3. Changes in the conductivity of fluid emerging from distal tubules in response to different experimental conditions. The results, expressed as sample/bathing saline ratio (S/R), show, from left to right: perfusion with standard saline, ouabain (10^{-3} \text{ mol L}^{-1}) added to the bath, ouabain (10^{-2} \text{ mol L}^{-1}) added to the perfusion fluid, ouabain (10^{-5} \text{ mol L}^{-1}) in the bath, ouabain (10^{-5} \text{ mol L}^{-1}) in the perfusion fluid, choline chloride perfusion, nitrate perfusion and cyclic AMP (10^{-2} \text{ mol L}^{-1}) added to the bath. Vertical bars represent means ± S.E.; the number of experiments is indicated in parentheses. Asterisks indicate those cases where the S/R ratio is not significantly different from 1.

The tubules were perfused with a chloride saline (Fig. 3). Although not conclusive, these results suggest that a chloride pump is not involved. To test the possible involvement of active absorption of sodium ions, we examined the effects of ouabain on the system.

**Effect of ouabain on salt absorption**

Ouabain is well known to exert a pronounced and specific depressing effect on the Na/K exchange pump in *in vivo* as well as *in vitro* preparations (Glynn, 1964), though there is a rather wide variation in the sensitivity of different tissues to this glycoside. The concentrations of ouabain necessary to inhibit transport range from less than 10^{-8} \text{ mol L}^{-1} to 10^{-3} \text{ mol L}^{-1}. With low concentrations of the glycoside the
degree of inhibition depends on the time the inhibitor is allowed to act. Furthermore, for frog skin, nerve and gall bladder (Diamond, 1962) there is a differential effect of cardiac glycosides, depending on the side of the preparation which is exposed to them, with the side towards which the net movement of Na⁺ is directed being the more sensitive.

With these findings in mind, we investigated the effects of ouabain on salt absorption by the distal tubules. During an initial control period of 1 h, standard saline solution was run through tubules bathed in the same saline solution. During this time the level of salt absorption was determined from the decrease in the conductivity of the samples. In eight experiments the bath was then changed for a saline solution containing ouabain at a concentration of $10^{-3}\text{mol}\text{l}^{-1}$ In a further eight experiments, ouabain at $10^{-3}\text{mol}\text{l}^{-1}$ was added only to the perfusion fluid. This relatively high concentration of ouabain was used first in order to find out whether it had any effect at all. In insect Malpighian tubules ouabain does not inhibit salt transport even at this concentration (Maddrell, 1969). In both sets of experiments the S/R ratio for conductivity was not different from 1, indicating that ouabain at $10^{-3}\text{mol}\text{l}^{-1}$ causes a 100% inhibition of salt absorption when presented on either side of the epithelium (Fig. 3). Ouabain acted very rapidly; the first sample taken 20 min after exposure to ouabain already showed no salt absorption.

Further experiments were carried out using ouabain at a concentration of $10^{-5}\text{mol}\text{l}^{-1}$. In 12 tubules where ouabain was applied only in the bathing solution the mean conductivity S/R ratio before the addition of the inhibitor was $0.84 \pm 0.02$ and after its addition it was $0.95 \pm 0.01$, i.e. there was about 70% inhibition of the salt uptake. With $10^{-5}\text{mol}\text{l}^{-1}$ ouabain only in the perfused fluid, in eight tubules, the respective values were $0.83 \pm 0.01$ and $0.91 \pm 0.01$, a level of inhibition of only 47% (Fig. 3). In both cases the effect was detected between 20 and 40 min after the inclusion of the inhibitor.

The results show, then, that ion absorption is strongly Na⁺ dependent, that nitrate ions can be substituted for chloride ions, and that ouabain strongly inhibits salt transport, particularly when applied to the outside surface of the tubules.

**Stimulation of salt and water transport by cyclic AMP**

To find out whether the epithelium of the distal tubule is under some kind of control, possibly hormonal, a series of experiments were carried out, in which $3',5'$adenosine monophosphate (cyclic AMP) at $10^{-2}\text{mol}\text{l}^{-1}$ was added to the medium bathing isolated tubules perfused with saline solution containing $^{14}\text{C}-\text{inulin}$. The basal level of tubular activity was established by analysis of the conductivity and the radioactivity of samples before the addition of cyclic AMP to the bath. In seven experiments the S/R ratio for conductivity was $0.73 \pm 0.03$ (Fig. 3) and for inulin concentration was $1.23 \pm 0.15$. The results showed that, on average, the net rate of salt absorption was $3.4 \pm 0.09 \text{nmol}\text{min}^{-1}\text{mm}^{-2}$, a value about four times higher than the basal level. The water movement was rather irregular but in some of the experiments absorption was enhanced by about two times. From these results it
seems very likely that the distal segments of the 4th and 5th pairs of nephridia are subject to alterations of membrane transport properties induced by hormones.

**DISCUSSION**

The osmoregulatory function of the excretory organs of the onychophorans has hitherto been known only from comparison of the blood and urine compositions and concentrations (Picken, 1936; Manton & Heatley, 1937; Campiglia & Lavallard, 1983a). Speculation as to the function of these organs, however, has been raised many times in papers concerning the histological and ultrastructural aspects of the nephridia (Gabe, 1957; Storch et al. 1978; Lavallard & Campiglia, 1979, 1983; Lavallard, 1981). In fact the morphological similarities of the 4th and 5th pairs of nephridia with the vertebrate nephron are remarkable. The podocytes in the coelomic vesicle, the brush border and the deep invaginations of the laterobasal membranes associated with mitochondria in the cells bordering the tubules all suggest the presence of such mechanisms as ultrafiltration, reabsorption and secretion of water and solutes.

The results presented in this paper establish some of the abilities of the distal tubules of the 4th and 5th pairs of onychophoran nephridia. The cells forming the wall of this segment possess very poor and irregular microvilli on the apical surface and on the basal surface they possess invaginations associated with mitochondria. These cells are rather similar to the cells of the thick ascending limb of Henle's loop of the mammalian nephron and the early distal tubule of the amphibian nephron. Not only are they similar in structure but they seem to have an analogous function in their ability to reduce the concentration of the intraluminal fluid. Fluid collected from the end of the distal tubules of the nephridia was found to have lowered conductivity, lowered osmotic concentration and lowered chloride content. These reductions are not caused by entry of water; our experiments in which changes of luminal concentration of inulin, a non-penetrating solute, were followed showed that relatively slow water movements did occur, but were from lumen to bath. The tubules evidently absorb a hyperosmotic fluid and this leads to the reduction of luminal concentration. These results have similarities with those observed for potassium chloride absorption in the lower Malpighian tubule of the insect, *Rhodnius* (Maddrell & Phillips, 1975), in which hyperosmotic KCl (900 mmol l⁻¹) solution is absorbed.

The mechanism of absorption of salt and water across the epithelium of the distal tubule of the onychophoran nephridium seems to be strongly influenced by the presence of Na⁺ ions as shown by the lack of conductivity change when the luminal fluid contains no Na⁺ ions. The absorptive process is not greatly dependent on the presence of a specific anion species, for chloride can be replaced by nitrate. Ouabain at a concentration of 10⁻³ mol l⁻¹ stops salt transport when in contact either with the luminal or the peritubular (basal) membranes. However, at 10⁻⁵ mol l⁻¹, ouabain inhibits about 70% of salt transport when added to the bathing medium and only 47% when in the luminal fluid. These results strongly suggest a model in which the
primary driving force responsible for salt absorption is a sodium/potassium exchange pump located on the peritubular (basolateral) surface of the cell membranes. The fact that ouabain still reduces transport when in contact with the luminal membranes, but to a lesser extent, could mean that ouabain can penetrate the cell–cell junctions and so affect Na/K pumps on the lateral cell membranes.

How, in detail, sodium extrusion at the basolateral membrane might lead to net NaCl flux from lumen to bathing solution cannot, of course, be described from our results. It will be interesting to test the possible involvement of NaCl co-transport, particularly across the luminal membrane, by applying drugs such as furosemide and bumetanide that block such co-transport (Frizzell, Field & Schultz, 1979).

Onychophorans are confined to humid environments and possess no defence against water loss through the integument. In fact, the osmoregulatory problem that they face is one of excess water, and, presumably, of retention of ions. We now find that the distal segments of the 4th and 5th pairs of nephridia have appropriate mechanisms for absorbing ions from the fluid passing through them, with little absorption of water. It is worth noting that the urine collected from the small nephridia is also hypo-ionic to (though iso-osmotic with) the blood (Campiglia & Lavallard, 1983a).

Finally, it is interesting that, like insect Malpighian tubules (Maddrell, 1980), the reabsorptive abilities of the distal nephridia are very likely under hormonal control. This is a subject which deserves further study, bearing in mind the relatively undeveloped state of the onychophoran nervous system.

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