THE PHYSIOLOGY OF PROTONEPHRIDIA

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by

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CONTENTS

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ABSTRACT

1. Present knowledge of the structure and function of protonephridial systems is reviewed.
2. The tapeworm, Hymenolepis diminuta, osmoconforms. Its protonephridia do not regulate tissue sodium or water content but possibly assist the tissues in conserving potassium.
3. The fluid in the ventral protonephridial canals contains salts, glucose, amino acids, proteins and the metabolic end products, lactate, succinate, urea and ammonia. Lactate is present in the highest concentration and is possibly the major excretory product of H. diminuta. More sodium than potassium is present in the fluid, suggesting an interstitial origin of the fluid.
4. Peristaltic bulges passing down the strobila of the worm move fluid along the canals and expel it at the posterior tip of the worm. The rate of peristalsis depends upon the CO_2 tension of the environment. At 6% CO_2, peristalsis is superseded by a ripple movement which is more efficient in moving the fluid.
5. Glucose, lactate and urea are absorbed from the canal lumen. Glucose absorption is carrier-mediated; lactate and urea absorption probably occur by passive diffusion.
6. The protonephridia of H. diminuta are not osmoregulatory organs. The evidence suggests that they are excretory organs, operating by filtration of interstitial fluid through the flame cells, followed by modification of the filtrate in the tubules and canals before its expulsion from the worm.

INTRODUCTION

Protonephridia are found in many lower invertebrates. They are generally characterised by their morphology and little is known about their physiology, although they are usually considered to be excretory or osmoregulatory organs. The first part of this thesis is a review of the subject, taken from literature published before August, 1971. Most of the experimental work presented later in the thesis has been published, or accepted for publication, before this date and is included in the review.

Experimental work began in October, 1968. The gross structure of cestode protonephridia had been described for most species but little was known about the fine structure. Their function was a matter for speculation: they have been described both as osmoregulatory organs and as excretory organs, but no experimental evidence was available for either function.

The rat tapeworm, Hymenolepis diminuta, was chosen for this study because it is easy to maintain in the laboratory. Its intermediate host is Tribolium confusum, the flour beetle, and its final host is the laboratory rat. H. diminuta has a well-developed protonephridial system, with large (up to 0.5 mm diameter), clearly visible longitudinal canals. It was therefore ideal for the type of experimental work envisaged.

Experiments were carried out in four main groups. It was first necessary to test the osmoregulatory ability of the worm before this function could be assigned to the protonephridia. Secondly, fluid from the protonephridial canals was sampled and analysed chemically to identify

possible excretory products. The factors governing the flow of fluid down the canals were next examined, since flow had not previously been demonstrated. Finally, the occurrence of absorption of some of the fluid constituents was studied, since an excretory organ would be expected to reabsorb some compounds, thus preventing unnecessary waste.

In the time available for the project, it was not possible to carry out a detailed study on the structure of the system. Observations on living worms and the results of light and electron microscopy are given in an Appendix. Reference to this may give a better understanding of the work involved in some of the experimental techniques described in the thesis.

Protonephridia - A Review of their Structure and Function

I Introduction

The morphological unit of the invertebrate excretory system is a discrete tubule termed a nephridium. Nephridia are subdivided into those having open inner ends, metanephridia, and those having closed inner ends, protonephridia. In turn, protonephridial terminal organs are of three morphological types: flame bulbs, which have a hollow cytoplasmic mass with a tuft of cilia within the hollow; flame cells which have a similar ciliary tuft but arising from a single cell; solenocytes, which are long, tubular cells with one or two flagella in their lumina.

Flame bulbs or flame cells are found in the phyla Platyhelminthes, Rotifera, Nemertea, Acanthocephala (in the order Archiacanthocephala only) and Endoprocta; solenocytes are present in Kinorhynchia, Gastrotricha, Priapulida, Annelida, Mollusca and in the class, Cephalochordata (lancelet fish).

Most detailed studies have been of the flame cells of Platyhelminthes and the flame bulbs of Rotifera. This review is concerned chiefly with these studies but reference will be made to work on other animals where relevant. Comparative reviews on the gross morphology of the excretory organs can be found in Hyman (1940-1967) and Barrington (1967); a specialized review on nephridia was written by Goodrich (1945).

II Flame cells. 1. Morphology.

The earliest descriptions of flame cells resulted from examinations of organisms by light microscopy. A flame cell

is of the order of 10μ long by 3μ wide. The light microscope, because of its limited resolution and magnification, is therefore an inadequate tool for study of these organs. The electron microscope was later used and detailed accounts of flame cell structure were then possible.

Thomas (1883) described the flame cell of Fasciola hepatica, the liver fluke, as being a conical cell with a large cilium beating like a flame within the cone. A similar description was given by Bugge (1902) for cestode flame cells. The canals leading from these cells were said to be lined with thin cuticle having either flattened epithelium below it or thin strands connecting epithelial type cells to the cuticle. Formation of new flame cells resulted from these epithelial cells sinking into the mesenchyme and there dividing into four or five cells. All but one of these differentiated into flame cells, the remaining one formed an intracellular channel which became the new tubule connecting the flame cells with the main canal.

Rediae and cercariae of Allassostoma parvum were also described as having triangular vibratile cones within the flame cell; a canal was seen running through the centre of the cone (Beaver, 1929). This canal was seen in flame cells of the turbellarian, Euplanaria maculata, in which the flame was sometimes frayed at its free end (Willey & Kirschener, 1938). The absence of further descriptions of the canal within the flame makes it probable that poor fixation was responsible for separation of the cilia within the tuft.

Further doubtful evidence from light microscopy was given by Coutelen (1931), studying hyatid cysts of Echinococcus. One type of flame cell observed had a spherical or ovoid

cytoplasmic body above the flame; the other type, in the same organism, had a piriform body with extending dendrites. Different planes of sectioning would possibly produce these "types" and further evidence is lacking to support these descriptions.

Growth and development of flame cells in F. hepatica was examined by Kawana (1940). A pair of flame cells was present in the fully developed embryo within the egg, one on each side of the organism. These remained in the free swimming miracidium and in the sporocyst. After the sporocyst had been in the snail host four days, a new flame cell was added to each side. This addition continued until there were four flame cells on each side of the sporocyst. By the time rediae appeared in it (8 - 10 days post-infection) the excretory system had posterior and anterior branches with 3 - 5 flame cells per branch. According to Kawana, the first indication of a developing redia was a pair of flame cells within a germ ball. These multiplied until there were fifty-nine pairs in the mature redia. The same process was repeated in the germ balls of the redia, until the free swimming cercaria had eighteen pairs of flame cells which remained in the metacercaria. After the metacercaria had excysted in the final host, the flame cells multiplied rapidly, until a very large number was present in the adult fluke. The main excretory vesicle of the mature fluke was formed by an enlargement of the upper descending region of the main excretory canal. No description of how the flame cells multiply was given by Kawana and there has been no corroborative work since.

Flame cell structure has been examined in in vitro

preparations of isolated specimens. Willey & Kirschener (1938) crushed the turbellarian, E. maculata, and observed the cilia to be tightly packed and well co-ordinated in the isolated flame cell. If the restraining wall was lost, however, the flame lashed wildly back and forth. Pantin (1947) isolated flame cells from the nemertine, Geonemertes dendyi. Viewed breadthwise, the flame filled most of the terminal chamber; viewed sideways, it appeared as a wave, the crests of which touched the chamber walls. The flame cells in living material were 17μ long by 4.5μ wide and in fixed material were 12 - 17μ long by 3.5μ wide. One nucleus was found in the cap.

In a study of adult F. hepatica, Stephenson (1947) could find no flame cells. He postulated that the smaller tubules of the excretory system ended in fat droplets, seen to be present in all parts of the system. He did not find the absence of flame cells surprising, because the adult excretory system was thought to have developed from the excretory vesicle of the cercaria, and the vesicle itself had no flame cells. The fate of the cercarial flame cells, known to be present in this larval stage, had not been determined. Although there were few papers on F. hepatica flame cells at this time, evidence did exist that adult liver flukes possess flame cells (Thomas, 1883; Kawana, 1940). These reports were obviously overlooked as no reference was made to them.

The structure of flame cells has been elucidated during the last decade by work using the electron microscope. This was done on many organisms and the flame cell seems to have basic similarities in them all. Fig. 1A illustrates the present knowledge of its structure.

FIG. 1. A. Longitudinal section through a flame cell from the miracidium of F. hepatica.

B. Part of the ribbed region of the flame cell wall.

C. Cross section through a tubule cell.

After Wilson, 1969.

Key: c - cilia, cr - ciliary rootlets,
d - desmosome, ir - inner rib,
l - leptotrich, m - mitochondrion,
n - nucleus, or - outer rib,
sc - smooth cytoplasm, t - tubule,
v - vesicles.

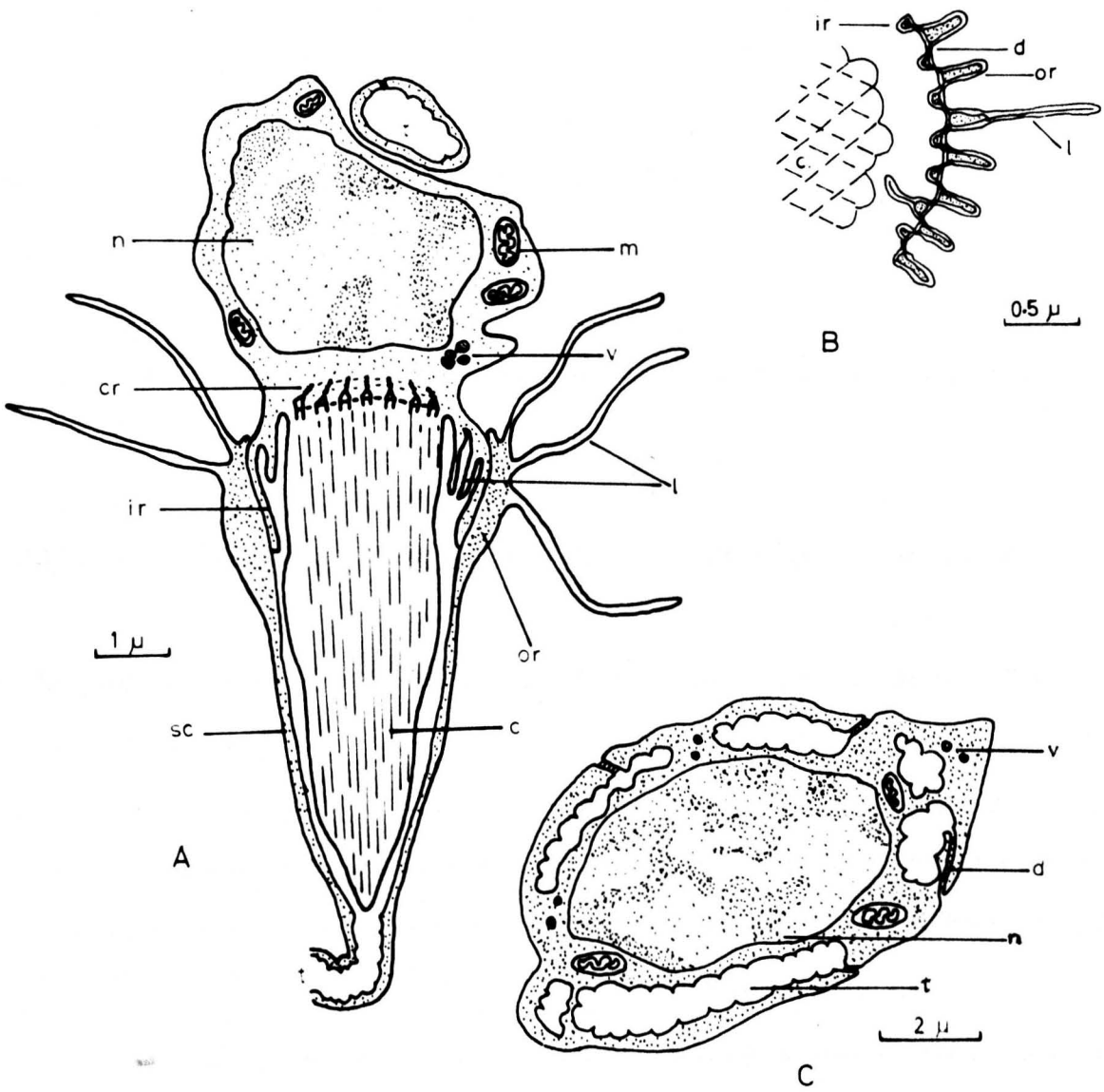

FIG. 1. Flame Cell.

Kümmel (1958) described the terminal organ of the miracidium of F. hepatica as a large cell with star-like processes emanating from its cap. These processes, however, were not observed by Wilson (1969). The flame cell cap cytoplasm of F. hepatica (miracidia and adults) contains one large nucleus, many mitochondria, vesicles, some Golgi membranes and endoplasmic reticulum (Pantelouris & Threadgold, 1963; Gallagher & Threadgold, 1967; Wilson, 1969). From the cap region long, narrow extensions arise which alternate and interdigitate with similar extensions from the first tubule cell (Fig. 1A) (Gallagher & Threadgold, 1967). These interdigitations form a ridged barrel in which the flame beats; they extend for 2 - 3μ starting approximately one third of the way down the flame cell (10μ long in the miracidium of F. hepatica). The exterior ridges project outwards by 0.5μ or more and the inner ridges are approximately 0.1μ wide (Fig. 1B) (Kümmel, 1958). The inner ridges arise from the cap region of the flame cell and the outer ridges arise from the tubule cell (Fig. 1A); they are held together along their lengths by a desmosome (Wilson, 1969). The ridges, or ribs, are semicircular in cross section, and have an electron dense core surrounded by a translucent matrix (Gallagher & Threadgold, 1967; Wilson, 1969). Arising from both the inner and the outer ribs are elongate cytoplasmic extensions.(See Fig. 1A). These are circular in cross section and have an electron dense microtubule within an annulus of translucent cytoplasm. Wilson (1969) termed these extensions "leptotrichs", after Kümmel (1964). They were long and undulating on the exterior of the flame cell but short and straight on the interior. Leptotrichs were observed by Gallagher & Threadgold (1967),

who called them "microvilli". Electron micrographs published by Morseth (1967) show them to be present in the flame cells of Echinococcus granulosus, although no reference was made to them; Howells (1969) also found them in the flame cells of Moniezia expansa.

Within the barrel formed by the interdigitating processes of the two cells is the tuft of approximately 100 cilia forming the "flame". The cilia arise from the nucleated cap region. Their rootlets extend into the cytoplasm and are fibres, with a periodicity of 700 Å. Ramifying from them are finer fibres of 300 Å periodicity. As they extend towards the cilium, the rootlets become hollow cylinders with 9 inner projections in cross section. Where the cilium separates from the mother cell, an interior thickening is found which reduces the lumen of the cilium. From this thickening arise the two inner microtubules which here are enclosed in a sheath like a 9 toothed cog-wheel. More distally the cilium shows the usual 9 + 2 arrangement of microtubule doublets. At their bases the cilia are separate, but they become tightly packed as they extend into the flame cell lumen. The central microtubules of all the cilia are aligned, suggesting some degree of co-ordination. These observations on flame cell cilia have been made by Kümmel (1959) on the miracidium of F. hepatica; Krudenier (1959), on Schistosoma mansoni and Tetrapapillatrema concavocorpa cercariae; Morseth (1967) on Echinococcus granulosus protoscolices; and Cardell (1962) on Himasthla quissetensis. In the latter organism, Cardell reported that the cilia were so close together that they formed a hexagonal array in cross section. This type of array was also observed in adult

F. hepatica flame cells (Pantelouris & Threadgold, 1963). The cilia in the tapeworm, Diphyllobothrium latum, were hexagonal in the centre of the flame but those facing the barrel wall had a circular outer edge (von Bonsdorff & Telkkä, 1966). In this organism, the peripheral microtubule doublets were arranged in opposite directions in adjacent cilia, equal numbers of each type of cilium being present in one flame. It was suggested that the beat may be three dimensional as a result.

The difference between cilia and flagella, it should be noted, is functional. Cilia move by lashing to and fro, while flagella have a characteristic wave movement travelling from base to tip (Sleigh, 1962). The "cilia" of flame cells show a wave formation in cross section so should, strictly, be termed "flagella". Since most authors describing these structures use the term "cilia", it has been adhered to throughout this review.

Rohde (1970) found that the flame cells of the aspidogastrid, Multicotyle purvisi, were similar to those of other Platyhelminthes except for their cilia. The ciliary membranes extended beyond the microtubules of the cilia and were embedded in the cytoplasm at the base of the barrel, so anchoring the flame at its tip. Ebrahimzadeh & Kraft (1971) described the flame cells of the cercaria of S. mansoni. There were five pairs of flame cells in the body and one pair in the tail. Their structure was similar to that described by Wilson (1969) for those of the miracidium of F. hepatica.

In the turbellarian, Dugesia tigrina, Wetzel (1962) found two types of flame cell. One third of those observed had thin walls, with pores approximately 400 Å diameter

between the lumen and the interstitium. The remaining two thirds were thick walled and did not appear to have such pores. Long microvilli, apparently similar to leptotrichs (Wilson, 1969) were seen around the flame cilia. Later work by McKanna (1968a) suggests that Wetzel misunderstood the structure of the terminal organ of D. tigrina. It was described by McKanna as being in the form of an elongate basket. Thin regions of the basket wall had fenestrations of 2 - 10 parallel slits, 350 Å diameter. The slits appeared to be bridged by 55 Å filaments spaced at 200 Å intervals. Eight thicker cytoplasmic columns, or ribs, containing 220 Å diameter microtubules, supported the thin regions of the basket. The columns formed bases for radial processes which prevented adjacent cells from occluding the pores. Thirty-five to ninety cilia formed the flame. These were surrounded by long microvilli, or leptotrichs, which arose from the luminal surface of the ribs along the length of the basket. Wetzel (1962) probably interpreted the fenestrated parts as the thin walled flame cells and the regions between the fenestrations as the thick walled type.

Cestode flame cells have not been examined as frequently as those of trematodes. The structure of the cilia in flame cells of Diphyllobothrium latum, described by von Bonsdorff & Telkkä (1966), was discussed earlier. The flame cells of M. expansa appear to be little different from those of trematodes (Howells, 1969). The cell body possesses one nucleus, mitochondria, vesicles, granular endoplasmic reticulum and some free ribosomes. The surface is deeply infolded. Whorls of approximately 50 incurving rods arise from the cell body; they were called flame cell rods. Each rod

contains a central microtubule. Secondary rods, probably synonomous with Wilson's (1969) leptotrichs, arise from the inner surface of the flame cell rods. Arising from the tubule cell are other rods, termed nephridial rods. These alternate and interdigitate with the outer row of the whorl of flame cell rods to form a ribbed barrel. The inner rows of flame cell rods are short, and extend in to the lumen. The nephridial rods are exterior to the flame cell rods. Along their lengths they are joined by desmosomes. The main difference between the description of M. expansa flame cells and those of F. hepatica is the manner in which the rods fuse at their ends. The flame cell rods curve inwards after reaching the edge of the nephridial cell. A desmosome joins the nephridial cell to the rod immediately before the latter curves. The nephridial rods were described as diverging before reaching the flame cell cap. Tonofibrils (regions of intercellular contact usually within a desmosome) connect the rods, before they diverge, with the cap cytoplasm. A pore was said to be produced by the divergence, thus making the interstitial compartment continuous with the flame cell lumen. The evidence for this pore is not convincing, however. The electron micrographs are not very clear and those showing the "pore" appear to be of oblique sections at the point in question. The cilia forming the flame of M. expansa have the usual 9 + 2 arrangement of microtubules but those on the outer edge of the flame appeared to have lost their central pair of microtubules.

For many years, the lumen of the tubule leading from the flame cell was postulated as being intracellular, while the lumina of the larger collecting ducts were intercellular

(Goodrich, 1945). Pedersen (1961) showed that the tubule walls in the planarian, D. tigrina, are made up of two cells, approximately half the lumen bounded by each cell. A desmosome is present where the cells meet. Within their cytoplasm are irregular nuclei, Golgi membranes and many vesicles. The tubule wall was said to be demarcated from the parenchyma by smooth membranes. Wetzel (1962) observed, however, that in this species the outer walls of the tubules are thrown into folds penetrating into the parenchyma. The presence of these folds, together with the numerous mitochondria in the cytoplasm, were taken to suggest modification of excretory fluid by the tubules. McKanna (1968b) agreed that two cells, joined by septate desmosomes, form the tubule walls of D. tigrina. The tubules lead into larger collecting ducts with cilia in their lumina and micropinocytotic caveolae in their walls.

The tubules of S. mansoni are lined by projecting septae and filaments arising from papillae in the walls (Senft et al. 1961). The projection of cilia and folds into the lumina of trematode tubules was reported by many workers. Cryptocotyle lingua has septae projecting into the lumen and pinocytotic vesicles in the walls (Krupa et al., 1969). F. hepatica adults have tubule lumina lined by irregular folds (Gallagher & Threadgold, 1967; Pantelouris and Threadgold, 1963) and the miracidium has similar projections in its tubules (Wilson, 1969).

The tubule walls of trematodes are similar in structure to those of D. tigrina. Single cells extend cytoplasmic encircling projections in adult F. hepatica. Where the arms meet, a desmosome is present to keep the fluid in the lumen

discrete from the interstitium (Pantelouris & Threadgold, 1963) (Fig. 1 C). Wilson (1969) described a similar structure for the tubules in the miracidium. The tubule cytoplasm contains nuclei, at least two per tubule, vesicles and small mitochondria. The cestode, M. expansa, has tubules with an apparently intracellular lumen since no desmosomes or lateral cell membranes can be seen (Howells, 1969). This suggests that the cestode tubules are slightly different from those of other Platyhelminthes. Another difference is the presence of bead-like microvilli lining the lumen of the tubules and collecting ducts in M. expansa. These were only found in the atrium of the miracidium of F. hepatica. Wilson (1969) described them as club-like. The atrium is the last part of the tubule in the miracidium and it opens to the exterior through a pore. The atrium is separated from the rest of the organism by desmosomes, suggesting that it is functionally discrete (Wilson, 1969). A similar separation by desmosomes was seen by Ebrahimzadeh & Kraft (1971). The excretory pore region of the cercaria of S. mansoni is maintained discrete by desmosomes.

The tubules of adult F. hepatica lead into larger collecting ducts and thence to an excretory bladder. No lateral membranes were observed in the collecting duct walls, which were postulated as being syncytial (Gallagher & Threadgold, 1967). Long lamellae, compacted into groups, line the luminal surface. Fat droplets were observed in the walls and in the lumen. Projections from underlying parenchymal cells extend to the bases of the duct cells but contact is prevented by a junctional complex which is probably a desmosome.

Cestode tubules lead into larger collecting ducts. Descriptions of the gross structure of the longitudinal canals and the junction between dorsal and ventral canals in the scolex can be found in Wardle & McLeod (1952) for most tapeworms. The excretory system of the horse tapeworm, Anoplocephala perfoliata, was described in detail by Lee & Tatchell (1964). Two types of system are found in this worm, a ventral system and a network system. The ventral system consists of a pair of longitudinal canals, one each side of the strobila, and a transverse canal connecting them at the posterior of each proglottis. The network system has a set of similar ventral and transverse canals and also a pair of dorsal longitudinal canals. Both systems joined in the scolex but not in the strobila.

In M. expansa, the flame cell tubules open into transverse canals at the posterior of each proglottis. The transverse canals are connected at each end with a ventral longitudinal canal opening to the exterior at the posterior of the worm. A pair of dorsal longitudinal canals is also present, connecting in the scolex with the ventral canals. These collecting canals are all lined by bead-like microvilli. The walls are made from a pavement epithelium with a well-defined basement membrane, through which nuclei protrude into spaces between the parenchymal cells. Around the nucleus are mitochondria, granular endoplasmic reticulum and probable pinocytotic vesicles (Howells, 1969).

An unusual type of excretory system was found in the turbellariam, Gnathostomula paradoxa (Graebner, 1968). The system consists of three cells, specially differentiated. The terminal cell has one cilium within a wall lined by eight

leptotrichs. The cilium protrudes into a hollow in the second cell, the canal cell. The basement membrane of the canal cell is broken at one point, near the hollow formed by the cilium, by the endoplasmic reticulum. This has very enlarged cisternae which form a twisting canal through the cell until joining an outlet channel. The outlet channel, lined with numerous microvilli, is in the third cell, the outlet cell, and opens on the exterior surface of the animal. A fourth cell, an accessory cell, was found in association with the terminal cell where it leads into the canal cell. The accessory cell has many invaginated membranes. The excretory system of Austrognathia **ri**edli, a member of the same family as G. paradoxa, is similar in most respects.

The protonephridia of the archiannelid, Dinophilus gyrociliatus, lies in the coelom between the intestine and the body wall (Brandenburg, 1970). The terminal organ is pear-shaped. **Its** nucleus is near the junction between the terminal cell and the tubule cell. A column of cytoplasm connects this part of the cell with the cytoplasm of the cap from which the cilia arise. These extend into the canal cell. The wall of the flame cell is made up of rods with a lamella between them. Unlike the flame cells considered earlier, these rods are not interdigitations between two cells, but are formed in the one cell, rather like the fenestrations of D. tigrina. Around the flame cell of D. gyrociliatus is a basement membrane and exterior to this is another membrane, separating the flame cell from the blood sinus into which it projects. The tubule leading from the terminal cell is formed by the encircling arms of a single cell, the arms joined by a desmosome. The canal cell nucleus is in the same region as

the flame cell nucleus. The two are opposite in a cross section because one side of the flame cell cytoplasm extends beyond its opposite side, forming an interlocking junction with the canal cell. The latter is lined by numerous microvilli before opening to the exterior of the animal.

2. Function

The function of the protonephridial system of Platyhelminthes has long been a subject for speculation. Wardle & McLeod (1952) stated:
"There is no experimental evidence that these organs are exclusively excretory ... there is strong presumptive evidence that ... they serve to regulate the water balance of the animal". von Brand (1952) contradicted:
"... no definite data on its participation in the elimination of water is available".

One of the earliest recorded physiological experiments was performed by Herfs (1922) on an unidentified cercaria from a Lymnaea sp. snail.

The bladder pulsations were observed when the cercaria was put into various solutions. In Lymnaea blood, a fixed rhythm was observed, the bladder "disappearing" after 15.7 sec. In Ringer solution, the time was reduced to 13.1 sec; in 50% Ringer, to 8.3 sec and in fresh water, to 6.3 sec. The faster emptying time as the medium became more dilute was taken to indicate that the kidney organ regulated leaching from the cercaria. A similar experiment, to observe the mature embryo of Schistosomum haematobium within the egg, was carried out by Reisinger (1923).

While the eggs were in urine, or an isotonic saline,

the flame cells did not flicker. On transferrance of the eggs to water, or hypotonic saline, the flame cells became active. The activity ceased if the eggs were returned to urine. Reisinger concluded that the flame cells regulated osmotic balance.

The flame cells of F. hepatica embryos begin to beat as soon as they have developed, slowing down as the egg matures. Light, probably indirectly, stimulates their activity prior to hatching but high intensity light inhibits them. The rate of beat is proportional to the temperature of the environment; it is not related to salinity of the medium (Wilson, 1967). The tubules leading from the flame cells collapse when the flame beat ceases, suggesting that, in this organism, the function of the flame is to provide a hydromotive force.

A similar conclusion was reached by Westblad (1924) after a study on the excretory system of the trematode, Galactosomum lacteum. The main excretory vessels and bladder of this fluke stained with alizarin; the smaller capillaries stained with neutral red. It was suggested that the capillaries secrete neutral red and the more proximal parts secrete alizarin. The excretory function was therefore thought to be contained in the walls of the ducts, so the function of the flame cell must be to provide hydromotive force and probably had little osmoregulatory activity. This conclusion was based on rather vague evidence since structural, rather than functional, differences may be sufficient to cause the differental staining.

The turbellarian, Gunda ulvae, in its normal habitat is subjected to sea and fresh water. According to Beadle (1934),

the regulation of water in its internal environment occurred in three stages: initial water in-flow causing the parenchyma to swell; water uptake from the parenchyma into the gut cells, with consequent resumption of the animal's normal movements; finally, decrease in the permeability of the surface of the animal to water. When the worm was transferred to sea water, the processes were reversed. It was concluded that the protonephridia play no part in osmoregulation in this animal. Another turbellarian, Gyratrix hermaphroditus, was described as having protonephridia adapted to the habitat (Kromhout, 1943). In fresh water forms, the flame cells, tubules and their related cells (paranephrocytes), an ampulla and an excretory pore were to be found. Brackish water forms had less distinct tubules and had neither paranephrocytes nor an ampulla. Marine forms had only flame cells remaining. It was suggested that, since only fresh water forms needed to osmoregulate, the ampulla must have this function. No further work on this organism was published, so it is possible that the three "forms" were actually three "species", or that the results were because of poor microscopic technique and purely coincidental.

Pantin (1947) found that the flame cells and ducts of the nemertine, Geonemertes dendyi, swelled if the animal was compressed while in fresh water. Flame activity was absent in partially dry animals but resumed when the nemertine was immersed in tap water. It was concluded that the upper part of the protonephridial system "secreted" water, shown by the swelling when compressed, and that the flame activity depended upon the water content of the internal environment of this species.

In an extensive review, Schopfer (1932) compared the

freezing point depressions of fluid from Ascaris spp., F. hepatica and M. expansa with their surrounding host fluids. In most cases the depressions were very similar, suggesting isotonicity between parasite and host. Experiments performed on Cysticercus tenuicollis showed that it lost weight in hypertonic saline and gained weight in hypotonic saline. The evidence therefore suggested that endoparasites are osmoconformers.

This type of weight change has been performed by many workers using different organisms. The tapeworm, H. diminuta, maintained a fairly constant weight in Tyrode's solution, varying appropriately in more concentrated or more dilute solution (Chandler et al., 1950). This worm was shown to gain water and lose salts in diluted Hank's saline in direct proportion to the dilution of the medium (Webster, 1970). Later work (Webster-unpublished) showed that reduction of the sodium content of the saline by 40%, while maintaining the osmotic pressure constant by addition of an appropiate quantity of mannitol, caused no water loss from the worm but the sodium loss from the tissues was again 40% after one hour. Sodium and water changes are therefore related only to the medium dilution, not to each other. F. hepatica behaved similarly in various concentrations and dilutions of Hedon-Fleig medium (Knox & Pantelouris, 1966), but addition of sucrose or maltose to normal Hedon-Fleig medium caused greater weight loss than addition of glucose, galactose or sorbitol. It was suggested that the first two, because they are not energy sources, will not cross the tegument as readily as the last three and so would remain in solution to exert an osmotic effect. F. gigantica was shown to change its body weight in proportion to the dilution or concentration of

Tyrode's solution, normal strength being isotonic with the fluke (Siddiqi & Lutz, 1966). This fluke also lost sodium and potassium to diluted medium in proportion to the dilution. The freezing point depression of an extract from F. gigantica and that of host bile were found to be equal. The authors concluded that the fluke "adjusted" osmotically by gaining salts and water across the tegument; their results indicate that the fluke actually conformed to its environment rather than "adjusted", which implies an active response. Another trematode, Gastrothylax crumenifer, from the reticulum of the water buffalo, was found to have no control over its body weight in sodium chloride solutions above or below 0.4 - 0.5 % (Goil, 1966).

Haematoloechus medioplexus, a trematode found in frog lungs, reduced its oxygen consumption when incubated in sodium chloride solution greater or less concentrated than 0.7 % (Bair & Peters, 1971). It was suggested that oxygen consumption could be a measure of osmotic activity in this fluke. This is the only evidence that metabolism may in any way be involved in osmotic changes.

Most evidence suggests that endoparasitic stages have few powers of osmoregulation, relying upon their host to maintain a constant environment. Free-living stages of these parasites and totally free living species are able to regulate their salt and water content to some degree, but whether by the protonephridia is not known. Adult parasites have, in many cases, well developed protonephridia, suggesting that they are functional. Their function may be that of excretion, as there is some evidence to indicate.

It has been shown that the flame cell is a complex

structure formed by two cells connected by interdigitating processes. These processes are separated from each other by desmosomes, which also keep the lumen of the flame cell discrete from the interstitial fluid of the organism. The ribbed region of the barrel has been postulated as a region for filtration of interstitial fluid, the flame beat being in some way concerned with fluid movement (Kümmel, 1958; Wilson, 1969; Howells, 1969). Kümmel (1965) presented a discussion of the possibilities for filtration. The criteria for its occurrence are, firstly, a filter separating two fluid compartments and, secondly, a hydrostatic pressure gradient across the filter between the compartments. The most likely place for such a filter in the protonephridial system is in the region of the interdigitated processes in the flame cell: this region was termed a "corona" for ease of description. Cells having such a corona, with cilia or flagella inside it, were collectively termed "cyrotocytes" (Kümmel & Brandenburg, 1961). Wetzel (1962), McKanna (1968a) and Wilson (1969) also postulated that the corona was the most likely place for filtration to occur. Howells (1969) suggested that the pores found at the top of the flame cell barrel of M. expansa, where the nephridial rods meet the flame cell cap, may be the region of fluid entry. This would not be filtration, however, as no barrier was apparent. Further evidence for filtration was the higher level of sodium compared with that of potassium in the protonephridial canal fluid of H. diminuta (Webster & Wilson, 1970). If interstitial fluid were filtered, it would be expected to have more sodium than potassium because cells conserve potassium in exchange for other cations; if fluid were secreted into the

protonephridial canals, the reverse ratio would be expected.

The pressure produced by the ciliary beat in the flame cell has been postulated as being great enough to cause filtration (Kümmel, 1965) but direct evidence is lacking.

Calculations were done using data from electron micrographs of flame cells and substituting this data into an equation quoted by Pappenheimer (1953) for the rate of flow of a liquid through a porous membrane (K. J. Woodward, unpublished). The equation is: $Qf = \frac{Kf.A}{\eta} \times \frac{\Delta P}{\Delta x}$ The variables will be described in the text following. The flame cilia occupy three quarters of the internal cross sectional area of the flame cell barrel. If the barrel is assumed to be cylindrical, its internal volume can be calculated. It is 19 cu.μ or 19×10^{-12} ml. Since the fluid space is only one quarter of this volume, the fluid volume can be derived. The fluid space is in three compartments because of the wave formation of the cilia. Cine photography enabled the time of formation of the fluid compartment at the top of the flame cell to be calculated. Hence Qf, the volume of fluid entering the flame cell per min, is obtained as 2.21×10^{-11} ml/sec. The fluid is assumed to be water, so its viscosity, η, is 8.93×10^{-3} dynes. sec. per sq. cm. The total area of the ribbed region of the barrel, A, can be calculated from electron micrographs and is 7.53×10^{-8} sq. cm. The distance across the ribs Δx can be measured and is 2.64×10^{-6} cm. Kf is a flow constant and is taken to be similar to that for the frog glomerular capillary membrane, 5.7×10^{-16} sq. cm. (Pappenheimer, 1953). By substitution in the equation above, ΔP, the pressure difference across the top of the flame cell barrel is calculated as 12.1 dynes/sq. cm., or 12.3 cms of water. This would be an adequate pressure for

filtration of fluid through the flame cell.

Once the fluid has entered the flame cell lumen, it is probably moved into the tubule by the wave action of the cilia. The tubules may have some function in modifying the fluid by secretion or resorption of materials. The many microvilli or folds in the lumina suggest possible reabsorption. From the tubules, fluid goes to the larger collecting ducts and then passes to the outside, either via a bladder or vesicle or, in tapeworms, directly from the ends of the ducts. Some work has been done on the function of the tubules in a few organisms.

The tapeworm, H. diminuta, was embedded in agar with its anterior tip in isotonic saline containing radioactive tracers. Its posterior tip was in isotonic saline alone. After 5 hr, Macpherson (1958) could detect no movement of the tracers along the worm. It was concluded that there was no transport of materials along the protonephridial canals. There was no reason to believe, however, that any of the radioactive tracers used - ^{32}P phosphate, ^{35}S methionine or ^{45}Ca chloride - would enter the canals. Webster (1971a) has shown that movement in the longitudinal canals of H. diminuta depends upon the peristaltic activity of the worm. Under the conditions used by Macpherson, no fluid flow would have occurred and hence no materials would have been transported.

The presence of lactate, urea and ammonia in the protonephridial canal fluid of H. diminuta indicates a probable excretory function for its protonephridia (Webster & Wilson, 1970). The quantity of each compound excreted depends upon the fluid flow down the canals and whether any selective

reabsorption occurs in them. Webster (1971b) demonstrated reabsorption of glucose, lactate and urea from the canal fluid. Glucose is reabsorbed by a carrier-mediated mechanism, lactate and urea are absorbed by probably passive means. This raised the question of whether the concentrations of compounds detected in the canal fluid by Webster & Wilson (1970) resulted from an equilibrium between influx from the smaller flame cell tubules and efflux across the canal wall. Such an equilibrium would make it difficult to calculate the excretory loss of compounds without knowing more about the dynamics of the fluid compartments of the tapeworm.

The presence of fat droplets in the excretory ducts of the trematode, F. hepatica, was reported by von Brand & Weinland (1925), Stephenson (1947), Pantelouris & Threadgold (1963) and Burren et al. (1967). Most of the small tubules have lumina full of lipid and the collecting ducts have walls lined by lipid. Burren et al. (1967) showed that lipid expulsion to the incubation medium was reduced when the flukes were ligated at each end before incubation. They postulated that the major route for lipid excretion by F. hepatica is the protonephridial system.

Other compounds have been detected in the excretory canals. A complex, protein-like, water insoluble product was found by autoradiography following incubation of the trematode, Cephalogonimus americanus, in ^{3}H-arginine (Pappas, 1971). Martin & Bils (1964) found calcareous concretions, formed from calcium carbonate and a little phosphate, deposited in concentric layers in the tubule lumina of the trematode Acanthoparythium spinulosum. The epithelium of the excretory

vesicle of Cryptocotyle lingua secretes membrane-bound granules (Krupa et al., 1969). No lipid has been observed (Howells, 1969) nor chemically detected (Webster & Wilson, 1970) in cestode excretory systems. Hedrick (1958) observed fat droplets deposited around the "osmoregulatory" system of H. diminuta, but not in the walls or lumina. Similar findings for another cestode, Raillietina cesticullus, were also given by Hedrick.

Alkaline phosphatase activity has been shown by several workers to be associated with protonephridia. G. dendyi and Rhynchodemus terrestris (nemetine and turbellarian respectively) have abundant enzymic activity in the convoluted tubule leading from the flame cell (Danielli & Pantin, 1950). Daughter sporocysts of a gorgoderid trematode show high enzyme activity in the tubule walls and on the flame itself (Coil, 1958). Miracidia of S. mansoni give no indication of such activity in the protonephridia, but the mature cercaria have intense activity in the bladder region (Dusanic, 1959). The sporocyst and cercariae of Cercaria bucephalopsis haimaena have alkaline phosphatase in the walls of excretory vacuoles, in the flame bulbs and in the tubules (James & Bowers, 1967). For many years, alkaline phosphatase was believed to be involved in secretion or absorption of materials, particularly sugars, but evidence has been accumulating against this hypothesis. The function of the enzyme, although often associated with sites known to transport materials, is virtually unknown.

The protonephridia of Platyhelminthes are probably excretory organs in the endoparasites but perhaps both osmoregulatory and excretory organs in free living organisms. The evidence suggests that body fluid is filtered through

the flame cell and the filtrate is modified in the tubules and collecting ducts.

III Flame Bulbs. 1. Morphology

The protonephridial system of Rotifera is, in many respects, morphologically different from that of Platyhelminthes. Each rotifer has two protonephridia, both leading via a bladder into a cloaca. The flame bulb has an apical end-plate from which cilia arise. The nucleus is in the wall of the tubule not, as in Platyhelminthes, in the apical cap (Goodrich, 1945).

Flame bulbs of Asplanchna spp. have been studied by Pontin (1964), Braun et al. (1966) and Warner (1969). Clément (1968) studied those of Notommatus copeus. The structures described in each case are very similar. The bulbs are usually fan-shaped, the widest part being the apical cap, and the narrowest where the bulb meets the tubule. At the widest part the bulb is 6 μ wide by 2 μ thick and it has an overall length of 10 - 12 μ. In A. priodonta one thick cytoplasmic channel connects the apical cap with the tubule (Braun et al. 1966). In A. brightwelli and N. copeus two channels, one at each side of the "fan" are found (Warner, 1969; Clément, 1968). The channels contain free ribosomes, rough endoplasmic reticulum or, in N. copeus, mitochondria.

The peripheral walls of the bulb are formed from a row of columns in scalloped formation, 3 - 5 columns per arc (Fig. 2B). They are 300 - 400 Å diameter bounded by a 100 Å membrane and interconnected by a thin, 50 Å, lamella (Warner, 1969). Near the centre of each arc is a cylindrical membrane-bound pillar. The pillars are 1000 - 2000 Å diameter

FIG. 2 A. Longitudinal section through a flame bulb from A. brightwelli.

B. Transverse section through flame bulb, below apical cap.

After Warner, 1969.

Key: c - cilia, col - column, cyt - cytoplasm, ms - membranous sheath, p - pillar, v - vesicles.

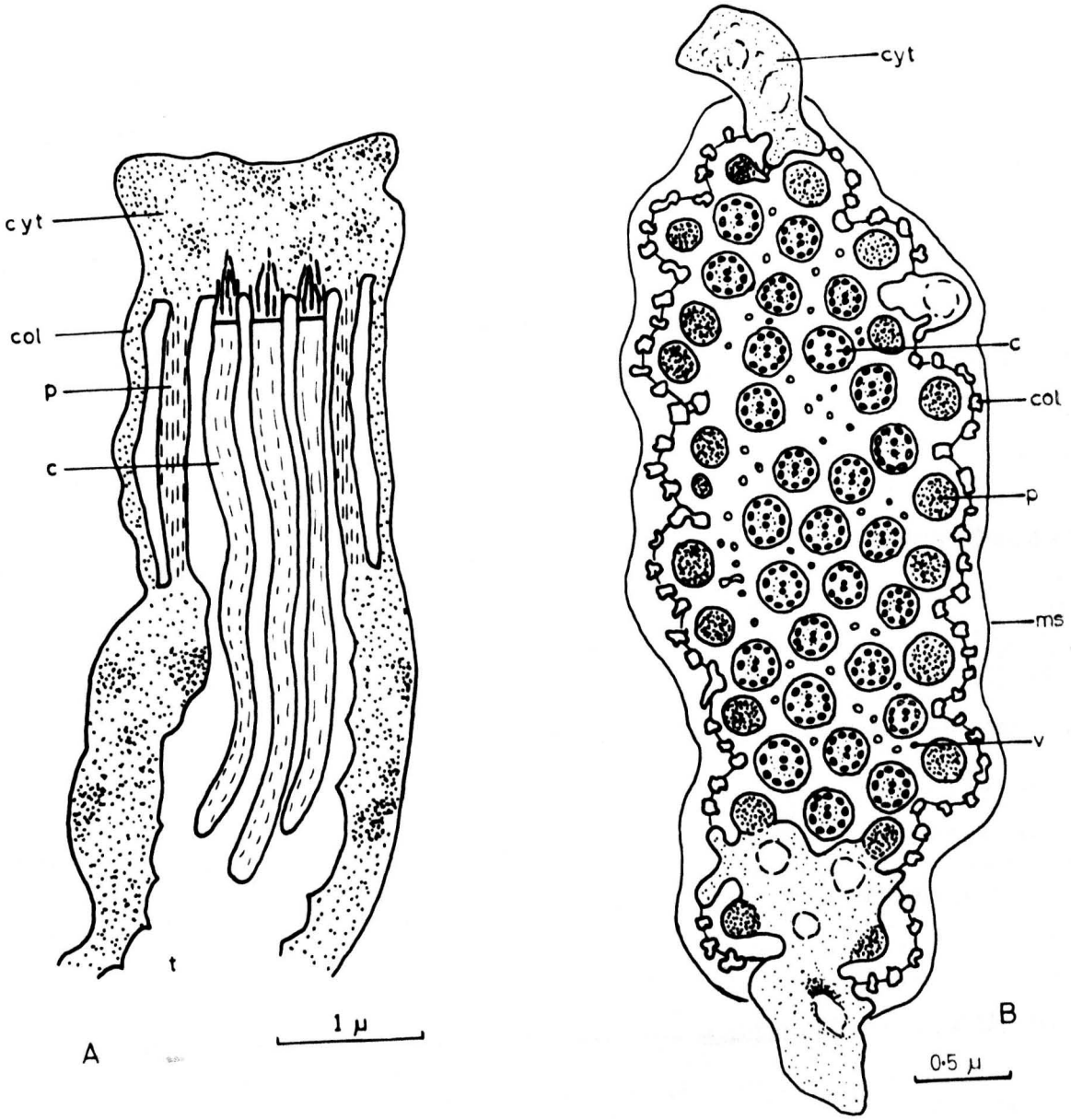

FIG. 2. Flame Bulbs.

and their matrix appears to be composed of many fine longitudinal microtubules. Each pillar is linked by a 25 Å fibril to each of the columns making the arc around it. As shown in Fig. 2A, the columns and pillars arise from the apical cytoplasm and fuse with the tubule cytoplasm. In the apical cap, the pillars are in contact with dense side arms arising from the basal bodies of the one or two external cilia (See Fig. 3). Similar side arms link these basal bodies with those of the flame cilia.

The flame cilia are fairly closely packed (Fig. 2B) but do not touch. Adjacent cilia are linked by fine fibrils. There are 30 - 36 in A.brightwelli (Warner, 1969), approximately 60 in A. priodonta (Braun et al., 1966) and more than 100 in N. copeus (Clément, 1968). Between the cilia and in the bulb lumen generally are many small vesicles which appear to have pinched off from the ciliary membrane. These vesicles remain in the bulb as they were never seen in the tubules (Warner, 1969).

Around the flame bulb is a membranous sheath continuous with the basement membrane covering the whole protonephridium. This was seen in all three species. Together with the intercolumnar membrane, it makes a barrier between fluid in the pseudocoel and fluid in the bulb lumen.

Mattern & Daniel (1966), working on Asplanchna, described the flame bulb as having a scalloped appearance in cross section. Around the whole bulb was a coating 200 Å thick, probably the membranous sheath (Warner, 1969), within which a membrane invaginated periodically to give a series of rail-like structures. Between these rails were spaces, said to be separated from the pseudocoel only by the outer coating. The rails could be traced to the level of the base of the cilia

FIG. 3 Protonephridium from *A. brightwelli*. (Arrows show direction of fluid flow)

After Warner, 1969.

Key: at - ascending tubule, b - bladder, ct - collecting tubule, dlr - distal loop region, dt - descending tubule, ec - external cilia, j - junction between cells, plr - proximal loop region.

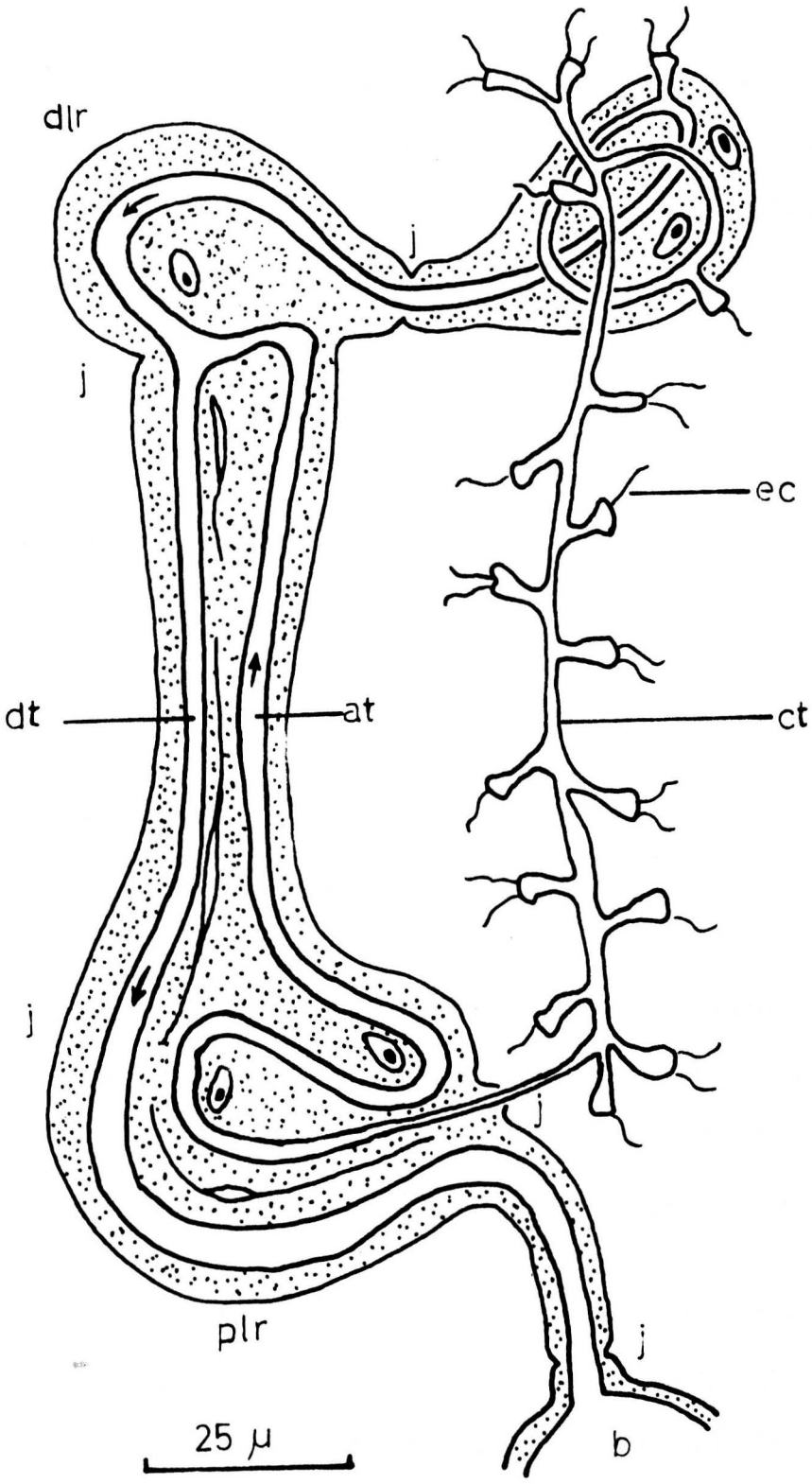

FIG. 3. Protonephridium of a Rotifer.

and were probably derived from the ciliary basal bodies. Comparison of these electron micrographs with those of other workers suggests that the "rails" are probably the columns forming the peripheral walls of the flame bulb.

The tubules of the protonephridium were thought to be syncytial and were so described by Braun et al. (1966) for A. priodonta. Clément (1969) observed some cellular junctions in the tubule walls and suggested that further work needed to be done to check whether the tubules were syncytial or not. Warner (1969) presented evidence that the tubules were made up of 3 - 4 multinucleate cells in A. brightwelli.

The flame bulbs of A. brightwelli lead into short ducts which empty into a collecting tubule. The cytoplasm of this tubule contains many mitochondria, a few Golgi complexes and vacuoles, free ribosomes and rough endoplasmic reticulum (Warner, 1969; Clément, 1969). Near the site of the flame bulb on the tubule was a nucleus. At each end the collecting tubule empties into the duct system leading to the bladder. This system is very complex and Fig. 3 is a two-dimensional guide. The proximal end of the collecting tubule cell is joined to the proximal loop tubule cell by septate desmosomes. The distal end of the collecting tubule united with an ascending tubule which was a continuation of that from the proximal loop region. The distal loop region, together with the ascending tubule, leads into a descending tubule and into the bladder. The collecting tubule, together with its flame bulbs, is a single multinucleate cell and is separated from other cells in the system by desmosomes. The ascending and descending tubules are divided into three distinct cytoplasmic channels, two of which form the descending tubule. The ascending tubule lumen is lined by an irregular membrane forming extensive

bulbous invaginations into the surrounding cytoplasm. These invaginations seem to pinch off to form vesicles in the cytoplasm. The outer cell membrane is infolded, but no vesicles are apparently formed. The descending tubule is made by two cytoplasmic channels, each bordering half the lumen, held together by septate desmosomes. The outer cytoplasmic channel is bounded by a membranous sheath similar to that around the flame bulb. The inner channel is apposed to the ascending tubule cell membrane but no structural links were observed. The tubule lumen is continued into the bladder by another cell joining the outer cytoplasmic channel of the descending tubule (Warner, 1969). A similar coiled tubule system was described by Pontin (1964) but in less detail.

In N. copeus, Clément (1969) observed the tubules to have numerous infoldings of the basal membrane with many mitochondria in association with them. He postulated that the infoldings were a site of active transport of liquid, presumably by pinocytosis, since no mechanism is known for active transport of water. Before joining the bladder, the tubule lumen increases and the walls contain numerous Golgi membranes and vesicles, ribosomes and granules. It was suggested that some secretion may occur in this region.

Little work has been carried out on the flame bulbs of Acanthocephala. Only species of the order Archiancanthocephala have protonephridia. There are two in each organism, appearing as a branching mass of 250 - 700 flame bulbs attached to a common duct. The bulbs have no nuclei, but there are usually three nuclei in the main branches or in the wall of the duct. The flame in each bulb is a linear row of cilia (Hyman, 1951).

2. Function.

The probable mode of function of flame bulbs is similar to that of Platyhelminth flame cells. The pillars, columns and membranous sheath were suggested by Warner (1969) as preventing collapse of the flame bulb under the pressure of filtration. This pressure was postulated as being provided by the turgor pressure of the pseudocoel (Braun et al.1966). Inhibition of the ciliary activity by nickel chloride prevented fluid movement in the protonephridia. The inhibition was reversed by washing, followed by addition of potassium chloride, after which the bladder pulsations returned to normal (Warner (1969). It was also noticed that rotifers kept in a ferritin suspension showed no sign of the particles in the protonephridia after 2 hours. In the stomach, pseudocoel and near the flame bulb sheath, however, abundant particles were present (Braun et al. 1966)

Response to the salinity of the medium by A. priodonta was found by Braun et al. (1966). Increase in the ionic concentration of the medium caused a reversible decrease in flame beat and output rate of the contractile bladder of both A. priodonta and A. brightwelli (Pontin, 1966). Increase in temperature of the medium caused an increase in beat and output rate, the increase being greater above 22°C than below. Pontin (1964) suggested that there was some correlation between flame bulb numbers and the surface area of the organism. This was based on the fact that male rotifers had a smaller surface area than females and also had fewer flame bulbs.

Micropuncture was performed by Braun et al. (1966) to sample the pseudocoelomic and excretory fluids of A. priodonta.

The pseudocoelomic fluid was hypertonic to it's surrounding medium and the excretory fluid contained less sodium than the body fluid. Rotifers therefore probably have some degree of osmoregulation. When transferred to distilled water in order to dilute the pseudocoelomic fluid, rotifers increased their rate of water removal via the bladder. Injection of ^{14}C-inulin into the pseudocoelom, followed by sampling of the excretory fluid using micro-pipettes, showed the normal filtration rate of a flame bulb of A. priodonta to be 8.3×10^{-12} l/min. This is of the same order of magnitude as the calculated value obtained by Woodward (unpublished) for flame cells, which was 2.21×10^{-11} cc/sec, or 1.33×10^{-12} l/min. The volume of a flame bulb is approximately 120 cu.μ. If the figure for the filtration rate is converted to similar units, 138 cuμ/sec flow through the flame bulb. This is slightly more than its own volume every second. The flame cell has a volume of approximately 19 cu.μ and the fluid flow rate is 22.1 cu.μ/sec. The flame cell, therefore, is also able to filter slightly more than its own volume every second. This correlation of fluid movement suggests that the two organs are equally efficient although morphologically different.

The evidence for rotifer protonephridia indicates that they are probably osmoregulatory organs. This function is more important to a free-living fresh water organism than to an endoparasite. The rate of water intake, in terms of terminal cell volume, is similar for flame bulbs and flame cells, yet parasitic Platyhelminthes have no control over their water content. Rotifers are very small organisms compared with Platyhelminthes, but their flame bulbs are almost seven times the capacity of flame cells. The actual water

turnover by the rotifer protonephridia is probably very much higher, compared with body size, than in the osmoconforming Platyhelminthes.

The archiacanthocephalan, Moniliformis dubius, parasitic in the rat ileum, was studied after known time periods in various salt solutions by Branch (1970). The worm swelled in hyperosmotic sodium chloride and potassium chloride solutions but this swelling was prevented when its metabolism was inactivated by temperature change. The anion accompanying the sodium or potassium ion could also prevent swelling if its diameter were larger than that of the hydrated pores of the plasma membrane. This suggested that the cation pump in this worm is not the usual sodium or potassium exchange for another cation as found in the erythrocyte. Acanthocephala seem to have little control over their osmotic concentration, thus behaving similarly to the endoparasitic Platyhelminthes.

IV Solenocytes. 1. Morphology.

Solenocytes are highly specialized protonephridia. They have only one flagellum in a long narrow tube, at the free end of which is a cell body (Goodrich, 1945). Solenocytes are usually found free in a fluid-filled space.

Brandenburg & Kümmel (1961) described the solenocytes of the polychaet, Glycera unicornis. The wall of the tube containing the flagellum has a fine lamella stiffened by 17 rods parallel to the axis of the tube. The tube, with its rods, is part of one cell and the flagellum runs through into a channel in a second cell (See diagram in Fig. 5).

The lancelet fish, Branchiostoma, has solenocytes with tubular walls made of 10 rods (See Fig. 4B) (Brandenburg &

FIG. 4 A. Part of protonephridial tree of *Priapulus*

After Kummel, 1964

B. Cyrtopodocyte of *Branchiostoma*.

After Brandenburg & Kummel, 1961.

Key: b - blood space, c - coelom, cp - cyrtopodocyte, k - top of kidney tubule, r - rods, tr - transverse rods.

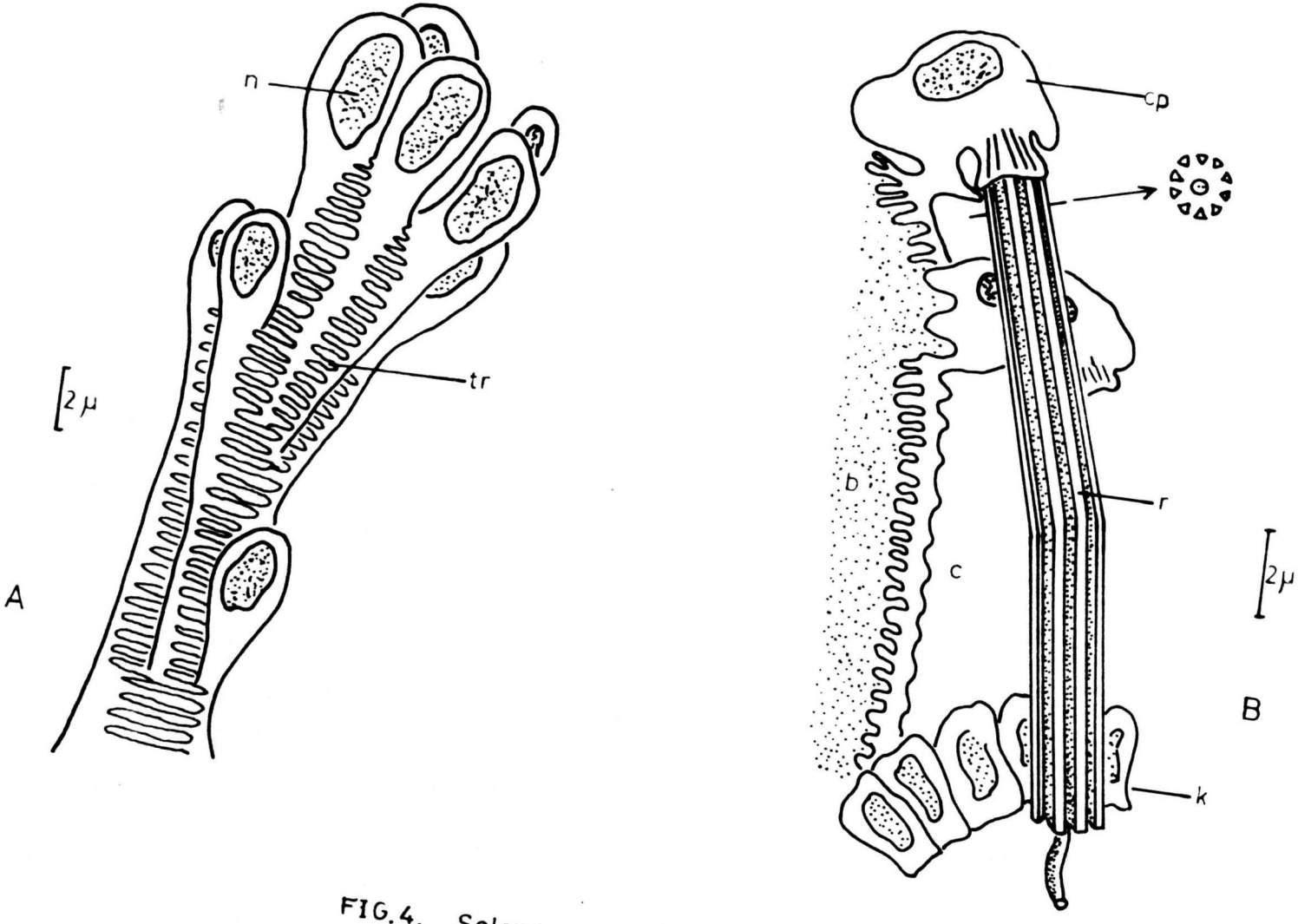

FIG. 4. Solenocytes

Kümmel, 1961; Nakao, 1965). These are triangular in cross section, the apex of the triangle pointing towards the centre of the tube. No lamella bridging the rods was observed. The head of the solenocyte is attached to the wall separating the coelom from the peribranchial space. Small processes from the head interdigitate with similar processes from the wall, which is called the ligamentum denticulum. Within it is a blood vessel, branches of which pass just beneath the basement membrane overlying the ligamentum. The basement membrane at these points is the only barrier between the blood and the head of the solenocyte. Thus the solenocyte head is similar to the podocytes of the vertebrate Bowman's capsule. Nakao (1965) postulated that materials may pass from the blood to the solenocyte by direct filtration across the basement membrane, or across nephridio-pores. Brandenburg & Kümmel (1961) termed the solenocyte a "cyrtopodocyte" because it has the characteristics, morphologically, of the podocytes of Bowman's capsule and also of a cyrtocyte. The tubule leading from the head runs through the coelom. Within this tubule the flagellum beats and it is probable that filtration of coelomic fluid occurs here. The renal tubule, into which the solenocytes empty, forms processes to penetrate the coelom wall and allow the solenocyte tubules to pass across it (Fig. 4B).

The gastrotrich, Chaetonotus, has a terminal organ composed of two juxtaposed tubes (Brandenburg, 1962). Each tube consists of eight rods with a fibrillar network running between them at right angles. A cytoplasmic mantle caps both tubes and processes extend from it. One flagellum is present in each tube. Kümmel (1962) found a similar double structure

in the turbellarian, Stenostomum, and in the entoproct, Urnatella gracilis. Stenostomum has two trellis-like structures separated by two cytoplasmic columns. Adjacent to one of these columns is a large nucleus. Twin flagella are present in close juxtaposition within each lumen. Urnatella is very similar, except that, instead of a trellis-like structure, the wall contains fine grids.

The solenocytes of Priapulus caudatus open into a very short duct, giving the protonephridium the appearance of a tree (Fig. 4A). The short duct empties into the nephridial sac of the priapulid through a filtering pad of long cells. These have microvilli on their exterior edge, forming a brush-border (Nørrevang, 1963). A second tree-like structure was found opening directly into the nephridial sac. The end bulbs of this structure were postulated to secrete the droplets found both in the tubule lumina and in the nephridial sac fluid. Similar protonephridial structure was found in P. caudatus and Halicryptus spinulosus (Kümmel, 1964). Each cell has a nucleus in its cap and one flagellum in its tubule lumen. The inner walls of the tubules are covered with short transverse bars between which is a fine lamella (Fig. 4A). Long, thin structures arise from the luminal side of the cap and surround the flagellum in the tubule. These were termed leptotrichs.

2. Function.

The function of solenocytes is probably the same as that of other protonephridia, either excretion or osmoregulation. Their mode of action is also probably the same. Brandenburg (1966) summarised the structures of the known terminal organs

and compared their regions of possible filtration. The solenocytes were included, as most have slits or grid-like structures in their tubule walls. No physiological work has been reported for solenocytes.

IV Inter-relationships of Protonephridial Types

The cells of choanoflagellates, choanocytes (or collar cells) of sponges and protonephridial terminal cells were postulated by Kümmel & Brandenburg (1961) as being merely variations of specific, morphologically defined cell-types. These they termed "basket flagellum cells" or "cyrtomastigocytes", shorted to "cyrtocytes". Their main characteristic is the possession of a flagellum tube with walls in the form of a basket for filtration. An evolutionary tree, given in Fig. 5, was drawn up by Kümmel (1962) to show how the various types of cell are related to each other.

According to Kümmel, the choanocyte of the sponge and the protozoan, the choanoflagellate, probably arose simultaneously from one common ancestor. From the choanoflagellate, the flame cells of Platyhelminthes and the solenocytes evolved, probably by parallel evolution. The flame cell gave rise to the terminal cells of the polychaets (e.g. Glycera) and, by divergent evolution, to the terminal cells of Stenostomum and Urnatella (Fig. 5).

The solenocytes of early lancelet fish probably evolved at the same time as flame cells and other solenocytes. At first they may have been exterior to the coelomic space but, during evolution, the coelom wall was penetrated and the solenocyte cell bodies became attached to the top of the coelom wall, adjacent to a blood vessel. To compensate for

FIG. 5. Evolutionary relationships of filtering excretory organs.

After Kümmel, 1962

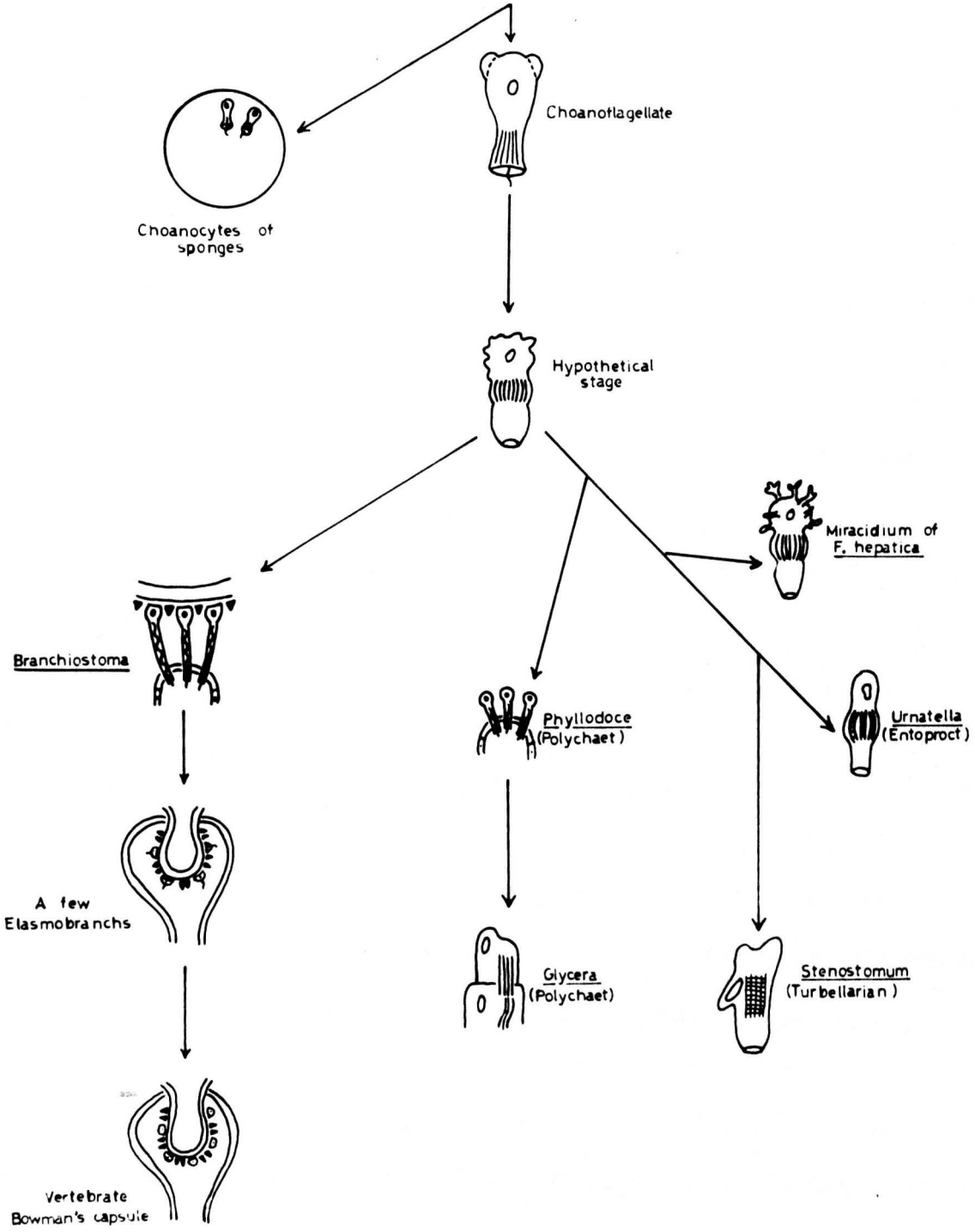

FIG.5. Evolution

the distance between the cell bodies and the renal tubule, long tubular structures evolved to bridge the coelomic space. These structures had regions for filtration of coelomic fluid and their cell bodies were in direct contact with blood spaces. The structure had characteristics of both a cyrtocyte and a podocyte and so has been termed a cyrto-podocyte.

Much of the tubular structure became reduced and contact with the renal tubule was lost in the evolutionary step to produce the filtering nephridium of some elasmobranchs (Fig. 5). These rudimentary podocytes eventually gave rise to the true podocytes of the Bowman's capsule in vertebrates. The blood vessel to which the earlier podocytes were attached became convoluted to form the glomerulus.

Clément (1968) also discussed similarities between protonephridia and mammalian kidney glomeruli. In the flame bulb of the rotifer is a basement membrane, an intercolumnar membrane and a radiating lamella, or fibril, linking columns with pillars. In the Bowman's capsule there are fenestrations between blood capillaries, a basement membrane and slit pores between the podocytes. Both organs are filters and there are three barriers in each case for the fluid to pass. Clément suggests that this is an example of convergent evolution.

In all the cyrtocytes examined, except in those of Lymnaea (a gastropod) larvae, a membrane or desmosome was found bridging the slits between the rods of the corona (Kümmel, 1965). In the exception given, a lamella was found overlying the whole corona. Such an overlying lamella was also present in the solenocytes of Urnatella, Pedizellina and Priapulida, supplementing the bridging lamella. Although

their permeabilities are unknown, the lamellae constitute a necessary requisite for filtration, a filter. A fluid-filled space was also found around all the terminal cells described.

Neither Kümmel (1965) nor Brandenburg (1966) discuss the relationship of rotifer flame bulbs with the other terminal organs. Although in many respects flame bulbs are similar to flame cells, their morphology is unique. The many variations of structure seen in the terminal organs of protonephridia suggest that, rather than divergent evolution occurring (as shown in Fig. 5), the evolutionary trend has been convergent. The organisms possessing protonephridia are diverse. There is no reason to believe that the phyla had the same type of terminal organ in their ancestors. As the need arose for a system to filter the body fluid, a specially differentiated cell evolved to cope. In this way, protonephridia could have arisen many times and each type would have morphological distinctions. By parallel, or convergent, evolution these types have adapted to become more efficient and so they have a tendency to resemble each other.

The main problems involved in studying protonephridia are those of size. The organisms possessing them are small; their protonephridia are microscopic. Until microtechniques are devised to overcome these problems, concrete evidence for the mode of action of flame cells, flame bulbs or solenocytes cannot be easily obtained and only hypotheses can be made.

V Summary.

1. The flame cells of Platyhelminthes are composed of two cells. One contains a large nucleus and the cilia forming

the "flame" arise from it. The other is part of the first tubule cell. The flame cell barrel is made of interdigitations of the two cells, joined together by desmosomes. The tubule lumina are intercellular, but formed from one cell with encircling arms joined by desmosomes. Cestodes have no apparent desmosomes in their tubule walls so the tubule lumina may be intracellular. The tubules of most Platyhelminthes are lined by folds, flagella or microvilli.

2. Flame cells probably function by filtering interstitial fluid across the ribbed region of the barrel (known as the corona). In free living Platyhelminthes, the protonephridia are probably osmoregulatory and excretory; in endoparasites they are excretory organs, because endoparasites are osmoconformers.

3. Flame bulbs of Rotifera are fan-shaped. The nucleus is in the tubule, not in the cap. The corona is composed of a row of columns in a scalloped formation; within each arc of of the scallop is a pillar. A membrane interconnects the columns and fibrils link the pillars to each column of their arc. The tubules leading from the flame bulbs are a complex system of 3 - 4 multinucleate cells. They empty into a contractile bladder.

4. Flame bulbs probably act as filtering organs. Rotifers are able to osmoregulate and the protonephridia probably have this function. They may also be excretory organs.

5. There are many morphologically different types of solenocyte. They have a long tubular structure within which are one or two flagella. At the free end a cytoplasmic cap containing a nucleus is present. Fenestrations piercing the walls of the tubules are probably the site of fluid filtration. Little physiological work has been done on solenocytes but their

function is probably the same as that of other proto-nephridia.

6. It has been postulated that the many types of terminal organ are related to each other by a divergent evolution from one ancestral type (Kümmel, 1962). The variety of the structures, however, suggests a convergent evolution from many different ancestors.

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THE OSMOTIC AND IONIC EFFECTS OF DIFFERENT SALINE CONDITIONS ON *HYMENOLEPIS DIMINUTA*

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Abstract—1. The increase in body weight of *Hymenolepis diminuta* in diluted Hank's saline is entirely due to the intake of water. There is some salt loss, but this is of negligible weight.

2. Salt loss and water intake are proportional to the dilution of the medium.

3. It is concluded that *H. diminuta* is an osmoconformer and that the protonephridial canals have no osmoregulatory function.

INTRODUCTION

A NUMBER of studies have been made on the rat tapeworm, *Hymenolepis diminuta*, in saline media. It is known that *H. diminuta* gains in body weight in diluted Tyrode solution, loses weight in concentrated solution and remains in equilibrium with normal strength solution (Chandler *et al.*, 1950). Similarly, the water content of the sheep tapeworm, *Moniezia expansa*, was found to be related to sodium chloride concentration and the presence of sugar and amino acids in the medium (Wardle, 1937). Siddiqi & Lutz (1966) carried out a more detailed study of osmotic and ionic regulation in the trematode, *Fasciola gigantica*. The body weight of this fluke increased in diluted Tyrode solution and decreased in concentrated solution. Since the tegument was found to be freely permeable in both directions to water and salts, they concluded that this was a method of osmotic adjustment. Their results, however, seem to indicate that the fluke has no control over its salt or water content (i.e. that it is an osmoconformer).

Little is known about whether *H. diminuta* has any powers of osmoregulation. This paper presents a study on the effects of dilute saline on *H. diminuta*, with reference to the function of the protonephridial system.

MATERIALS AND METHODS

H. diminuta was maintained in the laboratory rat, each host having up to ten worms. Mature worms used for the experiments were obtained by sacrificing the rat with a blow on the head and cervical severing. The ileum was removed, slit and the worms washed off into Hank's saline (after Paul, 1965) at 37°C. The large number of worms per rat was necessary to ensure that all worms in one experiment had the same initial environment.

In order to find the osmoregulatory powers of the worm, changes in body weight after incubation in known saline dilutions at set time periods were compared. The first experiment was performed in the balanced saline described by Read *et al.* (1963), but as it was found to be hyperosmotic to the worms at normal strength it was discarded in favour of

Hank's saline. This initial experiment also showed that worms in more than 50% diluted saline disintegrated after 40 min incubation, so dilutions of Hank's saline to only 80 and 60% of original concentration were used; all dilutions were made with distilled water.

Worms were given a preliminary incubation for one hour in normal saline to allow adaptation to external conditions. Throughout the experiments the worms were shaken gently in a water bath at 37°C. After the preliminary incubation, the worms were carefully removed from the medium, blotted on thick filter paper to remove excess moisture, and the initial weight determined on a Mettler balance (Model H16) to 0·01 mg. Blotting and weighing took less than 30 sec and the worms were handled as little as possible.

The weighed worms were transferred to the various diluted salines, all at 37°C, and reweighed at 20-min intervals for 1 hr. Results were calculated as percentage loss or gain in body weight.

Weight changes were fairly substantial in dilute saline and it was possible to resolve the changes into water intake and cation loss. Worms were given a preliminary incubation as before, then two worms were removed, blotted and weighed in porcelain crucibles which had been predried at 105°C for ½ hr and kept in a desiccator until used. The remaining worms were transferred to 60% saline and two removed at 20-min intervals for 1 hr to be weighed as before. All weighed worms were dried overnight at 105°C then reweighed to give the dry weight. The water content was calculated as a percentage of fresh weight. The change in percentage water was calculated as a percentage of the initial water.

The dried worms were ashed in a muffle furnace at 550°C for 2 hr, the ash dissolved in 1 ml 2 N HCl and diluted to 25 ml. Sodium and potassium ion concentrations were determined on an EEL flame photometer (Mark II) using suitable NaCl and KCl standards for calibration. The percentage changes in percentage sodium and potassium were calculated as for water.

It was necessary to show that the composition of the worms with respect to water and cations remained constant in undiluted saline, and to see if any relationship existed between the water and cation composition of the worm and the dilution of the medium. Worms were removed from the rat and divided into two groups. The smaller group was rinsed, blotted and weighed. The larger was given a preliminary incubation for 1 hr, then a few worms were removed for blotting and weighing while the remainder was transferred to 100, 80 and 60% Hank's saline for a further hour. These were then blotted and weighed. Weighing, drying, ashing and cation determinations were carried out on all samples as described previously. Water, ash, sodium and potassium concentrations were calculated as a percentage of fresh weight.

RESULTS

The percentage change in body weight of *H. diminuta* after different periods of time in diluted salines is shown in Fig. 1. The percentage changes in composition after the same time periods in 60% Hank's saline are shown in Fig. 2. From the data for these figures the percentage changes in weights of water, sodium and potassium can be calculated for comparison with the percentage change in weight of the worm. These calculated results are given in Table 1 as mean percentage change ± standard error. As the weights of sodium and potassium were of the order of 0·1–0·2 mg and the weight of water was of the order of 70–90 mg, the change in weight of the worm must be entirely due to water intake. The means of percentage change in weight of worm and of water were compared for each of the time intervals using Student's *t*-test. The values of *t* for the 20-, 40- and 60-min intervals were, respectively, 1·149, 4·473 and 0·232. These show no significant

difference between the means at the 5 per cent level, confirming that water intake is responsible for the increase in body weight.

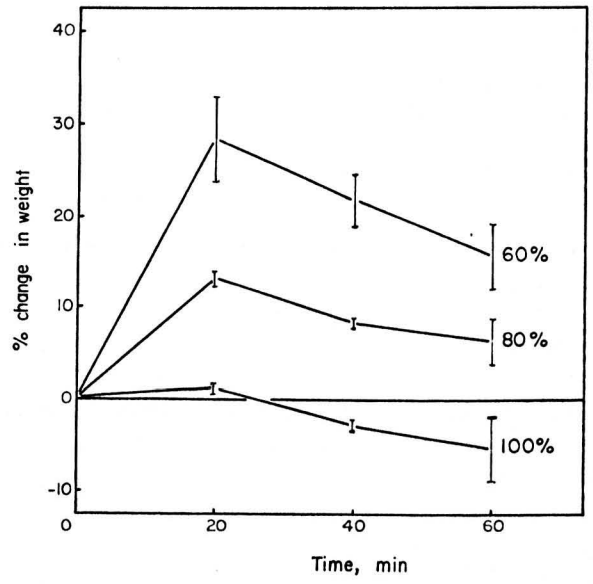

FIG. 1. Percentage change in weight of *H. diminuta* at 20-min intervals in undiluted (100%) Hank's saline and in saline diluted to 80 and 60% of original concentration.

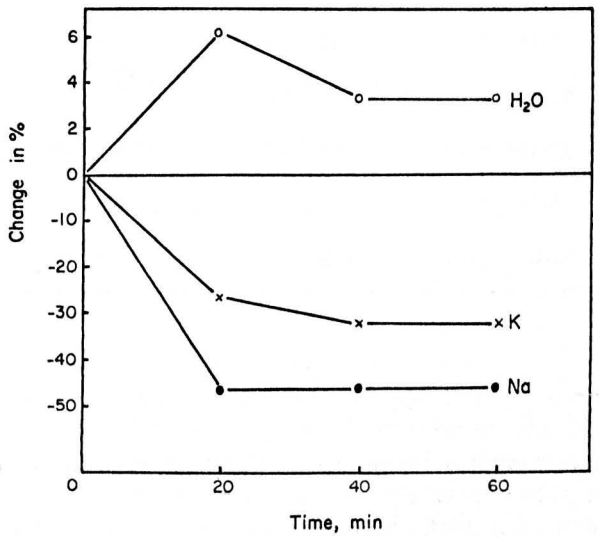

FIG. 2. Percentage changes in percentage water, sodium and potassium at 20-min intervals in Hank's saline diluted to 60% of its original concentration.

TABLE 1—PERCENTAGE CHANGES IN WEIGHT OF WORM, WATER, Na AND K IN 60% HANK'S SALINE (MEAN ± S.E.)

Time (min)	Percentage change in weight of			
	Worm	Water	Na	K
20	28·03 ± 4·69	35·91 ± 4·99	−24·04 ± 0·96	−9·26 ± 5·56
40	21·72 ± 2·95	25·70 ± 3·21	−35·90 ± 2·56	−18·52 ± 3·70
60	15·62 ± 3·48	17·18 ± 5·78	−40·07 ± 1·61	−22·22 ± 3·70

The water, ash, sodium and potassium percentages after different incubation treatments are given in Table 2. Comparison of the means for percentage water in worms from the rat, after an hour's preliminary incubation and after a further hour in undiluted saline using Student's *t*-test on pairs of means, shows there to be no significant difference between the treatments at the 5 per cent level. The composition of the worms, therefore, remains constant in undiluted saline, but changes as the saline becomes more dilute. There is a decrease in the ash present and sodium and potassium ions are lost to the medium in proportion to the medium dilution.

TABLE 2—PERCENTAGE COMPOSITION OF *H. diminuta* WITH DIFFERENT TREATMENTS (MEAN ± S.E.)

Treatment	H_2O (%)	Ash (%)	K (%)	Na (%)
From rat	76·31 ± 0·39	1·51 ± 0·03	0·30 ± 0·02	0·14 ± 0·00
Preliminary incubation	74·77 ± 0·93	1·29 ± 0·02	0·30 ~~0·03~~ ± 0·01	0·13 ± 0·01
1 hr undiluted saline	74·49 ± 0·07	1·29 ± 0·03	0·31 ± 0·01	0·15 ± 0·01
1 hr 80% saline	76·78 ± 0·39	1·15 ± 0·09	0·27 ± 0·01	0·13 ± 0·01
1 hr 60% saline	80·04 ± 0·53	0·81 ± 0·48	0·19 ± 0·01	0·11 ± 0·01

DISCUSSION

These experiments on *H. diminuta* in different saline dilutions show that any changes occurring can be accounted for by water and salt movement across the tegument, such movements being proportional to the dilution of the bathing medium. Three phases in adaptation to a new osmotic environment can be seen from these results. The first phase is the entry of water, lowering the internal osmotic pressure of the worm. The second phase is loss of salt, mainly sodium, causing further osmotic pressure lowering and leaving the worm with an excess of

water compared with the medium. The third phase is exit of this water and consequent loss in body weight of the worm. By the time this final loss of water has occurred, the loss in sodium is approximately 40 per cent, corresponding to the 60% Hank's saline used for the incubation. Loss of potassium is also in proportion to the dilution of the medium but only 20 per cent is lost. This is to be expected, as potassium is usually actively conserved within cells while sodium is pumped into the intercellular fluid.

Since the worm has no apparent control over either its water or cation composition, it must be an osmoconformer, and the protonephridial system cannot have an osmoregulatory function. It has been suggested in an earlier paper (Webster & Wilson, 1970) that the protonephridial system has an excretory function because the fluid in the ventral canals of the system has a high lactic acid content.

The changes in weight and cation composition of *H. diminuta* are similar to those observed in *Fasciola gigantica* by Siddiqi & Lutz (1966). It would seem that some helminth parasites have lost the ability to osmoregulate, perhaps because they are in an osmotically constant environment where regulation is unnecessary.

SUMMARY

1. *H. diminuta* increases in body weight in diluted Hank's saline by the intake of water. Salt loss accompanies this intake, but the ions are of negligible weight.

2. Since the water intake and salt loss are proportional to the dilution of the medium, *H. diminuta* must be an osmoconformer.

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Key Word Index—Hymenolepis diminuta; osmoregulation; salt and water intake; rat tapeworm; tapeworm.

Addendum to "Osmotic and Ionic Effects of Different Saline Conditions on *Hymenolepis diminuta*".

INTRODUCTION

It has been shown (Webster, 1970) that *Hymenolepis diminuta* loses 40% of its tissue sodium and 20% of its tissue potassium when incubated for 1 hour in 60% Hank's saline. The 60% saline was obtained by adding distilled water to normal saline, so diluting all the salts equally. Another set of experiments, presented here, was undertaken to determine the effects on the worm of diluting only the sodium or potassium content of the saline. Mannitol was added to similarly diluted saline in order to maintain the osmotic pressure at its original level. By this means, it was hoped to determine whether sodium and potassium movements are independent of water movements across the worm surface. Mannitol was used because it is a non-toxic, low molecular weight carbohydrate which can be used to maintain the osmotic pressure of the medium without affecting the worm.

MATERIALS AND METHODS

The experiment was carried out in two parts. The first part was to test the effects of sodium dilution on the worm, the second was to test the effects of potassium dilution.

For each experiment, three solutions were used. These were normal Hank's saline (after Paul, 1965), Hank's saline with 60% of the sodium chloride (or 60% of the potassium chloride) specified, and thirdly, the corresponding 60% saline with sufficient mannitol added to compensate for the omitted salt. The quantity of mannitol was calculated on the basis

that the salt left out would have constituted a certain number of osmoles, so an equal number of osmoles of mannitol must be added to keep the osmotic pressure constant. The diluted salines will be referred to as 60% Na (or K) saline and 60% Na (or K) saline plus mannitol.

The method was the same for both experiments. Two worms were removed from the rat, their protonephridial canal fluid sampled in the manner described by Webster & Wilson (1970), then the worms were blotted gently. Their fresh weights were determined by weighing them individually in predried, preweighed porcelain crucibles. Another two worms were removed from a different rat and incubated individually in 25 ml Hank's saline each. Two more rats were sacrificed to obtain two worms for incubation in separate 25 ml aliquots of 60% Na (or K) saline and two worms for incubation in separate 25 ml aliquots of the corresponding 60% saline plus mannitol. The incubation was for 1 hour and the worms were gently shaken in a water bath at 37 - 38°C throughout that time.

After the incubation, the worms were removed one at a time from their media. Their protonephridial canal fluid was sampled, then they were blotted and transferred to crucibles for fresh weight determination. Weighings were carried out to an accuracy of 0.01 mg. on a Mettler balance (Model H16). All worm samples were dried overnight at 105°C and their dry weights determined. Water content was expressed as a percentage of fresh weight. The worms were ashed at 550°C for 2 hr in a muffle furnace and the cooled ash dissolved in 1 ml 2N HCl. This was made up to 25 ml with distilled water and the sodium or potassium content of the worm tissues was then determined. One µl aliquots of the protonephridial canal fluid were diluted to 1 ml for cation

determination. An EEL flame photometer (Mark II) was used for all cation determinations, with appropriate NaCl or KCl standards. The ion concentrations were expressed as a percentage of fresh weight, in the case of tissues, or as µg/µl for the canal fluid. The tissue ions could also be expressed as mEq/l tissue water, since the weight of the ion present and the water content of the tissues were known.

RESULTS

The water content and the sodium ion concentration of the whole worm and of the canal fluid are given in Table 1 for the different salines tested. The percentage changes in these levels after the 1 hr incubations, using data for worms from the rat as initial concentrations, are given in Table 2. Unfortunately, because of the technical difficulties of the experiment, the worm numbers used in each saline were low, so statistical comparison is not practicable.

TABLE 1. Water and sodium composition of worm tissues and canal fluid after 1 hr in different salines. (Mean)

Conditions	Worm tissues % H_2O	% Na	mEq Na/l tissue H_2O	Canal Fluid µg Na/µl
From Rat (control)	73.99	0.18	102	3.5
Normal saline	68.81	0.16	100	4.3
60% Na saline	81.89	0.11	59	2.1
60% Na saline + mannitol	73.56	0.12	72	3.1

TABLE 2. Percentage change in composition of worm tissues and canal fluid during 1 hr. incubation.

Saline	Percentage change in Tissue H_2O	Tissue Na	Canal Fluid Na
Normal	-7.0	-11.1	+22.9
60% Na	+10.7	-38.9	-25.7
60% Na + mannitol	-0.6	-33.9	-11.4

It can be seen from Table 1. that there is no change in the tissue water content of the worms after incubation in 60% Na saline plus mannitol, but in the 60% Na saline, without the osmotic pressure compensation, the water content of the worm rises. The slight water deficit between worms from the rat and worms incubated in normal saline may indicate that the saline is not quite iso-osmotic with H. diminuta tissues or that there is a slight variation among worms. The results for canal fluid sodium show similar trends. There is little difference in the sodium concentration of the fluid after the incubation in 60% Na saline plus

mannitol, but there is quite considerable reduction of sodium in 60% Na saline alone. These facts, and the apparent increase in canal sodium concentration after incubation in normal saline, suggest that canal sodium may be dependent upon tissue water content. If the worm loses water, as in normal saline, the canal sodium concentration rises. If the worm gains water, as in 60% saline, the canal sodium concentration falls. The correlation between tissue water and canal sodium is also seen after incubation in 60% Na saline plus mannitol. In this case neither the tissue water nor the canal sodium concentration alters.

The sodium concentration of the tissues falls slightly after incubation in normal saline, but in both 60% Na salines, in spite of the addition of mannitol, sodium is lost from the worm. In the case of the 60% Na saline, the loss is almost 40%, i.e. the dilution of the sodium in the medium. In the mannitol saline, the loss is not quite so great, but it is of the same order of magnitude and sufficient to show that sodium loss is independent of water movement.

The results for the potassium experiment are given in Tables 3 and 4. As for the sodium experiment, the number of worms per treatment were small and statistical analysis is not practicable.

TABLE 3. Water and potassium composition of worm tissues and canal fluid after incubation in different salines (Mean)

Conditions	Worm tissues % H_2O	% K	m Eq K/l tissue H_2O	Canal fluid µg K/µl
From rat (control)	77.57	0.28	92	0.77
Normal saline	77.12	0.30	98	0.37
60% K saline	74.68	0.25	87	0.25
60% K plus mannitol	74.38	0.24	81	0.35

TABLE 4 Percentage change in composition of worm tissues and canal fluid after 1 hr. incubation.

Saline	Percentage change in Tissue H_2O	Tissue K	Canal fluid K
Normal	-0.58	+7.14	-51.9
60% K	-3.73	-10.71	-67.5
60% K + mannitol	-4.11	-14.29	-54.6

The results show that there is little water loss from the worms after incubation in any of these salines. This is probably because the osmotic effect of omitting 40% of the KCl from Hank's saline is very small. Hank's saline contains 0.4 KCl per l., so a decrease of 40% of this value is almost negligible, compared with a similar decrease it its NaCl content. Normal Hank's saline contains 8 g NaCl/l; a 40% decrease gives, therefore, an appreciable osmotic effect, as shown by the results in Table 1.

The potassium concentration of the canal fluid falls considerably after incubation in any of the media. This decrease is not related to tissue water because the water content changes only slightly. The tissue potassium concentration falls by 10 - 14% when the worms were incubated in 60% K saline, irrespective of mannitol addition. It is significant that the decrease is much less than the potassium dilution factor. A similar result was obtained by Webster (1970) when worms were incubated in saline diluted to 60% of its original concentration by addition of water. It suggests that the worms can regulate potassium loss to some degree.

DISCUSSION

Previous work demonstrated that H. diminuta lost tissue sodium to Hank's saline diluted by 40% (Webster, 1970). The loss was approximately 40% of the tissue sodium. At the same time, because the osmotic pressure of the saline had been lowered by the dilution, the worms gained water. The results presented here show that sodium loss from the worm depends upon the sodium concentration of the medium. Sodium is lost independently of water movements, since the worms lose 33% of their sodium to 60% Na saline plus mannitol, even though there was no change in the water content of the worm tissues.

The results for potassium also correlate with those of the previous work. Potassium loss from the worm tissues is only half of the sodium loss for a similar medium dilution. This suggests some degree of control of potassium loss.

The variations in sodium and potassium concentrations in

the protonephridial canal fluid can probably be explained by similar variations in these ion concentrations in the interstitial fluid of the worm. The results indicate that the level of sodium in the canals is related to the water content of the worm tissues. They also show that the canal potassium level falls by 50 - 60% when the worm tissues lose some potassium to the incubation medium. The fluid in the protonephridial canals, because of its greater level of sodium than potassium, is thought to have an intercellular origin (Webster & Wilson, 1970). This is consistent with the hypothesis that the flame cells filter interstitial fluid. If the interstitial fluid were becoming diluted with respect to sodium, as would be expected when water enters and sodium leaves the worms in diluted saline, the filtered fluid in the canals would reflect such a dilution. Similarly, if the worm tissues were actively conserving potassium from the interstitial fluid, there would be a reduction in the potassium being filtered across the flame cell. It is possible that the fall in the interstitial fluid potassium concentration may alter the potassium gradient between interstitium and canal lumen so causing uptake of potassium by the canal cells.

The protonephridial canals of H. diminuta can be said to have no active role in sodium or water regulation. The worm, however, is able to regulate its potassium content, but whether this regulation is carried out by the worm tissues or by the canals it is not possible to state.

SUMMARY

1. H. diminuta loses sodium to the bathing medium in proportion to the dilution of the sodium in the medium. The loss is independent of water movement which itself depends upon the osmotic pressure of the medium.

2. Potassium loss is less than sodium loss for an equal dilution of the medium potassium. This may be because the tissues conserve potassium in exchange for other cations.

3. The concentrations of sodium and potassium in the protonephridial canal fluid appear to be related to their concentrations in the interstitial fluid. When water enters and sodium is lost from worms in medium diluted with respect to sodium, there is less sodium in the canal fluid. The converse is also probably true, that if water leaves the tissues of worms in hyperosmotic medium, the interstitial fluid becomes more concentrated and so does the canal fluid. Following the reduction of the potassium concentration of the medium, the worm tissues conserve potassium so lowering the interstitial fluid potassium level and also that of the canal fluid.

4. H. diminuta osmoconforms. Its protonephridia play no role in water or sodium balance. Potassium balance is regulated but whether by the interstitial cells or by the canal cells cannot be shown.

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THE CHEMICAL COMPOSITION OF PROTONEPHRIDIAL CANAL FLUID FROM THE CESTODE *HYMENOLEPIS DIMINUTA*

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Abstract—1. A micropuncture technique was used to sample fluid from the longitudinal protonephridial canals of *Hymenolepis diminuta*.

2. The fluid samples were analysed and quantitative results are given for freezing-point depression, pH, dry material, ash, Na^+, K^+, Cl^-, total CO_3^{2-}, glucose, lactic acid, total nitrogen, soluble protein, amino acids, ammonia and urea. No lipids were detected.

3. The results are consistent with the suggestion that the protonephridia have an excretory function.

INTRODUCTION

THE FUNCTION of the protonephridial system of cestodes has long been a subject for speculation (Wardle & McLeod, 1952). Unpublished work on *Hymenolepis diminuta*, the cestode used in this investigation, showed that the worm had no control over the osmotic concentration of its body fluids in different dilutions of Hank's saline.

Many workers have incubated *H. diminuta* in balanced salines for known time periods and analysed the medium for excretory products. This method does not differentiate between loss across the tegument by diffusion and loss by the protonephridial canals. Campbell (1963) reported the production of ammonia and urea, confirming earlier work by Fairbairn *et al.* (1961). Lactic acid was shown to be the predominant end product of carbohydrate metabolism in *H. diminuta* by Read (1956) and Laurie (1957), but Fairbairn *et al.* (1961) reported that succinic and acetic acids were produced in greater quantity than lactic acid. If the protonephridial canals have an excretory function, it would be expected that some of these compounds would be present in the fluid.

This paper presents the results of an investigation into the composition of the fluid in the longitudinal protonephridial canals of *H. diminuta*. The method for collection of fluid was adapted from that of Shipp *et al.* (1958) for micropuncture of single proximal tubules of *Necturus* kidney. Micropuncture was feasible because the tapeworm has canals up to 0·5 mm wide.

MATERIALS AND METHODS

Hymenolepis diminuta was maintained in the laboratory rat, each host having only one or two worms. Mature worms used for fluid collection were obtained by sacrificing the rat with

a blow on the head and cervical rupture. The ileum was removed, slit and the worms washed off into Hank's saline (after Paul, 1951) at 37°C.

The object of the sampling technique was to obtain intact worms as quickly as possible and remove fluid from the canals without contamination by tissue fluids or medium. The apparatus is shown in Fig. 1. The worms were kept in the Perspex bath filled with Hank's saline, maintained at 37°C by a warm water jacket with a constant flow of water from a Tecam Tempunit. The posterior part of each worm was fastened to the cork shelf with a strip of Parafilm held by entomological pins.

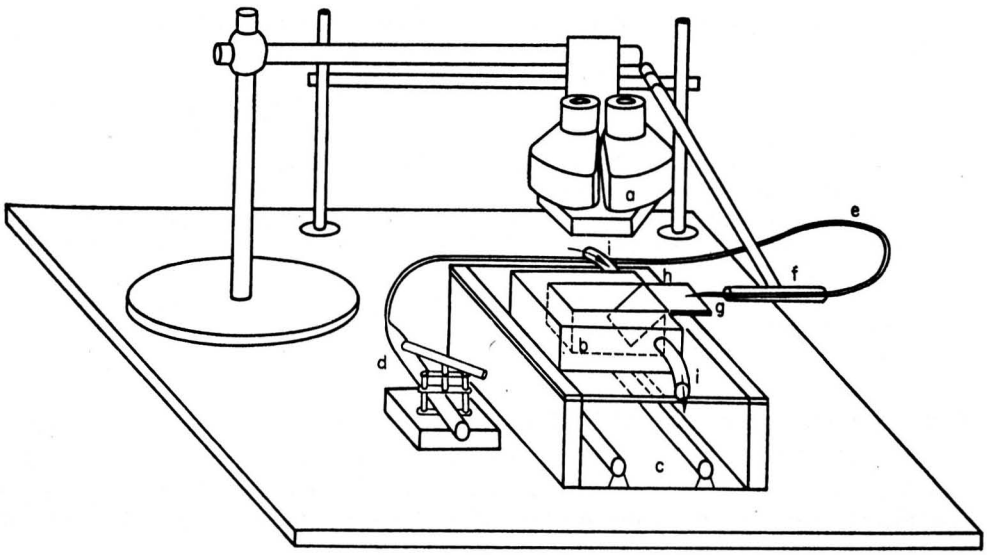

FIG. 1. Diagram of apparatus. Key: a, microscope; b, worm bath; c, fluorescent tubes; d, oil reservoir and screw clamp; e, fine polythene tube; f, guide for polythene tube; g, micropipette; h, Perspex and cork shelf; i, water inlet and outlet.

Micropipettes were made by pulling out 10-μl Drummond microcaps in a micro-Bunsen flame to give a tip diameter of approximately 100 μ. These were mounted in the fine polythene tube.

The principle of the sampling technique was to block the longitudinal canals of the worm to cause a build-up of fluid which could then be withdrawn. Risella 33 (Shell-Mex & B.P. Ltd.), a fairly viscous, colourless oil, was stained overnight with Sudan black B and filtered (Shipp *et al.*, 1958). The polythene reservoir was filled with this oil and connected to the tube holding the micropipette. Oil was squeezed along the fine tube to the tip of the micropipette with the screw clamp, which had a fine pitch for accurate control. Any slight change in pressure on the reservoir caused by this clamp was reflected in oil movements at the tip of the micropipette. In this way injection of oil and withdrawal of fluid was precisely controlled.

The part of the worm on the Perspex shelf was carefully blotted dry with tissue paper. Slight pressure was exerted on the oil in the reservoir to let a trickle of oil out of the micropipette, which was then inserted into the ventral longitudinal canal for a distance of two or three proglottides. An oil droplet was injected which flowed across the transverse ducts to the canal on the opposite side, so blocking the whole system. The pipette was withdrawn, still with slight positive pressure on the reservoir to avoid taking in bathing medium. The

worm was left for 30 sec to undergo peristalsis and fluid accumulated anterior to the oil droplet. The oil in the micropipette was adjusted until it was just at the tip, then the pipette was re-inserted about half-way along the oil droplet. The tip was pushed through the oil into the fluid and the pressure reduced on the oil so that fluid was withdrawn into the micropipette. When the micropipette was full of fluid, the tip was pulled back into the oil droplet and a little oil drawn in to seal the fluid against contamination. The pressure was adjusted to prevent further fluid movement and the micropipette was withdrawn. The oil seal was expelled onto dry tissue paper and the fluid expelled into micro test-tubes, approximately 15×2 mm. The samples were sealed at the base of these tubes by a drop of oil expelled above the fluid to prevent evaporation. Centrifugation caused any free oil droplets in the fluid to coalesce at the surface so that the layer could be removed carefully by a capillary attached to a vacuum pump when the fluid was to be used. Known aliquots of fluid were measured out using Drummond microcaps.

The time taken for collection of fluid was approximately 30 min from beginning to end. Fluid was always freshly collected for analyses.

Analysis of fluid

Freezing-point depression was determined on 0·1 μl droplets of fluid using a micro-method adapted from that of Gross (1954) to employ direct temperature measurement (R. S. V. Pullin, personal communication).

The pH was measured on 2 μl of fluid using microelectrodes G 252C and K 150 (Radiometer, Copenhagen).

Dry weight determinations were carried out on a Cahn Gramelectrobalance. Samples of fluid (1 μl) were expelled onto predried, preweighed aluminium foil discs of 5 mm diameter. These were left overnight in a desiccator with silica gel as drying agent, then reweighed. Control discs were included.

Ash was determined in 1-μl samples. The procedure was followed as for dry weight, but the samples were expelled onto platinum foil. When dry, they were ashed at 550°C for 1 hr, cooled in a desiccator and reweighed.

Sodium and potassium ion concentrations were determined by flame photometry (EEL, Mark II machine) on 1-μl samples diluted to 1 ml. Standard NaCl and KCl solutions were used to calibrate the machine.

Chloride ion concentration was determined by the method of Shales & Shales (1941) with a Beckman/Spinco 153 Microtitrator. Samples (5 μl) were titrated undiluted.

Total carbonate, which includes gaseous CO_2, carbonic acid and bicarbonate ions, was determined conductimetrically by a modification of the method of Prop (1954) scaled down to take 5-μl samples (R. S. V. Pullin, personal communication).

Free sugars were detected qualitatively by thin-layer chromatography on 0·25 mm thick cellulose layers. Samples (5 μl) were run, together with standards, in ethyl acetate–66% isopropanol (65:35). Sugars were detected using aniline phthalate reagent (Stahl, 1965).

Glucose concentration was determined enzymically by the method of Salomon & Johnson (1959). Optical density was measured at 635 mμ on a Beckmann Microcolorimeter. Samples (2 μl) were diluted with 200 μl of distilled water to give better mixing when reagents were added.

Organic acids were detected by thin-layer chromatography on 0·25 mm silica gel-G layers. The solvent system was 95% ethanol–25% ammonium hydroxide–water (75:12:9); acids were detected with aniline xylose reagent (Randerath, 1964). As a check, another solvent system, benzene–methanol–acetic acid (45:8:4), was employed. Acids were detected with bromophenol blue (Petrowitz & Pastuska, 1962).

Lactic acid concentration was determined colorimetrically by the method of Hullin & Noble (1953). Samples (1 μl) were diluted to 5 ml during protein precipitation and 1-ml aliquots of this were used for colorimetry. The colour was read at 560 mμ on a Bausch & Lomb Spectronic 20, used for all following readings of optical density.

Total nitrogen was determined by the method of Jacobs (1964), scaled down to analyse 2-μl samples. Optical density was read at 570 mμ.

Non-protein nitrogen was attempted by the same method, after deproteinization, but the method was not sensitive enough for such small samples.

Soluble protein was determined by the method of Lowry *et al.* (1951) in 3-μl samples. The optical density was read at 750 mμ.

Amino acids and ammonia concentrations were determined quantitatively on a Technicon Auto-Analyzer with 0·1 μM of each amino acid as standard. Samples of 16 and 20 μl were used.

Total α-amino acids were determined colorimetrically in 2-μl samples of fluid by the method of Stein & Moor (1954) using leucine as a standard. Optical densities were read at 570 mμ.

Urea was detected chromatographically on 0·25 mm thick silica gel-G layers using the solvent system phenol–water (150 : 50 w/w) and stained with Ehrlich's reagent (after Smith, 1960). Quantitatively, urea concentration was determined by the method of Fawcett & Scott (1960) using 2-μl samples. The optical density was read at 550 mμ.

Fluid was extracted for lipids with chloroform–methanol (2: 1) and the extract chromatogrammed on 0·25 mm thick silica gel-G layers. For classes of lipid, the solvent system was hexane–diethylether–acetic acid (80:20:1) (Ginger & Fairbairn, 1966); iodine vapour was used for detection. For phospholipids, chloroform–methanol–water (65:25:4) was employed; ammonium molybdate–perchloric acid reagent and Dragendorff's reagent were used for detection (Wagner *et al.*, 1961).

RESULTS

The freezing-point depression, determined on twenty-seven samples of canal fluid from fourteen worms, had a mean ± standard error (S.E.) of $-0{\cdot}74 \pm 0{\cdot}03$°C. Using standard NaCl solutions for calibration, the fluid was calculated to be equivalent to $1{\cdot}15 \pm 0{\cdot}04$% NaCl.

The pH of the fluid increased from approximately 4·5 to an equilibrium value of $6{\cdot}32 \pm 0{\cdot}07$ within 10 sec when the fluid was exposed to air. The equilibrium value is the mean ± S.E. of twelve determinations on fluid from five worms.

The dry weight, ash and ion concentrations are given in Table 1.

TABLE 1—DRY WEIGHT, ASH AND ION CONCENTRATIONS OF CANAL FLUID

Material	Mean ± S.E. (mg/ml)	No. of determinations	No. of worms
Dry weight	$27{\cdot}1 \pm 0{\cdot}72$	10	4
Ash	$6{\cdot}8 \pm 0{\cdot}52$	5	3
Na^+	$3{\cdot}2 \pm 0{\cdot}05$	5	4
K^+	$0{\cdot}7 \pm 0{\cdot}05$	5	4
Cl^-	$1{\cdot}8 \pm 0{\cdot}11$	8	7
Total CO_3^{2-}	$0{\cdot}27 \pm 0{\cdot}06$	7	5

Standard glucose, in thin-layer chromatography, had an R_f value of between 0·11 and 0·13. Samples of fluid contained a compound with an R_f value of 0·13, which was identified as glucose and was the only detectable sugar. The mean

concentration of glucose in nine samples from four worms was 1·5 ± 0·31 mg/ml by colorimetric determination.

Only a single compound was detected during chromatography for organic acids. This had an R_f value in the range 0·40–0·54 in ethanol–ammonium hydroxide–water and 0·15–0·17 in benzene–methanol–acetic acid. The R_f of standard lactic acid was in the range 0·48–0·50 and 0·19–0·24 in the respective solvent systems. To check the discrepancy, a sample of lactic acid was run, at the same time as a mixed sample of lactic acid and canal fluid, in benzene–methanol–acetic acid. There was no separation of the mixture and the R_f value for both samples was 0·15. The compound was identified as lactic acid, and colorimetric determination on ten samples of fluid from three worms gave a mean lactic acid concentration of 9·5 ± 0·70 mg/ml.

Estimation of total nitrogen in ten samples of fluid from four worms gave a mean ± S.E. of 0·87 ± 0·11 mg/ml.

Soluble protein determined colorimetrically on seven samples from three worms had a mean concentration of 3·52 ± 0·17 mg/ml.

Amino acids detected by the Auto-Analyzer are tabulated in Table 2. The mean total concentration was 2·01 ± 0·40 mg/ml. Ammonia present in the same samples had a mean concentration of 0·04 ± 0·005 mg/ml.

TABLE 2—AMINO ACID COMPOSITION OF CANAL FLUID (mg/ml)

Amino acid	Worm 1	Worm 2	Worm 3	Mean
Threonine	0·10	0·11	0·04	0·08
Serine	0·20	0·19	0·09	0·16
Glutamic acid	0·10	0·04	0·15	0·10
Proline	0·24	0·17	0·06	0·16
Glycine	0·11	0·13	0·07	0·10
Alanine	0·92	0·97	0·39	0·76
Valine	0·04	0·05	0·02	0·04
Methionine	0·01	Little	Little	<0·01
Isoleucine	0·04	0·02	0·01	0·02
Leucine	0·08	0·08	0·05	0·07
Tyrosine	0·10	0·10	0·10	0·10
Phenyl alanine	0·03	0·04	0·01	0·03
Histidine	0·08	0·07	0·05	0·07
Lysine	0·05	0·05	0·05	0·05
Taurine ?	0·10	0·10	0·05	0·08
Ornithine ?	0·18	0·16	None	0·17
Four unknowns	0·08	0·08	0·08	0·08

α-Amino acids determined colorimetrically in six samples from four worms had a mean concentration of 2·1 ± 0·26 mg/ml.

Urea was detected chromatographically. Standard urea solutions yielded a yellow spot of R_f value 0·68, identical with the Ehrlich-positive spot in the sample.

Colorimetric determination in twenty-four samples from five worms gave a mean urea concentration of 0·5 ± 0·04 mg/ml.

No classes of lipids could be detected in the extracted canal fluid. Unextracted samples yielded a blue spot in ammonium molybdate–perchloric acid reagent, but this could be any phosphate compound.

DISCUSSION

H. diminuta has been shown (L. A. Webster, unpublished) to have no control over the osmotic concentration of its body fluids. In diluted Hank's saline worms gained water and lost salts over a period of time. If *H. diminuta* is an osmoconformer, the fluid in the protonephridial canals would be expected to have the same osmotic pressure as that of the environment of the worm. The canal fluid had a freezing-point depression of −0·74°C, equivalent to 1·15% NaCl. Schopfer (1932) measured the freezing-point depression of sheep intestinal fluid and found it to be −0·82°C, equivalent to 1·3% NaCl. It is possible, therefore, that the worm is in osmotic equilibrium with its host and the alternative hypothesis, that the protonephridia may have an excretory function, must be examined.

The presence of lactate, urea and ammonia in the canal fluid is indicative of an excretory function for the protonephridial system. These compounds have already been detected in incubation media by Read (1956), Laurie (1957), Fairbairn *et al.* (1961) and Campbell (1963). Lactate and urea are less likely to diffuse across the tegument than the smaller ammonia molecule, so the protonephridia may be a more probable route for their excretion. The presence of urea as an excretory product may indicate a low water turnover rate in the worm since it is less toxic than ammonia. The ornithine cycle enzymes for production of urea are present in *H. diminuta* (Campbell, 1963), so the urea detected is probably not of host origin.

Lipids were not detected in the canal fluid. Howells (1969) reported that no lipid droplets were to be seen in the protonephridial canals of *Moniezia expansa*. Trematode protonephridia, on the contrary, contain many lipid droplets which are expelled through the excretory pore (von Brand, 1966).

In order to complete the organic analysis, amino acid and glucose levels in the canal fluid were determined (2·01 and 1·5 mg/ml respectively). The total amino acid concentration for the whole worm was calculated from data of Goodchild & Dennis (1966) as 67·4 mg/ml. The level in the canal fluid is approximately 3 per cent of this total. Most of the individual amino acids are in the same proportions in the fluid as in the whole worm, with the exception of alanine. Alanine is unusual because it accounts for 38 per cent of the total amino acids in the canal fluid, yet only for 6 per cent of the total in the worm. Similar calculations are not possible for glucose, but the rate of loss of both amino acids and glucose will be dependent on the rate of fluid flow in the canals.

The dry material in the canal fluid was 27·1 mg/ml. The sum of the means of the constituents analysed is 24·0 mg/ml. The difference could result from cumulative experimental errors arising from technical difficulties in analysing such small quantities of fluid. Alternatively, some compound may have been overlooked, but

the difference is so small that it is assumed the major constituents of the fluid have been identified.

When canal fluid is exposed to air the pH rises from approximately 4·5 to equilibrium at 6·32. This rise in pH may be caused by effervescence of excess gaseous CO_2, suggesting a poor buffering capacity of the fluid.

To check whether the pH of the protonephridial fluid could be reproduced by the ions detected, and that a buffering system had not been overlooked, a simulated fluid was made up with ions in the same proportion as in the true fluid. The ion concentrations are given in Table 3, together with the respective pH values of the fluids. The most feasible buffering system detected by analysis was the carbonic acid/bicarbonate system. The presence of small amounts of protein in the fluid might also have some buffering effect. The discrepancy in pH between simulate and canal fluid may suggest the presence of minor acidic components not yet identified.

TABLE 3—ANIONS AND CATIONS OF TRUE AND SIMULATED FLUID (m-equiv./ml)

	Na^+	K^+	NH_4^+	Lactate$^-$	HCO_3^-	Cl^-	pH
True fluid	140	18	2	107	4	51	6·32
Simulate	140	18	2	107	4	49	7·25
Totals in true fluid	Cations 160			Anions 162			

It is conventional to assume that all the CO_2 dissolved in water reacts to form carbonic acid. In fact, only 0·33 per cent of the CO_2 dissolved is in the H_2CO_3 form (Robertson, 1965). The Henderson–Hasselbalch equation for the buffering system $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$ is $pH = pK + \log (HCO_3^-)/(H_2CO_3)$, where pK for the reaction at 37°C is 6·10. The ratio of (HCO_3^-) to (H_2CO_3) can be calculated for different pH values, and from this ratio the percentage of total carbonate present attributable to H_2CO_3 and CO_2 can be calculated. At the lower fluid pH of 4·5, 97·4 per cent of the total carbonate is dissolved CO_2 and H_2CO_3; at the equilibrium pH of 6·32, 37·6 per cent is in this form. The remainder in each case is HCO_3^-.

The partial pressure of CO_2 (pCO_2) in the fluid can also be found using the total carbonate figures (see Table 1) to calculate the percentage of bicarbonate present. This value can be substituted in the modified Henderson–Hasselbalch equation: $pH = pK + \log (HCO_3^-)/a.pCO_2$ (where $pK = 6{\cdot}10$; a, the solubility coefficient of CO_2 at 37°C = 0·0334) (Robertson, 1965). The pCO_2 at pH 4·5 is calculated as 120 mm Hg. This is higher than the pCO_2 of rabbit intestine, which is 20–40 mm Hg (Campbell, 1933). The calculation suggests that the deep tissues of the worm have a higher pCO_2 than rat intestinal lumen.

The foregoing conclusions are consistent with the protonephridial system having an excretory function. In order to prove this hypothesis, it is necessary to show that the flame cell is an organ of filtration and that the filtered fluid is modified by selective secretion and/or reabsorption in the canals. Kümmel (1958) and Wilson

(1967) have provided circumstantial evidence that the flame cell is a site of filtration, and Howells (1969) has shown that the protonephridial canals of *Moniezia expansa* are lined with microvilli which, he postulates, may function in secretion and/or reabsorption. Further circumstantial evidence is provided by the ratio of sodium to potassium in the canal fluid determined in this study, which is indicative of an intercellular, rather than intracellular, origin of the fluid. This suggests filtration and not secretion, because secreted materials would come from inside the cells and so be expected to have higher potassium levels relative to sodium.

If the body surface were the only route for excretion, then the rate of diffusion from the deeper tissues would limit the loss of excretory products. Flame cells in *H. diminuta* appear to be distributed all over the central tissues but are rarely found outside the mesenchymal muscle layers.

SUMMARY

1. A method for obtaining fluid from the longitudinal canals of *H. diminuta* was described.

2. The canal fluid was analysed quantitatively for freezing-point depression, pH, dry weight, ash, cations, anions, sugars, organic acids, nitrogenous compounds and lipids.

3. The results were discussed with reference to the probability of the protonephridial system having an excretory function.

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Key Word Index—Hymenolepis diminuta; chemical composition; protonephridial fluid.

Addendum to "The Chemical Composition of Protonephridial Canal Fluid ..." Further Work on Succinate and Lactate

INTRODUCTION

After incubation of Hymenolepis diminuta for 3 hr in saline gassed with 5% CO_2: 95% N_2, Fairbairn et al. (1961) found that, of the total acids excreted by the worm, succinate accounted for 55.9% and lactate 9.5%. Using similar incubation conditions, Read (1956) and Laurie (1957), respectively, found that lactate accounted for 80 - 95% and 37 - 38% of the total excreted acids. The only apparent difference between the techniques was the age of worms used. Read and Laurie used worms several weeks old. Fairbairn et al. used worms 14 - 15 days old, but demonstrated that succinate is the major acid excreted even by 3 month old worms. There seems no reason, therefore, for the difference in findings.

The protonephridial system of H. diminuta is probably the excretory route for these acids. If so, their ratio in the canal fluid should show which is the major acid excreted. Webster & Wilson (1970) were able to detect only lactate in canal fluid taken from worms straight out of the rat. In order to check whether incubation in the presence of CO_2 is necessary for succinate to appear in the canals, further experiments, presented here, were carried out.

The concentration of lactate in the canal fluid has been determined by the method of Hullin & Noble (1953) (Webster & Wilson, 1970). This colorimetric method may detect compounds other than lactate, so producing a higher

value than the actual concentration present. Recently, a method specific for lactate has been devised (H. Leese, personal communication). It is more sensitive than the colorimetric technique, detecting 0.005 μ mole/ml instead of 0.1 μ mole/ml. The reagent used is lactate dehydrogenase in the presence of NAD^+. Lactate is oxidised by the enzyme, the hydrogen released reacts with the NAD^+ to form NADH and this is measured fluorimetrically, the molecular ratio of NADH to lactate being 1:1. Since this method will detect lactate only, further samples of canal fluid were analysed to obtain a definite value for the lactate concentration in the canals.

MATERIALS AND METHODS

Removal of worms from the rat, apparatus for sampling fluid from their protonephridial canals and the procedure involved were as described by Webster & Wilson (1970). The saline in the worm bath of the sampling apparatus was at 38°C and gassed continuously with 5% CO_2 : 4% O_2 ; 91% N_2, to simulate intestinal gas levels (Webster, 1971).

In order to investigate the possible presence of succinate in the canal fluid, six worms were incubated for 3 hr in the gassed saline before fluid samples were taken. One or two μl aliquots of the fluid samples were spotted onto 0.25 mm silica gel-G layers, together with succinate and lactate standards. The plates were developed in 95% ethanol - 25% ammonium hydroxide-water (75:12:9); acids were detected with aniline xylose reagent (Randerath, 1964).

A check on the sensitivity of thin-layer chromatography for succinate detection was considered necessary. Two

solutions were tested, one of succinic acid, the other of sodium succinate, both containing 3 µg succinate ion/µl. This was thought to be of a similar order of magnitude to the possible succinate concentration in the canal fluid. Sodium succinate was tested because succinate in canal fluid would be expected to be in the salt form, and may give a different Rf value from the acid form. Five-µl of each solution were spotted onto thin-layer plates as before, together with a 50 µg succinate standard. The plates were developed and acids detected as described above.

It was hoped to use succinic dehydrogenase as a specific reagent for succinate, in order to obtain a definite value for its concentration in the canal fluid. The enzyme is, however, extremely unstable and cannot be obtained commercially. Attempts to isolate it from beef heart mitochondria, by the method of Bernath & Singer (1962), failed, probably because of inadequate facilities for temperature control.

Lactate analysis was carried out on fluid sampled from the canals of six worms. One-µl aliquots of the fluid were diluted with 3 ml 5% perchloric acid then neutralized with 0.6 ml 30% K_2CO_3 solution. The suspension formed was centrifuged to precipitate the protein from the sample and the supernatant divided into two aliquots for the analysis. This was carried out in the Technicon Auto-Analyzer.

RESULTS

Six fluid samples for organic acid detection produced spots having Rf values of 0.55 - 0.61. Those for lactate standards were in the 0.55 - 0.64 range and there was no

significant difference between them at the 5% level (t = 0.32, 12 degrees of freedom). Five of the samples produced spots having Rf values of 0.32 - 0.36. Those for succinate standards were in the 0.25 - 0.36 range and there was no significant difference between these ranges at the 5% level (t = 1.91, 11 degrees of freedom). Fluid from worms incubated for 3 hrs in saline gassed with 5% CO_2 : 4% O_2 : 91% N_2, therefore, contains lactate and succinate.

The succinate spots from the canal fluid were always less clearly visible than the lactate spots, although standards containing equal quantities of each were equally visible. There is probably less succinate than lactate in the canal fluid.

The sensitivity limit of thin-layer chromatography for succinate detection is approximately 10 µg. The 15 µg succinate test spots were only faintly visible while the 50 µg standard was clearly visible. Comparison of the 15 µg spot with those produced by 2 µl of canal fluid suggests that the fluid may contain less than 5 µg succinate/µl of canal fluid. This is corroborated by the succinate spots appearing smaller than the lactate spots from fluid samples.

The concentration of lactate in the canal fluid, given by the enzymatic method, is 6.440 $\pm$ 0.114 mg/ml (Mean $\pm$ S.E. for 12 samples from 6 worms). This mean was not significantly different, at the 5% level, from the mean of 9.5 $\pm$ 0.7 mg/ml obtained by Webster & Wilson (1970) (t = 1.605, 12 degrees of freedom). Similar concentration ranges were found in each analysis: colorimetry gave a range of 7-14 mg/ml and the enzymatic method gave 3-14 mg/ml. There is, therefore, wide variation among worms in the quantity of lactate excreted.

DISCUSSION

The relative concentrations of lactate and succinate in the canal fluid appear to confirm the results of Read (1956) and Laurie (1957), suggesting that lactate is the major acid excreted by H. diminuta. It was unfortunate that succinic dehydrogenase could not be isolated and a definite value for the fluid succinate concentration obtained. The presence of succinate in the canal fluid, however, supports the hypothesis that the protonephridial system of H. diminuta has an excretory function (Webster & Wilson, 1970).

Worms incubated in aerobic conditions excrete less total acid than worms in anaerobic conditions (Fairbairn et al.,1961). Fluid sampled by Webster & Wilson (1970) was from worms straight out of the rat, but the worms were incubated aerobically during the sampling. It is possible that this short incubation was sufficient to decrease the acid production and so decrease the succinate concentration in the canal fluid. Since the succinate level in fluid from worms incubated anaerobically for 3 hr is at the limit of chromatographic detection, and probably less than 5 µg/µl, it is not surprising that succinate was not detected by Webster & Wilson (1970).

SUMMARY

1. Succinate was detected in protonephridial canal fluid of H. diminuta after incubation of worms for 3 hr in saline gassed with 5% CO_2: 4% O_2: 91% N_2. The size of the succinate spot detected by thin layer chromatography suggests that its concentration in the fluid is less than that of lactate.

2. Enzymatic analysis for lactate in protonephridial canal

fluid shows there to be 6.440 ± 0.114 mg/ml. This mean is not significantly different from the mean of 9.5 ± 0.7 mg/ml obtained previously by colorimetry. The enzymatic analysis is more sensitive.

3. The presence of succinate in the canal fluid supports the hypothesis that the protonephridia of H. diminuta are excretory organs. The major acid excreted by this system is probably lactate.

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THE FLOW OF FLUID IN THE PROTONEPHRIDIAL CANALS OF *HYMENOLEPIS DIMINUTA*

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Abstract—1. Two types of activity were observed in *Hymenolepis diminuta*. The more common peristalsis is superseded by rapid ripples as the pCO_2 is increased.

2. Fluid flow in the protonephridial canals in turn depends upon these activities.

3. The pressure exerted by the fluid in the canals and the volume of these canals were measured.

4. The mean rates of excretion of lactate, urea and ammonia were calculated as 19·4, 1·02 and 0·08 μg/mg dry wt. per hr respectively. It is probable that the canals are the main excretory route for lactate and urea.

INTRODUCTION

VERY FEW studies have been carried out on cestode movement. Waves of expansion in a tetrarhynchid larva, *Nybelinia surmenicola*, found in fish, were described by Wardle (1934). *In situ*, there were observed 4 expansions of the larva/min, while in double Locke solution 24–30 expansions/min were seen. It is not stated how observations were carried out *in situ* without changing any conditions. A more detailed study was carried out by Rietschel (1935) on *Catotaenia pusilla*, a cestode from mice, in Ringer solution. Waves of contraction were seen to move backwards from the anterior with a speed dependent on temperature. Cutting the nerve cords and excretory canals did not interrupt the flow of waves posterior to the cuts, thus they are not dependent upon nervous impulses or hydrostatic pressure for their propagation.

The only study carried out on fluid movement in the longitudinal protonephridial canals was by Macpherson (1958) on *Hymenolepis diminuta*. After embedding the main part of the tapeworm in agar to prevent movement, the anterior tenth of the worm was dipped into isotonic saline containing ^{32}P phosphate, ^{35}S methionine or ^{45}Ca chloride; the posterior tip was dipped into isotonic saline. The whole was kept at 38°C for time periods of 45 min–5 hr, after which it was shown that no movement of the labelled materials had occurred. There are two major criticisms of this work: firstly, if the evidence presented in this paper is correct, the embedding of a worm to prevent its moving would also prevent fluid from flowing; secondly, there is no reason to suppose that any of the labelled compounds would be transported in an excretory system.

The present work gives evidence to support the theory that fluid flow depends upon worm movements and also that the longitudinal canals of *H. diminuta* have an excretory role.

MATERIALS AND METHODS

H. diminuta was maintained in the laboratory rat, one or two worms per host. The worms were obtained by sacrificing the rat with a blow on the head and cervical rupture. The ileum was slit and the worms washed off into Hank's saline (after Paul, 1965) at 37°C. This was done as quickly as possible to minimize any effects of changes in gas tensions in the worms' environment.

Unpublished observations had shown that the flow of fluid down the protonephridial canals is related to the peristaltic activity of the worm. In order to further investigate this, it was considered necessary to have worms in conditions as near those in the intestine as possible. Various levels of O_2 and CO_2, within the ranges found in the ileum, were bubbled through the saline bathing the worm and the effects on the activity of the worm observed. The intestinal levels of the gases were taken from Campbell (1933), who measured the CO_2 level in the ileum of the cat and rabbit as 35–60 mm Hg and the O_2 level to be 20–40 mm Hg; and from Rogers (1949), who measured the O_2 level of the rat ileum as 8–32 mm Hg, or about 4 per cent. If 30 mm Hg is equivalent to 4 per cent, then the CO_2 range measured by

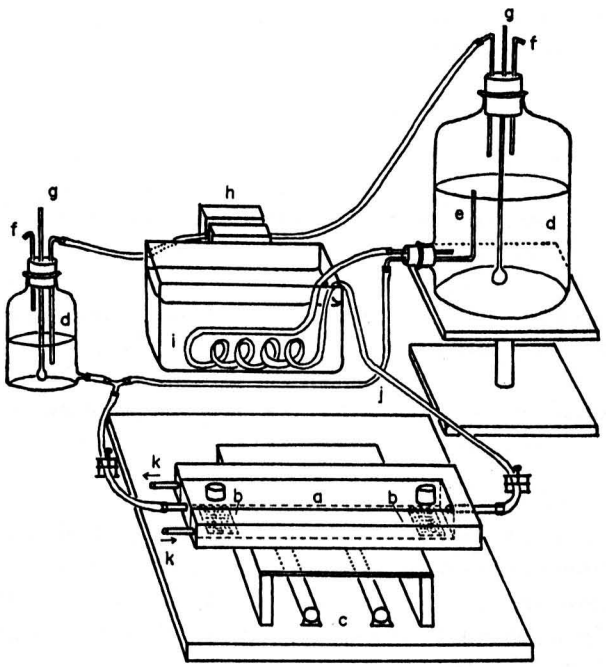

FIG. 1. Diagram of apparatus. Key: a, Perspex worm bath at 37°C; b, cork pieces to which worm pinned; c, fluorescent tubes; d, reservoir of Hank's saline; e, overflow to maintain constant level; f, gas outlet; g, bubbler for gas mixture; h, peristaltic pump to return saline from small to large reservoir; i, glass coil in water bath to warm saline to 37°C; j, polythene tubing to conduct saline; k, inlet and outlet to water jacket at 37°C.

Campbell is 6–8 per cent. These percentage values were used for the experiments, the remaining volume made up by N_2.

The continuous flow apparatus used is shown diagrammatically in Fig. 1. The worm bath was a closed box approximately one foot long, surrounded by a water jacket to maintain a constant temperature within the bath. Holes of 2·5 cm dia. cut in the lid at each end allowed access to the interior of the bath; these were sealed with bungs during an experiment. The worm was pinned to pieces of cork below the holes such that the saline flow was towards the posterior of the worm, simulating intestine contents. The saline reservoirs were gassed with N_2 for 1 hr to remove all other dissolved gases. The O_2 level required was then bubbled through for 6–10 min to equilibrate.

For the observation of peristaltic activity at different gas tensions, a strip of mm graph paper was placed under the worm bath. The speed of peristalsis, in mm/sec, was measured as the time for one peristaltic bulge to move 20 mm measured on the graph paper. The frequency was measured as the number of peristaltic bulges to pass a given line on the graph paper in one minute.

To check the type of activity found *in vivo*, a rat was anaesthetized as described by Smithers & Terry (1969), the intestine carefully opened and the worms observed.

For observation of fluid flow down the longitudinal canals, the same continuous flow apparatus was employed, but the worm bath was open-topped to allow access for injecting the worms. A Perspex lid was placed over the top while the gases equilibrated in the system, but the lid had to be removed periodically in order to observe the worms with a microscope. A cork shelf, to which the middle portion of a whole tapeworm was pinned, was fitted over one end of the worm bath. The equipment used for the injection of oil droplets into the protonephridial canals to act as a seal was the same as that described in an earlier paper (Webster & Wilson, 1970). In this experiment a suspension of Aquadag (colloidal graphite) was injected into the canal after passing the tip of the micropipette through the oil droplet. The worm was ligatured at the point of injection to prevent backflow and then pinned freely in the worm bath to incubate. The time for the carbon particles to pass from the place of injection to the posterior of the worm was taken and the length of the worm from injection point to the posterior tip was measured. From these the speed of maximum fluid flow in mm/sec could be calculated. The speed of peristalsis in each worm used for fluid flow measurement was also calculated so as to have both speeds taken under exactly comparable conditions. The efficiency of peristalsis in causing fluid flow could then be calculated as: speed of fluid flow × 100/speed of peristalsis. The gas tensions used in this experiment were 4% O_2 : 4% CO_2 : 92% N_2 and 4% O_2 : 6% CO_2 : 90% N_2, chosen because two types of activity had been observed at these tensions.

Because the canal fluid flowed with an overall direction towards the posterior, it was thought that there may be an overall pressure within the canals and an experiment was devised to measure it. The apparatus used was the same as for the previous experiment, with the worm pinned to the cork shelf by its posterior end, but a manometer was substituted for the oil reservoir with its screw clamp. The manometer was attached to the polythene tubing holding the micropipette. The manometric solution was 1% aqueous sodium cholate coloured with methylene blue. The tip of the micropipette was filled with Sudan black B stained oil. The micropipettes were calibrated for capillarity before and after measuring the pressure in the canals. This was done by dipping the pipette tip under the surface of the bathing saline and adjusting the manometer height (i.e. the difference in height between the two arms of the manometer) to prevent any movement of oil in its tip. This procedure was repeated after inserting the micropipette into the protonephridial canal. The pressure exerted by the canal fluid was calculated as the manometer height necessary to prevent oil movement in the micropipette inserted into a canal minus the height necessary to overcome capillarity. The pressure in the canals was measured in worms at the two gas tensions given above. The accuracy of the apparatus was checked by inserting the micropipette through a rubber membrane covering the horizontal piece of an L-shaped tube. The vertical part of

the tube contained a measured height of water and the manometer measured this height with a less than 2 per cent error.

Knowing the speed of fluid flow down the canal, it should be possible to calculate the rate of loss of fluid from the worm if the volume of a given length of worm were known. In order to measure the volume, inulin (carboxylic acid—C14) (supplied by the Radiochemical Centre, Amersham, Bucks.) was used as a tracer, because it has a high molecular weight (> 5000) and is not normally metabolised. A standard solution registering 3500 counts/min per μl was made up of inulin in Hank's saline. Micropipettes were calibrated by drawing up a measured 1 μl of distilled water and marking the level of the meniscus with a felt-tipped pen. The pipettes were dried, then samples of the radioactive solution were drawn up to the mark and expelled on to planchettes for counting. An oil seal was injected into the canals at the anterior end of a posterior piece of tapeworm approximately 100 mm long. The tip of a micropipette containing a measured quantity of ^{14}C inulin solution was inserted beyond the oil seal and the solution injected into the canals. A ligature was tied to prevent backflow through the injection hole, and another was tied at the posterior tip to prevent loss of radioactive material to the bathing medium. After incubation for 1 min under a gas tension of 4% O_2 : 6% CO_2 : 90% N_2, the posterior end of the worm was pinned to the cork shelf and a sample of the canal fluid removed. One-μl aliquots of the samples were expelled on to planchettes for counting. The ^{14}C inulin samples and the canal fluid samples were counted on a Nuclear Chicago planchette counter with a 32 per cent efficiency for counting ^{14}C. The length of worm between the ligatures was measured after relaxing the worm in cold saline for a few minutes then laying it on mm graph paper. The volume of the canal system for the length measured was given by:

$$\text{Volume } (\mu l) = \frac{\text{counts/min in injected solution}}{\text{counts/min per } \mu\text{l. in collected fluid}}.$$

All volumes were corrected to μl./100 mm length for direct comparison between worms.

RESULTS

Little change in speed or frequency of peristalsis was observed when the oxygen tension was raised by 1 per cent increments to 4 per cent, as shown in Table 1. With the pO_2 constant at 4 per cent and the pCO_2 raised by 1 per cent increments, a

TABLE 1—SPEED AND FREQUENCY OF PERISTALSIS UNDER VARYING O_2 AND CO_2 TENSIONS

Gas tension (N_2 to 100%)	Speed (mm/sec)	No. readings	Frequency (bulges/min)	No. readings
1% O_2	2·7 ± 0·14	16	14·5 ± 0·76	6
2% O_2	2·3 ± 0·12	19	14·4 ± 0·72	7
3% O_2	2·2 ± 0·15	16	14·2 ± 0·48	6
4% O_2	2·3 ± 0·14	15	14·4 ± 0·81	5
4% O_2 : 2% CO_2	2·1 ± 0·09	16	13·8 ± 0·37	5
4% O_2 : 3% CO_2	1·8 ± 0·13	13	11·4 ± 0·40	5
4% O_2 : 4% CO_2	1·4 ± 0·15	8	10·0 ± 1·24	6
4% O_2 : 5% CO_2	2·0 ± 0·35	5	10·7 ± 0·88	3
4% O_2 : 6% CO_2	—*		—*	
4% O_2 : 7% CO_2	1·4 ± 0·23	3	—*	

Values given as mean ± standard error.
*No reading possible.

slight decrease in both speed and frequency were observed up to 5 per cent (Table 1). When the level was raised to 6 per cent the activity of the worm changed completely, the peristaltic movements being superseded by a ripple-motion passing rapidly along the strobila. Under the conditions of this experiment it was not possible to measure the ripple speed. Beyond 6 per cent there was a return to peristalsis, though it was more intermittent than before. All the readings in Table 1 were taken on posterior sections of worms: the anterior showed no variation in its peristaltic activity under any gas tension used, but the activity was too rapid to be measured by the above technique.

An anaesthetized rat was carefully opened and the worms observed *in situ* in the intestine. It was observed that the worms moved in a manner similar to that described above for worms in 4% O_2: 6% CO_2: 90% N_2—the anterior made rapid peristaltic movements, while the posterior showed a ripple-motion travelling backwards along the worm. Unfortunately, the effects of the anaesthetic and of air on the worms could not be assessed, but it was noticed that the peristaltic activity of the rat intestine had ceased under anaesthetic.

In a preliminary experiment for the measurement of the speed of fluid flow in the protonephridial canals, Aquadag (colloidal graphite) was suspended in saline and injected into the canals. The black particles could easily be seen under the binocular microscope and their progress down the worm was noted. At the crest of a peristaltic wave (i.e. in the widest part of the bulge) the canals were seen to open wide, but in the trough of the wave they were constricted. As the peristaltic bulges moved down the worm the canal fluid was carried along with them (see Fig. 2).

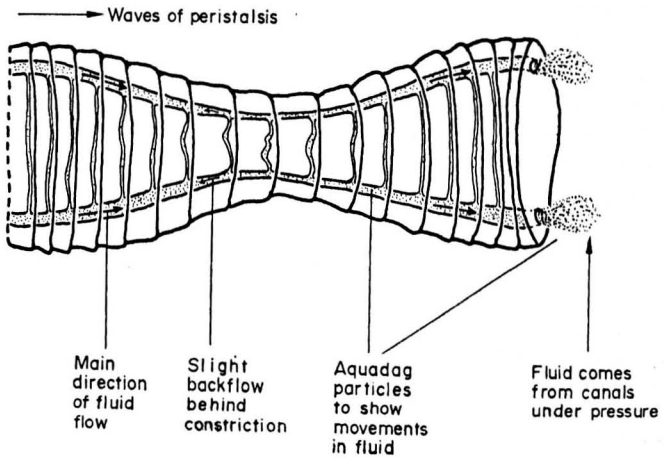

FIG. 2. Diagram of the posterior end of *H. diminuta* to show the movement of fluid in the canals in relation to peristaltic activity.

At the posterior end, the opening of the canals were not visible in a resting phase, but when a bulge reached the end the edges of the proglottid were pulled apart and the canal openings exposed. Small quantities of fluid were released from them in

droplets, 1 droplet/bulge, so the flow was discontinuous, dependent upon the rate of peristalsis.

The speed of fluid flow was measured as the time taken for the graphite particles to be moved from the point of injection to their first appearance in a droplet released from the posterior. This gave the maximum speed. The peristaltic speed was measured in the same worm and from these two speeds the efficiency of the system was calculated. It was not possible to measure the mean canal fluid speed because the particles became evenly distributed throughout the canals. The results measured are given in Table 2 for two gas tensions. Four worms were used

TABLE 2—SPEED OF FLUID FLOW, SPEED OF "PERISTALSIS" AND PERCENTAGE EFFICIENCY OF "PERISTALSIS" IN CAUSING FLUID FLOW

Gas tensions	Fluid speed (mm/sec)	"Peristalsis" speed (mm/sec)	Efficiency (%)
4% O_2 : 4% CO_2 : 92% N_2	0·12 ± 0·02	0·66 ± 0·04	18·4 ± 3·49
4% O_2 : 6% CO_2 : 90% N_2	0·56 ± 0·25	1·04 ± 0·14	50·8 ± 11·63

Peristalsis changes to a ripple motion at 6% CO_2. Values are mean ± standard error.

for each gas mixture. The speed of peristalsis under 4% O_2 : 6% CO_2 : 90% N_2 in this experiment was actually the speed of the ripple movements previously described. It was possible to measure the time for the ripples to move 5 mm with an accurate stop watch. The difference between the speed of peristalsis observed in this experiment and the speeds observed in the previous experiments at the same gas tensions can probably be explained by the slightly different conditions present in this experiment. Although a Perspex lid was kept on the worm bath most of the time, and the gases bubbled through the reservoirs were in the same proportions as before, it was not possible to ensure that gases did not diffuse into or out of the bathing medium during its passage through the warm bath. It was necessary, therefore, to measure the fluid flow and peristalsis in the same worm to obtain a true comparison for calculation of efficiency.

Pressure was measured in the canals of four worms for each of two gas mixtures: the results are given in Table 3. Student's *t*-test on the results showed that the means were not significantly different from each other at the 5 per cent level of

TABLE 3—PRESSURE IN THE CANALS

Gas tension	Pressure (cm H_2O)	No. readings
4% O_2 : 4% CO_2 : 92% N_2	2·8 ± 0·3	16
4% O_2 : 6% CO_2 : 90% N_2	2·1 ± 0·3	15

Values are mean ± standard error.

probability ($t = 1{\cdot}48$ with 29 degrees of freedom). This can probably be explained by the dependence of fluid flow upon peristalsis, and that the insertion of a micropipette causes the worm to become stationary around that point. The results, however, indicate that a positive pressure, greater than capillarity, is exerted towards the posterior of the worm. Capillarity was in the range 7–11 cm H_2O depending on the diameter of the micropipette, but constant for each.

The approximate counts/min from the ^{14}C-inulin injected were 3000, and from the fluid sampled after a minute in the worm there were 150 counts/min per μl. From this it was calculated that the mean volume ($\pm$ S.E.) of the protonephridial canal system in a 100 mm length from the posterior end of a tapeworm was 19·9 $\pm$ 3·5 μl.; seven worms were used for the calculations, the range was 9–37 μl.

Correlating the values for volume with that for speed of fluid flow, determined at a gas tension of 6% CO_2 : 4% O_2 : 90% N_2, it is possible to calculate the approximate mean fluid output and the minimum fluid output from the protonephridial system. The speed of flow was 0·56 mm/sec with a 50 per cent efficiency. At this speed, the time necessary for a particle to move 100 mm would be 2·98 min. The mean volume of the canals in a piece of worm 100 mm long is 19·9 μl., therefore, assuming 100 per cent efficiency, 19·9 μl. will be emptied from the worm in 2·98 min. The fluid output will be 6·7 μl./min. But the system is only 50 per cent efficient, so the fluid output will be 3·4 μl./min. The minimum output can be calculated similarly, using 9 μl. (the minimum measured volume) as the canal volume of a 100 mm length of worm. Nine μl. will be emptied in 2·98 min, therefore the output is 3·0 μl./min at 100 per cent efficiency. Allowing for only 50 per cent efficiency, the minimum fluid output from the worm is 1·5 μl./min.

DISCUSSION

As the pCO_2 is increased, the consequent increase in the speed of peristalsis until only ripple movements are observed can probably be explained by the importance of CO_2 in the metabolism of *H. diminuta*. Fairbairn *et al.* (1961) showed that in 5% CO_2 : 95% N_2 *H. diminuta* increased its glucose utilization by seven to eight times that in N_2 alone, and the CO_2 was found to stimulate carbohydrate metabolism generally. The reactions involving CO_2-fixation are essential for forming the four-carbon dicarboxylic acids (oxaloacetic, malic, fumaric and succinic) of the Krebs's cycle, which is incomplete in *H. diminuta* (Scheibel & Saz, 1966). The formation of malate from pyruvate is accompanied by oxidation of $NADPH_2$ to NADP and the subsequent conversion of malate to succinate through the intermediate fumarate causes oxidation of the phosphopyridine nucleotides reduced during glycolysis. The presence of CO_2, therefore, stimulates the synthesis of the Krebs's cycle intermediates and hence the production of high-energy phosphates necessary for metabolic processes. The increase in activity as the CO_2 tension rises suggests an increase in available energy and so is consistent with this theory.

A slight positive pressure was recorded in the canals acting towards the posterior. This may assist the flow of fluid down the smaller collecting tubules leading

from the flame cells by causing a slight suction at their junctions with the large canals.

The higher rate of metabolism caused by 6% CO_2 must produce an increase in the rate of formation of excretory products. The more rapid peristalsis at this CO_2 level hastens the expulsion of fluid containing these excretory compounds, such as lactate, urea and ammonia (Webster & Wilson, 1970). The concentrations of these compounds are 9·5, 0·5 and 0·04 μg/μl. respectively. In conjunction with the mean fluid-output rate of 3·4 μl./min, and the minimum fluid-output rate of 1·5 μl./min, these concentrations can be used to calculate the approximate mean and minimum rates of excretion of the compounds from the canals. In order to standardize the excretory rate to μg/mg dry wt. per hr, the dry weight of a whole adult worm was taken to be 100 mg (unpublished data). The calculated excretory rates are given in Table 4. Moss (1970) incubated *H. diminuta* under anaerobic conditions and

TABLE 4—EXCRETORY RATES OF LACTATE UREA AND AMMONIA (μg/mg dry wt. worm per hr)

Compound	Mean rate	Minimum rate
Lactate	19·4	8·6
Urea	1·02	0·45
Ammonia	0·08	0·04

analysed the ammonia nitrogen accumulated in the medium after a known period of time. The result was 23 μg ammonia N excreted/g worm per 30 min. If the mean excretory rate calculated in this paper is expressed in similar units, only 6·2 μg ammonia N/g worm per 30 min are released. Fairbairn *et al.* (1961) and Read (1956) give values for the excretory rate of lactate and Campbell (1963) gives a value for the excretion of urea. The calculations necessary to change the rates expressed in this paper into the units given by other authors would be based on too many unknown factors, so making such comparison of little value. It seems possible that the larger molecules, lactate and urea, are excreted by the protonephridia, while the smaller molecule, ammonia, can diffuse freely across the tegument. This would explain the lower excretory rate for ammonia when compared with the result of Moss.

SUMMARY

1. Fluid flows in the protonephridial canals of *H. diminuta* from anterior to posterior.

2. The fluid movement is produced by the propagation of peristaltic waves also moving anterior to posterior.

3. The nature and rate of peristalsis are dependent upon the pCO_2 of the medium. Up to a certain pCO_2 there is an increase in peristalsis, and hence an increase in fluid flow.

4. The fluid in the canals contains excretory products; the system is probably

an important route for the elimination of lactate, urea and, to a lesser extent, ammonia.

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Key Word Index—Hymenolepis diminuta; protonephridial fluid; fluid flow; excretion; peristalsis.

Absorption of glucose, lactate and urea from the protonephridial canals of *Hymenolepis diminuta*.

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ABSTRACT

1. Glucose, lactate and urea are absorbed from the protonephridial canal fluid of Hymenolepis diminuta.

2. Absorption of glucose is carrier-mediated. At normal concentrations in the fluid, 80% of the glucose is absorbed.

3. Absorption of lactate is passive: At normal concentrations, 40% is absorbed.

4. Absorption of urea is probably passive: At normal concentrations, 50% is absorbed.

5. The absorption rates of these compounds compare favourably with their rates in the mammalian kidney tubule. It is suggested that a filtration-resorption mechanism operates in the protonephridia.

INTRODUCTION

The cyclophyllid tapeworm, Moniezia expansa, has protonephridial tubules and collecting ducts lined by epithelium with bead-like microvilli on its luminal border. The cytoplasm of the epithelium contains numerous mitochondria, extensively developed Golgi complexes and endoplasmic reticula (Howells, 1969). Another cyclophyllid, Hymenolepis diminuta, contains the metabolic end-products lactate, urea and ammonia in the fluid from the longitudinal protonephridial canals (Webster & Wilson, 1970). Glucose and amino-acids are also present. It seems likely that the lining epithelium of the protonephridial canals is active in modifying the fluid by either absorption or secretion of some of these compounds.

The purpose of the study reported here was to investigate the absorption of glucose, lactate and urea from the canals of H. diminuta. The principle of the method used was injection into the canals of radioactively labelled inulin and, in the same solution, a test compound labelled with a different radio nuclide. After a one minute incubation, fluid was sampled from the canal and its radioactive constituents counted. Inulin was used to measure the dilution of the injected solution by the canal fluid. It is not metabolised, nor absorbed, it is not toxic and has a low molecular weight. The radioactive labels used were ^{14}C and ^{3}H (T). Their decay products have different energy levels and so can be

counted simultaneously in a solution, using a scintillation counter. The ratio of ^{14}C-inulin to ^{3}H-test compound in the injected solution can thus be compared with their ratio in the canal fluid after a known period of incubation. Since it is unlikely that inulin would be absorbed, any change in the ratio would be due to absorption of the test compound. The radioactive compounds used were of high specific activity so that the actual quantity of labelled compound present made a negligible contribution to the concentration of the solution. They were therefore added as required to a solution containing a known concentration of non-radioactive glucose, lactate or urea. This non-radioactive solution acts as a carrier for the radioactive compounds, at the same time allowing the concentration of the test material to be varied as required.

MATERIALS AND METHODS

Mature worms were removed from the rat ileum into Hank's saline as described in an earlier paper (Webster & Wilson, 1970). The apparatus used for all micro-injection into and fluid sampling from the canals was the same as that described by Webster & Wilson, the only variation being the gassing of the saline in the worm bath with 5% CO_2: 4% O_2: 91% N_2, to simulate intestinal gas levels. These gas tensions were chosen because they cause a fairly rapid peristaltic rate down the worm and so induce fluid flow in the canals (Webster, 1971).

Injection micropipettes were calibrated approximately. A 1 µl aliquot of water was drawn into the micropipette and the meniscus marked with a felt-tipped pen. The water was expelled and the micropipette dried. The radioactive solution to be injected was then drawn up to the mark. A sample of the solution was expelled into 10 ml of Bray's solution (Bray, 1960) in a scintillation vial for counting. This was repeated to give triplicate samples to work out the initial ratio.

A piece of worm approximately 100 mm long was selected from the posterior end of the whole tapeworm and the anterior part of this piece pinned to the cork shelf of the worm bath. A droplet of oil was injected into the anterior in order to block the canals on each side. This block extended along 4 or 5 proglottides. The micropipette was then removed and exchanged for a calibrated one, into which a 1 µl sample of the radioactive test solution was drawn. The tip of this micropipette was inserted into the canal, through the oil droplet and the solution expelled into the canal fluid. A further oil droplet was injected to prevent back leakage as the micropipette was withdrawn. Ligatures were tied round the worm, one at a point posterior to the opening made by the micropipette, another at the posterior tip of the piece of worm. These two ligatures, made with suture thread, prevented any loss of radioactive materials from the worm while it was left for one min in the saline bath. This allowed the radioactive solution to flow the full length of its canals and mix with the fluid already present.

During the incubation period, the calibrated micropipette was exchanged for an uncontaminated one. The piece of worm was then pinned to the cork shelf by its posterior end and fluid sampled from the canals. Samples were expelled into microtest tubes together with a droplet of oil to seal the fluid from the air. Sampling time was approximately 4 min, an unavoidable source of variability between worms. The actual time that some of the injected sample was in the worm was between one and five min.

The piece of worm was put into predried, preweighed, porcelain crucibles to be dried at 105°C overnight for determination of its dry weight. One µl aliquots of the fluid sample were taken, using Drummond Microcaps, and mixed with 10 mls Bray's solution. All fluid and calibration samples, together with vials for counting background radiation, were counted on a Nuclear-Chicago Unilux II scintillation counter for 10 min. Nine or ten separate pieces of worm were used for each concentration of all compounds tested.

Absorption of glucose from the canals was tested by injection of a solution containing 0.1 µCi of D-glucose-6-T, 0.0065 µCi of inulin (carboxylic acid - C14) and 0.5 - 8 µg carrier glucose per µl. Inhibition of glucose absorption by phloridzin was tested using the same concentrations of ^{14}C-inulin and ^{3}H-glucose, together with 2 µg carrier glucose and 10^{-5}, 10^{-4} and 10^{-3} M phloridzin per µl. Inhibition of glucose absorption by its isomer, galactose, was tested by replacing the phloridzin

with 8 µg galactose per µl.

It was not possible to obtain commercially made ^{3}H-lactate or ^{3}H-urea to use in conjunction with ^{14}C-inulin. Instead, ^{14}C-lactate and ^{14}C-urea were used with ^{3}H-inulin. Before using the ^{3}H-inulin in experimental work, however, a check was carried out using 0.15 µCi inulin-T and 0.003 µCi of the ^{14}C-inulin per µl of solution. Their ratio did not change after 1 min incubation in the canals of the worm. (Comparing the mean ratio obtained with a theoretical ratio of 100, $\underline{t}$ = 1.57 with 10 degrees of freedom; there was no significant difference at the 5% level). This meant that the two types of inulin could be used interchangeably without affecting the results.

Absorption of lactate and urea from the canals was tested using 0.1 µCi inulin-T, 0.002 µCi of either L-lactic acid-C14 (u), sodium salt, or urea-C14, together with 1-16 µg carrier sodium lactate or 0.5-2 µg urea as appropriate.

All injected solutions were made up in Hank's saline (minus 0.1 g glucose/100 ml). Radio chemicals were obtained from Radiochemicals Ltd., Amersham, Bucks., England.

The calculations are based on the assumption that, if no absorption occurred from the canals, the dilution of the inulin and the radioactive test will be the same. If any of the radioactive test is absorbed, the same proportion of the non-radioactive moiety (or carrier) will also be absorbed, since no distinction will be made by the canal cells. The percentage of, for example, glucose remaining in the canal after the incubation is given by:

$$\frac{\text{Counts/min } ^{14}\text{C-inulin injected}}{\text{Counts/min } ^{14}\text{C-inulin in sample}} \times \frac{\text{Counts/min } ^{3}\text{H-glucose in sample}}{\text{Counts/min } ^{3}\text{H-glucose injected}} \times 100.$$

From this the percentage of labelled glucose absorbed and hence the µg total glucose absorbed per mg dry weight of worm tissue can be derived. The calculations are similar for ^{3}H-inulin with ^{14}C-lactate or ^{14}C-urea.

RESULTS

The results for the absorption of glucose from the protonephridial canals are shown in Fig. 1., each point representing the mean ± standard error for 10 replicates. When 0.5 µg glucose/µl was injected, 82.9 ± 4.6% of that injected was absorbed; when 8 µg/µl were injected, 41.4 ± 5.1% was absorbed. The curve of the graph is possibly indicative that the mechanism of uptake is becoming saturated, suggesting carrier-mediated transport. The addition of phloridzin to injection solutions containing 2 µg glucose/µl, of which 78.2 ± 5.3% would normally be absorbed, confirmed this suggestion, as the results shown in Table 1 illustrate. Phloridzin was chosen because, at low concentrations, it is a specific inhibitor of the glucose carrier (Parsons et al. 1958). At higher concentrations (10^{-3}M and above), it interferes with oxidative phosphorylation mechanisms of the cell and so abolishes any type of active transport. Since the volume of the canals in a 100 mm length from the posterior end of the worm is approximately 20 µl (Webster, 1971), any concentration of a compound added would be diluted by one in twenty;

FIG.1. Absorption of Glucose from the Canals

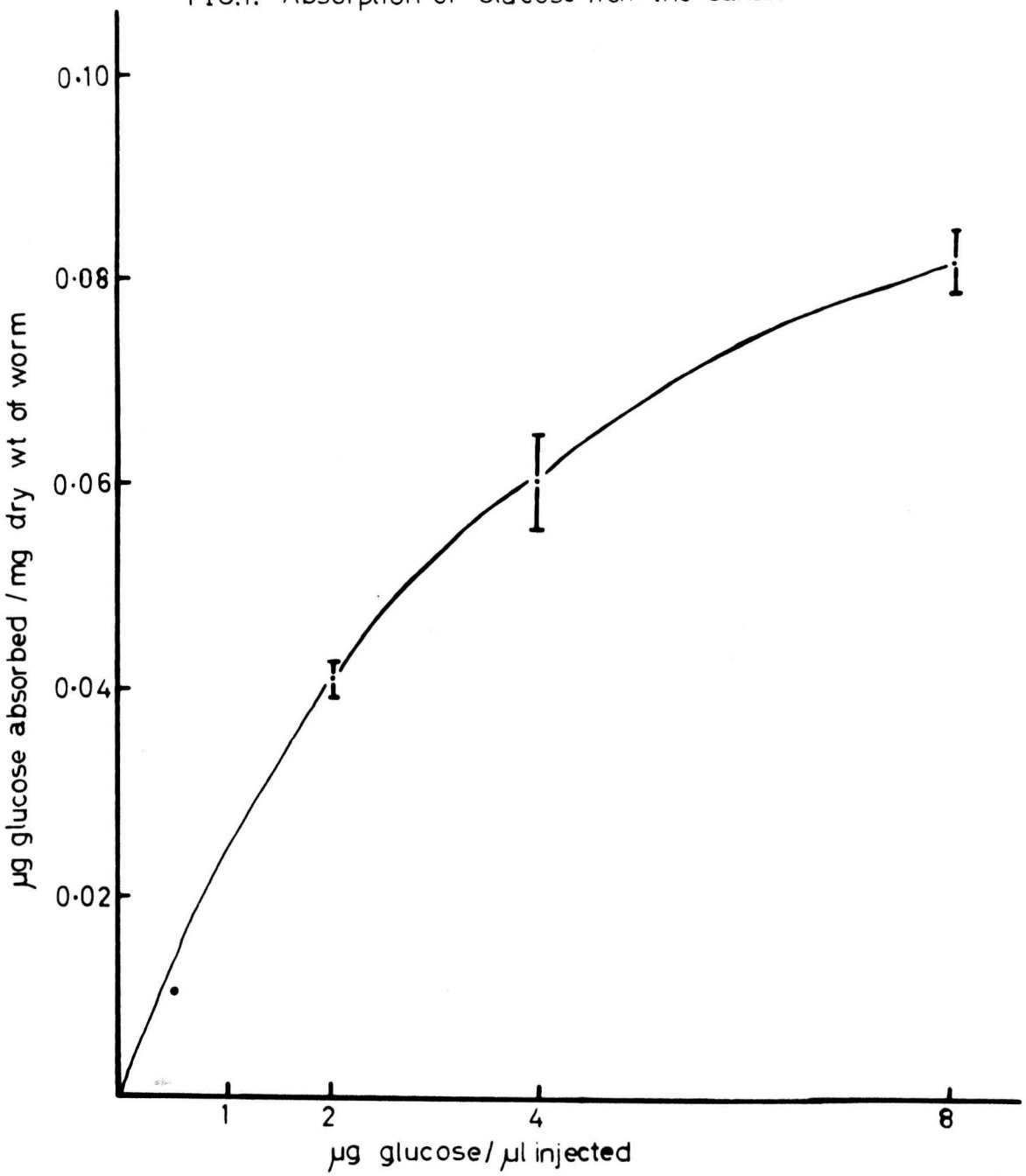


TABLE 1. The effect of phloridzin on absorption of glucose from the protonephridial canals. 2 µg glucose/µl were injected.

Concentration of phloridzin (M)	µg glucose absorbed per mg. dry wt.	Inhibition %
0	0.042 ± 0.003	-
10^{-5}	0.030 ± 0.003	28.6
10^{-4}	0.030 ± 0.004	28.6
10^{-3}	0.021 ± 0.003	50.0

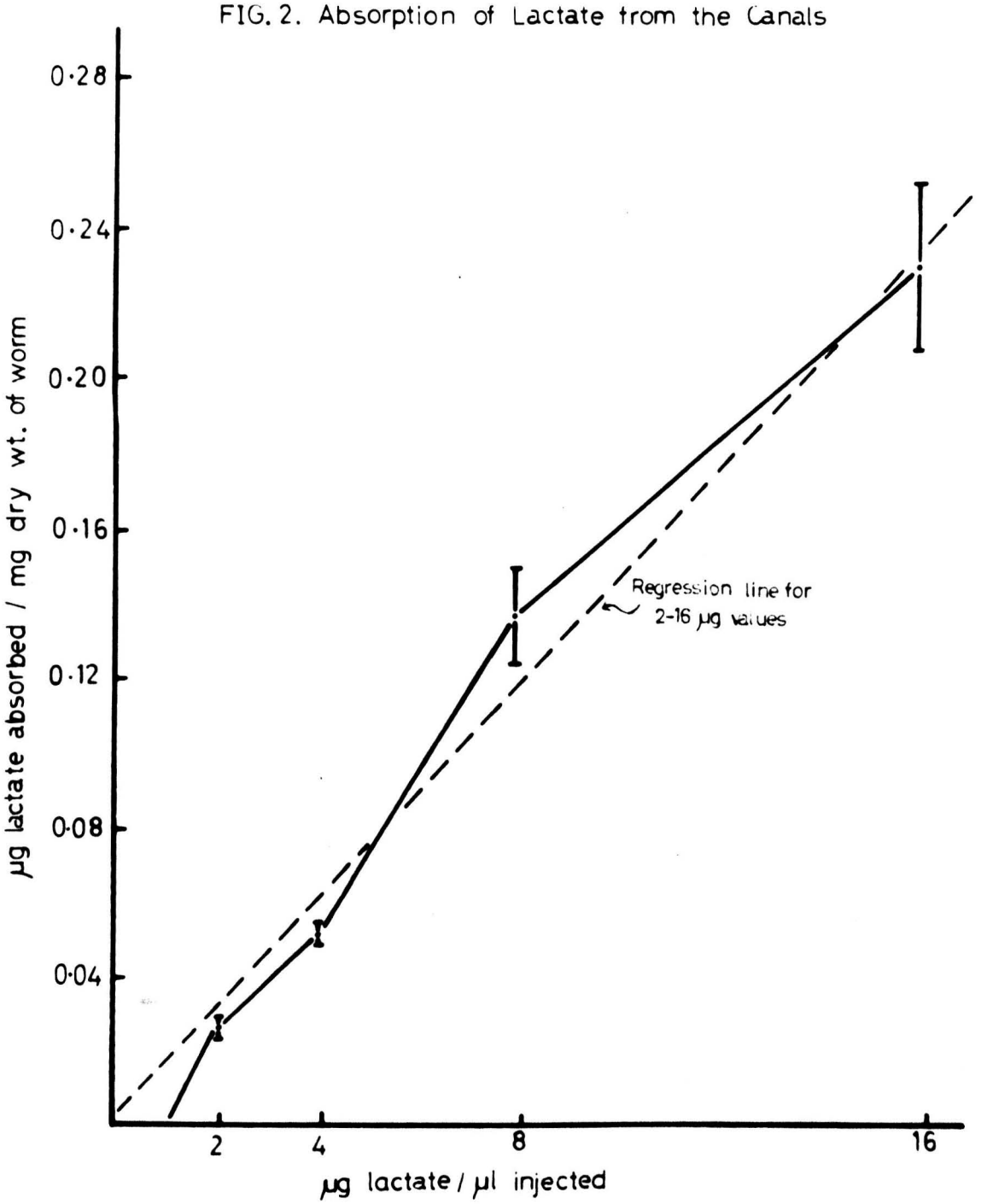
Values are mean ± S.E. for 10 worm pieces.

i.e., if 10^{-3}M phloridzin were injected, it would be diluted to 0.5×10^{-4}M during incubation. Thus the concentrations used in these experiments were never high enough to affect the metabolism of the cells but were sufficient to inhibit glucose transport. In all cases the µg glucose absorbed per mg dry weight of worm were significantly different at the 5% level from the value obtained in the absence of phloridzin. Glucose transport from the protonephridial canals is therefore probably carrier-mediated.

In the presence of 8 µg galactose/µl, the absorption of glucose from a 2 µg/µl concentration injected was 79.5 ± 3.6% (or 0.038 ± 0.005 µg/mg dry wt.) compared with 78.2 ± 5.3% (or 0.042 ± 0.004 µg/mg dry wt.) in the absence of galactose. These values are not significantly different at the 5% level ($t = 0.68$, with 17 degrees of freedom).

The results for the absorption of lactate are shown in Fig. 2. The points shown represent mean ± S.E. for 10 worms. At the injected concentration of 1 µg lactate/µl, the values obtained for the comparison of the ratios were not significantly different at the 5% level from those obtained by testing ^{3}H-inulin against ^{14}C-inulin. There was therefore no absorption of lactate at this level. At the other concentrations used, 37 - 43% absorption occurred. A regression line ($y = 0.0143x + 0.003$) was drawn for the 2-16 µg/µl range, showing clearly (Fig. 3) that lactate absorption is directly proportional to concentration, i.e. that absorption is passive over the range tested.

FIG. 2. Absorption of Lactate from the Canals



The results for the absorption of urea are given in Table 2. The experiments were carried out with worms from rats fed ad libitum, but the results showed no apparent increase in absorption rate with respect to injected concentration. Since differences were noticed between groups of worms taken from different rats, an experiment was performed to discover whether urea absorption depends upon the metabolic state of the worm. Two rats having worms of identical ages were taken, one was starved overnight and the other fed ad libitum. Both were sacrificed at precisely 10.0 a.m. (on consecutive days) and worms injected with 1.5 µg urea/µl. The results for the worms from the two rats are shown in Table 3. The results are significantly different at the 5% level and suggest that absorption of urea depends upon the gradient between canal lumen and the cells of the canal wall.

DISCUSSION

The normal level of glucose found in the longitudinal canals of H. diminuta is 1.5 µg/µl (Webster & Wilson, 1970): at this level 80% of the glucose present in an injected test solution is absorbed. The carrier mechanism does not become saturated until in excess of 8 µg glucose/µl are present in the fluid. In the mammalian kidney, the threshold concentration for plasma glucose (the level at which it first appears in the urine because the absorption carrier is saturated) is 180-200 mg/100 ml (Pitts 1968).

TABLE 2. Urea absorption at different concentrations injected

Injected (µg/µl)	Absorbed (µg/mg dry wt. worm)	% Absorption
0.5	0.008 ± 0.001	51.2 ± 4.7
1.0	0.020 ± 0.002	43.7 ± 4.9
1.5	0.012 ± 0.003	25.0 ± 5.5
2.0	0.022 ± 0.005	34.8 ± 5.7

Values are Mean ± S.E. for 10 worm pieces.

TABLE 3. Urea absorption in fed and starved worms, 1.5 µg/µl injected

	Absorbed (µg/mg dry wt. worm)	% Absorption
Fed worms	0.013 ± 0.003	29.0 ± 4.4
Starved worms	0.049 ± 0.006	52.2 ± 2.4

Values are Mean ± S.E. for 6 worm pieces.

This is the same as 1.8 µg/µl, suggesting that the tapeworm canals, with their much higher saturation level, can absorb larger quantities of glucose than the mammalian kidney tubules.

The results obtained here showing galactose to have no effect on glucose absorption are similar to those of Phifer (1960), who found that galactose at twice the glucose concentration present did not inhibit glucose uptake across the tegument. Read (1961), using galactose concentrations in excess of five times that of glucose, demonstrated competitive inhibition of glucose uptake across the whole worm. Since the absorptive mechanisms of the canals in the worm are likely to be similar to those in its tegument, perhaps an even higher galactose level than the one used might have inhibited glucose transport in the canals.

The absorption of lactate is here shown to depend on the concentration present in the fluid, suggesting passive uptake at the levels tested. Since the range tested exceeded the level in the canal (9.5 µg/µl, Webster & Wilson, 1970) it is probable that uptake normally occurring is passive. At the canal concentration, 37 - 42% of the lactate will be absorbed.

The absorption of urea, appears to depend on the concentration gradient between canal lumen and the cells lining the canal. This is based on the hypothesis that worms from a starved rat will not be metabolising protein and amino acids as rapidly as those from a fed one, hence the urea concentration in the tissues of the starved worms will be lower than the urea concentration in the tissues of the

fed worms. If an identical urea concentration were then injected into the canals of both types of worm, it would be expected that the concentration gradient between canal lumen and canal cells would be greater in the starved worms than in the fed ones. Since the rate of passive diffusion of a compound depends upon the concentration gradient, more urea ought to diffuse out of the canals of the starved worm and into the tissues than out of the canals of the fed one. The results of the experiment showed that this, in fact, happened. The starved worm absorbed almost twice as much urea from the canals as the fed one. This suggests that urea absorption depends upon the concentration gradient and is probably passive over the range tested. At the concentration normally found in the canals (0.5 µg/µl Webster & Wilson, 1970) 45 - 55% of the urea will be absorbed. Comparison of this with similar data for the mammalian kidney (Pitts, 1968) shows the system to be less inefficient than it appears. The kidney tubules reabsorb 40% of the urea filtered through the glomerulus. The mechanism of uptake in the kidney is passive.

Similar comparison cannot be made for lactate since it is normally metabolised further by the mammal, but in a partially anaerobic environment it is not metabolised by the worm.

Previous work (Webster, 1970) has shown that H. diminuta has little control over its salt and water content. The protonephridial system, therefore, cannot have an osmoregulatory function. Because the canal fluid contains the metabolic end-products,

lactate, urea and ammonia, Webster & Wilson (1970) postulated that the protonephridia have an excretory function. Morphological evidence led Wilson (1969) to conclude that the flame cell is an organ of filtration. The presence of glucose, amino acids and more sodium than potassium, in the canals of H. diminuta (Webster & Wilson, 1970) indicate a probable interstitial origin for the fluid. It is likely that fluid bathing cells would have a higher concentration of sodium than potassium and would also contain nutrients such as glucose and amino acids. A direct filtration of this fluid through the flame cells would explain the relative concentration of such compounds in the canal fluid. If the latter were secreted by cells, the concentration of potassium would be higher than sodium and, probably, there would be little secretion of glucose or amino acids.

Webster (1971) showed the rate of loss of lactate and urea from the protonephridial canals of H. diminuta to be 19.4 and 1.02 µg/mg dry weight per hr. respectively, but the absorption factor had been overlooked. It is not possible to calculate the rate of excretion from the canals because it is not known whether the concentrations measured in the canal fluid represent a steady state between influx from flame cells and efflux back into the tissues. The main problem in studying tapeworm excretion is that, although absorption from the canals can be examined, it is not possible to look at differential distribution of compounds between intracellular fluids and interstitial fluids. A means of distinguishing between these two compartments is needed before the fluid dynamics of the tapeworm can be properly understood.

SUMMARY

1. Glucose is absorbed from the protonephridial canals of H. diminuta by a carrier-mediated mechanism. 80% of the glucose normally present is absorbed.

2. Lactate and urea are probably absorbed from the canals by passive means, 40 and 50% respectively being absorbed at normal canal concentrations. The absorption of urea compares favourably with that of the mammalian kidney tubule.

3. The protonephridia of H. diminuta are postulated as having an excretory function operating on a filtration followed by resorption basis.

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Key Word Index - Hymenolepis diminuta; protonephridial canals; absorption; glucose; urea; lactate.

CONCLUSIONS

The experimental results have been discussed at the end of each group of experiments and also with respect to the present knowledge of protonephridia, in the review at the beginning of the thesis. All that now remains is to correlate the conclusions from each experiment and from them form a hypothesis about the physiology of the protonephridial system of H. diminuta.

H. diminuta is unable to regulate either water intake or salt loss when incubated in diluted Hank's saline, both varying in proportion to the dilution factor. Potassium loss is less than sodium loss, suggesting a mechanism for regulating potassium content. If only the sodium content of Hank's saline is decreased, worms lose sodium to the saline in proportion to the decrease, and water enters the tissues. Addition of mannitol to this sodium-decreased saline, to maintain the osmotic pressure at its normal level, prevents water entering the worms, but does not affect sodium loss. Sodium and water movements across the worm surface are therefore related, not to each other, but to the concentration of the environment. If the potassium content of Hank's saline is similarly decreased, worms lose potassium to the saline but the loss is smaller than the sodium loss observed previously.

As water enters the tissues from the saline with decreased sodium content, the sodium concentration of the protonephridial canal fluid falls. The converse is also true, that loss of water from the tissues causes an increase in canal fluid sodium concentration. Similarly, incubation of worms in saline with decreased potassium content causes

a rapid fall in the potassium concentration of the canal fluid. It is suggested that these variations in canal fluid concentrations are directly due to similar changes in interstitial fluid. This would be expected if the flame cells were filtering the interstitial fluid into the tubules, from where the fluid passes into the main canals.

The protonephridial canal fluid contains the metabolic end-products lactate, succinate, urea and ammonia. Salts glucose, amino acids and proteins were quantitatively determined in the fluid, but no lipid was detected. More sodium than potassium is present, again suggesting an interstitial, rather than an intracellular, origin for the fluid. The presence of metabolic end-products may indicate an excretory function for the protonephridia. Lactate, having the highest concentration, is probably the major excretory compound of H. diminuta.

Peristaltic bulges passing along the strobila of the worm push pockets of fluid down both ventral longitudinal canals. At the posterior tip, these pockets are expelled from the worm. The rate of peristalsis is dependent upon the CO_2 tension of the external environment of the worm, the rate increasing with increase in tension, up to 6% CO_2, when a ripple movement supersedes the bulges. This ripple is more efficient at moving fluid down the worm than the peristalsis proper. Since the higher level of CO_2 stimulates the metabolism of H. diminuta (Fairbairn et al., 1961) more excretory products will be formed by the worm tissues. These products will be removed more quickly by the enhanced activity of the worm.

Absorption occurs in the canals. Numerous microvilli line the luminal surface of the canal walls (See Appendix) and these are probably the sites of absorption. Glucose is absorbed by a carrier-mediated process, as shown by the inhibition of absorption in the presence of phlorizin. Lactate and urea are probably absorbed by passive diffusion.

An attempt was made to calculate the rates of excretion of lactate, urea and ammonia. The calculations did not take into account either the occurrence of absorption or the fact that more fluid may still have to enter the canals from proglottides posterior to the sampling point. The values obtained from the calculations may not, therefore, be the real excretory rates of the compounds, but with the data available, it is not possible to calculate them more accurately.

The problems involved in a study on protonephridia are the size of the organs, the absence of a circulatory system in close proximity to them and, in Platyhelminthes, the absence of a coelom or pseudocoelom. Braun et al. (1966) injected a suspension of ferritin into the pseudocoelom of a rotifer and observed that the particles did not pass into the protonephridial system. They therefore postulated that the rotifer flame bulb filters pseudocoelomic fluid. Injection of ^{14}C-inulin into the pseudocoelom, followed by the timing of its rate of entry into the protonephridial system, also allowed the filtration rate of the flame bulb to be estimated. Such direct experiments are highly impracticable using tapeworms because their flame cells filter interstitial fluid, which is virtually inaccessible to micropipettes. The lack of a circulatory system in close

proximity to the flame cells also makes direct experimentation difficult. In mammals, blood flows through the Bowman's capsule and filterable materials pass through the glomerulus into the kidney tubules. Solutions injected into the blood can therefore have detectable effects on the composition of the fluid in the tubules. Since the tapeworm has nothing similar to the blood system, such work is impossible. Platyhelminthes are virtually unique in regulating the interstitial space directly.

Flatworms are also unique in another respect. Their external surface is a living tegument and their internal environment is in direct equilibrium with their external environment across this surface. This equilibrium was demonstrated by the water, sodium and potassium movements in the osmotic experiments. It is maintained by diffusion of materials into, and out of, the worm but, because the diffusion path from the deep tissues is too large, the protonephridial system is necessary to remove materials, particularly excretory products, from these deeper tissues. The evidence for this comes from the position of the flame cells; these are mostly found in the parenchyma tissue deep in the worm.

When studying the protonephridial system, three fluid compartments should be considered. These contain the cellular, interstitial and protonephridial fluids. Other fluid compartments, such as the uterus, are present in the worm but these correspond with the transcellular fluids of mammals (e.g. cerebrospinal, intraoccular or pleural fluids) and have no structural continuity with the protonephridial system. The possible inter-relations of the three relevant fluid compartments are shown in Fig. 1. There are experi-

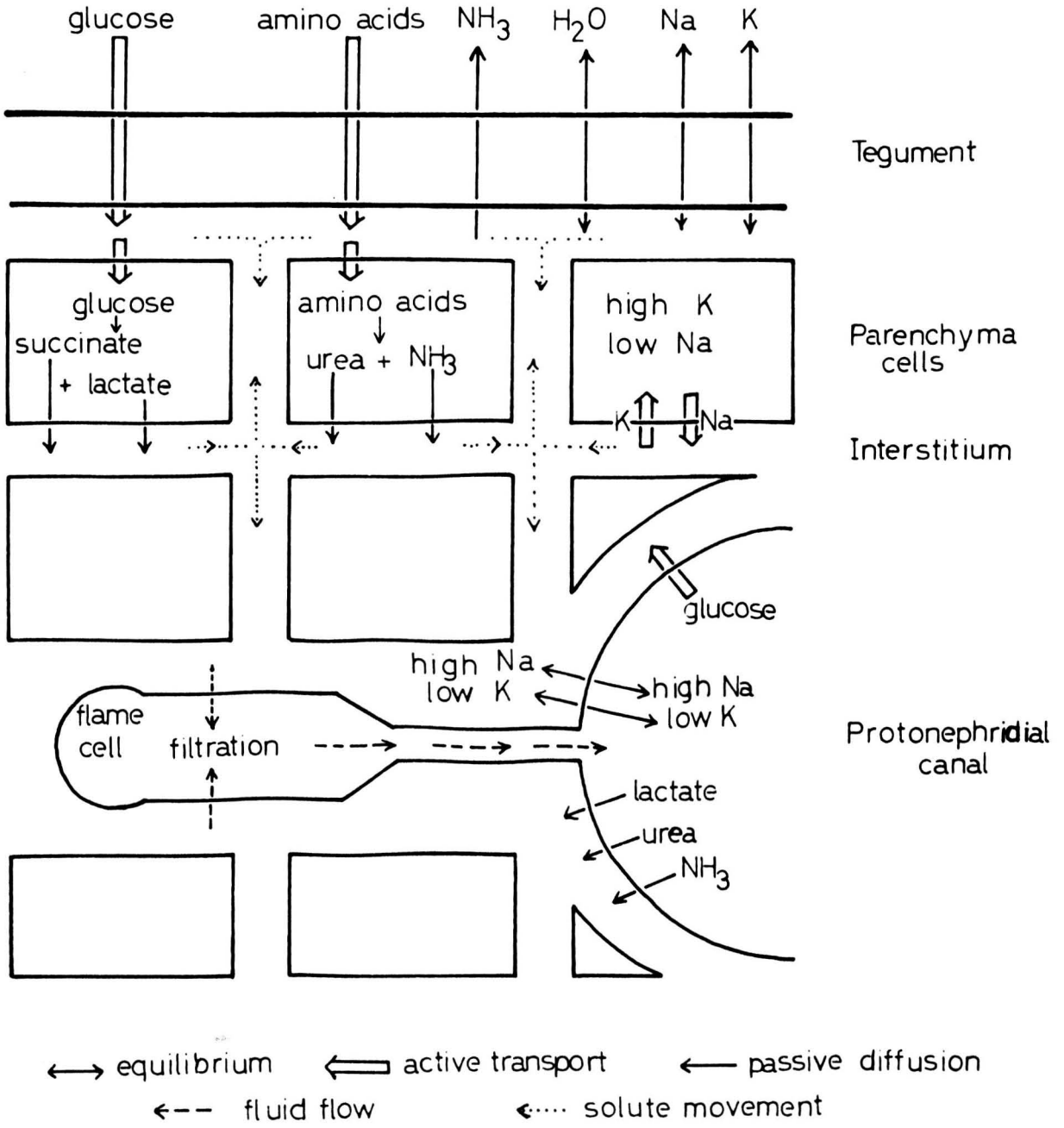

FIG.1. Inter-relations of Fluid Compartments

mentally demonstrated equilibria across the tapeworm tegument for sodium, potassium and water. The tegument actively transports glucose, amino acids and other food materials from its external environment (Read, 1966). These pass into the tegumental cells, then into the interstitial fluid and from there are taken up by other cells of the worm. In these cells, glucose is metabolised to lactate and succinate, which will diffuse out into the interstitial space. Amino acids are used in protein synthesis within the cells, but are also metabolised, leading ultimately to the formation of urea and ammonia. These will also diffuse into the interstitium. Some of the excretory products in the interstitial space will diffuse across the tegument but in the deeper tissues, where the diffusion path is long, many of the metabolic end-products will be filtered across the flame cells. The end-products will then appear in the protonephridial canals. Since glucose, amino acids and other metabolites are probably also present in interstitial fluid, they will also be filtered and hence will be present in the protonephridial canals.

The fluid inside the cellular compartment is usually high in potassium and low in sodium. This compartment is in equilibrium with the interstitial fluid and maintained so by the sodium pump of the plasma membranes. This pump actively conserves potassium in exchange for sodium and other cations. Since the interstitial fluid is presumed to be low in potassium and high in sodium, and it is this fluid which is filtered by the flame cells, these cations are in a similar ratio in the protonephridial fluid. Across the protonephridial canal walls there will be an equilibrium

between luminal and interstitial sodium and potassium. Glucose absorption occurs, returning the metabolite to the interstitial space where it can be absorbed by the metabolising cells. Urea and lactate are absorbed passively, probably forming equilibria with the interstitial fluid. These compounds, however, will not be metabolised, but will either return to the flame cell for filtration or be lost by diffusion across the tegument.

With the techniques available at the present time, much of the fluid dynamics described above are conjectural. It is not practicable to isolate cellular fluid from interstitial fluid or even to isolate a sample of interstitial fluid from a tapeworm. If this were possible, it would be comparatively easy to compare directly the compositions of interstitial and protonephridial fluids. It would then be possible to determine the concentration gradients across the canal walls and hence define the absorption of glucose, urea and lactate as active or passive. So far absorption mechanisms have been defined on indirect evidence only.

Theoretically, stopped-flow perfusion techniques could be applied to tapeworm protonephridial canals. The method would involve injecting a heavy mineral oil into the canals, in order to block the fluid movements along several proglottides. A test solution could then be injected into the centre of the oil block. The oil should prevent the test solution from being contaminated by canal fluid. After a known time period, the test solution could be sampled and analysed to determine the effect of canal activity upon its concentration. Unfortunately, while it is comparatively easy to inject an oil block over several proglottides, it is not as easy to prevent movements of the worm from splitting

the block into several smaller ones. If the worm movements were to split the block while a test solution was in position, the solution would be contaminated with canal fluid and the experiment rendered useless. This stopped-flow technique could probably be developed, with a certain amount of care, and using controlled conditions. The results would give much information about the effect of the canal wall activity on the luminal fluid concentration.

It has been shown that fluid moves from the anterior to the posterior of the worm along the ventral canals. Such fluid movement would make the canals an ideal transport system, carrying compounds from the scolex or juvenile proglottides to the maturing or gravid proglottides. No experimental work has been performed to test this hypothesis, but the canal system does form one of the few continuous structural links along the strobila of the worm.

The nature of protonephridial function is intimately concerned with the fluid dynamics of the tapeworm as a whole organism. Until the fluid compartments within the worm can be examined separately, protonephridial physiology is likely to remain enigmatic.

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APPENDIX

Some observations on the structure of the protonephridial canals of *Hymenolepis diminuta*

INTRODUCTION

Many living specimens of *Hymenolepis diminuta* were observed before performing experimental work. Pieces of worms were embedded in wax, sections of these were cut and examined using a light microscope. The size and position of the protonephridial system of the worm were thus determined.

The protonephridia of the sheep tapeworm, *Moniezia expansa*, were studied by Howells (1969). The tubules and canals of this worm were lined with bead-like microvilli, which were postulated as the possible sites of absorption or secretion of materials. Having shown that *H. diminuta* is able to absorb materials from its longitudinal canals (Webster, 1971), an electron microscopical study was carried out in the hope of finding similar microvilli in this worm.

The observations from living worms and the results of light and electron microscopy are presented here, in order to clarify some of the experimental techniques described in the thesis.

MATERIALS AND METHODS

Living worms bathed in Hank's saline were examined by eye and with the aid of light microscopes. Pieces of worms were fixed in 40% formalin - 0.9% NaCl (1:10), embedded in paraffin wax and 10 μ sections cut on a Cambridge rocking microtome. Sections were stained by the method of Coutelen (1931).

For electron microscopy, 17 day old worms were removed from the rat and rinsed in cold, Hank's saline. Small pieces

were cut from anterior, middle and posterior parts of worms and fixed for 4 hr at 4°C with 6% glutaraldehyde in 0.1M phosphate buffer at pH 7.4, containing 3% sucrose and 2 mM $CaCl_2$. After washing in buffer several times, the pieces were post-fixed in 1% aqueous OsO_4 for 1 hr at 4°C. They were then dehydrated with ethanol at room temperature and stained for 30 min in 1% uranyl acetate in 70% ethanol followed by a further 30 min in 1% uranyl acetate in 90% ethanol. Final clearing was in propylene oxide after which the worm pieces were embedded in araldite which was polymerised at 60°C. Sections were cut using a Huxley ultramicrotome and collected on grids coated with formvar and carbon.

Specimens were stained on the grids with lead acetate (Reynolds, 1963) for 15 min, then washed with consecutive streams of 0.02 N NaOH and distilled water for 30 sec in each. Specimens were examined in an AEI EM6B electron microscope, operating at 60 KV.

Further sections of worm were cut at 1.0 µ and stained on glass slides with toluidine blue. These were examined by the light microscope and photographed with a Zeiss photomicroscope.

RESULTS

The living tapeworm is 45 - 60 cm long, white and fairly opaque. If the worm is positioned on a sheet of glass above a light source, the protonephridial canals can be seen quite easily, because they are more translucent than the rest of the body.

In the anterior of the worm, there are two pairs of longitudinal canals, as shown in Fig. 1. The larger, ventral

FIG. 1. Anterior of H. diminuta

A. Drawing from a wax section through an anterior proglottis

B. Diagram to show canals in the living worm.

Key: dc dorsal canal; sr seminal receptacle; t testis; u uterus vc ventral canal

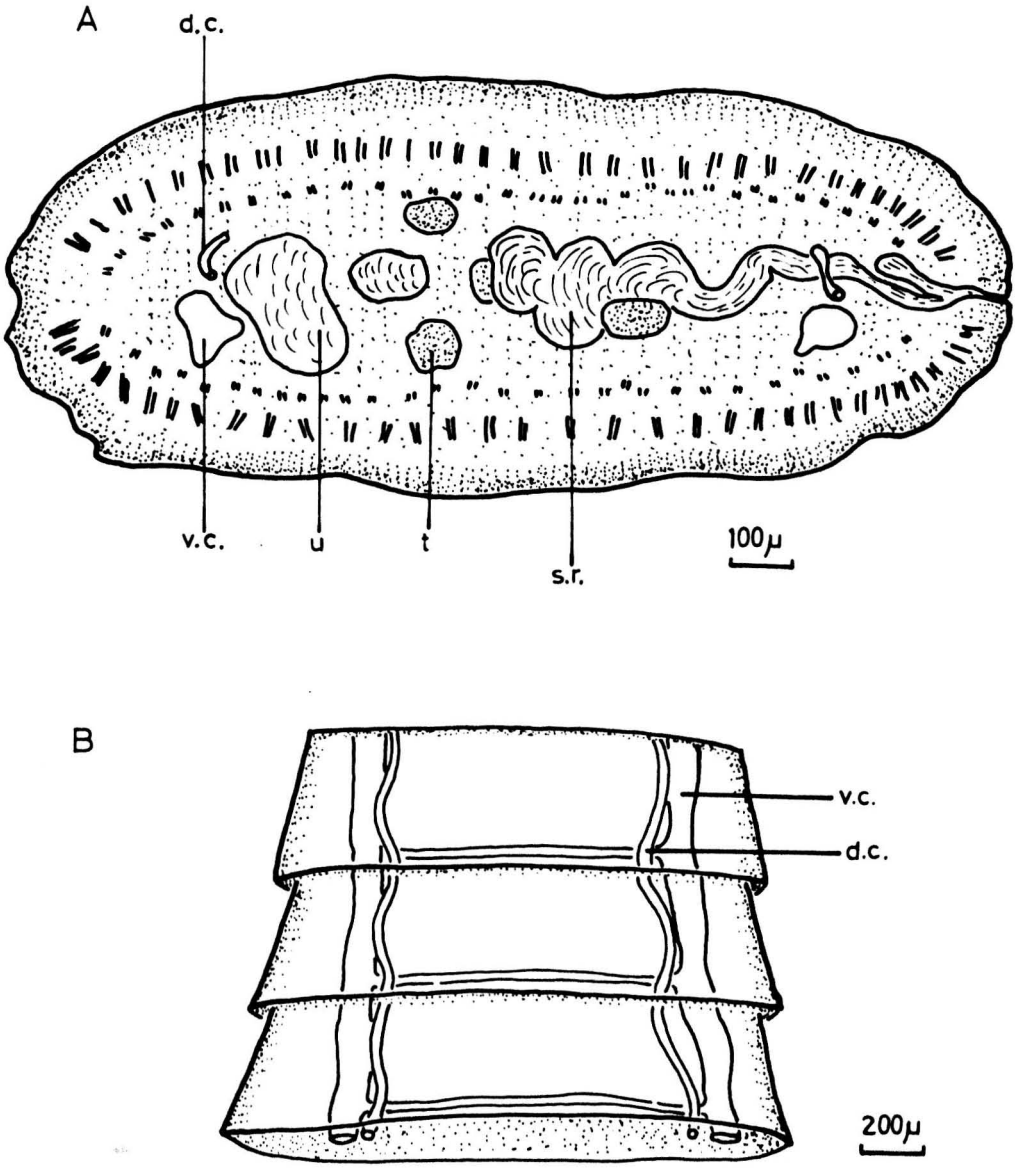


FIG.1. Anterior

canals are connected at the posterior of each proglottis by a transverse canal. Above the ventral canals, and slightly to the interior with respect to the worm, is a pair of undulating dorsal canals. In the anterior, the ventral canals are approximately 100 μ diameter, the transverse canals are 20 - 30 μ diameter and the dorsal canals 40 μ diameter.

In the middle of the worm, both pairs of canals are present. Plate 1 shows that the ventral canal has become oval in cross section, measuring across its two axes approximately 300 μ x 150 μ. The dorsal canal is still 40 μ diameter.

In the posterior of the worm, most organs have degenerated leaving only uterus, which is extensive and full of eggs. The ventral longitudinal canals are present (Fig. 2) and are 300 - 500 μ across their larger axis and 150 μ across their narrower axis. In cross section, their boundary is often irregular because the gravid uterus exerts pressure from all directions upon it. Transverse canals of approximately 100 - 150 μ diameter connect the ventral canals. The dorsal canals have not been found: this may be because they have ended somewhere in the middle region of the worm or they have been obscured by the many branches of the uterus. The ventral canals open at the posterior tip of the worm, emptying their fluid into the external environment.

The only junction observed between the dorsal and ventral canals is in the scolex. A ring vessel lies within the region of the suckers, as shown in Fig. 3. The canals connect individually with this ring and all the vessels are approximately 40 μ diameter in this region.

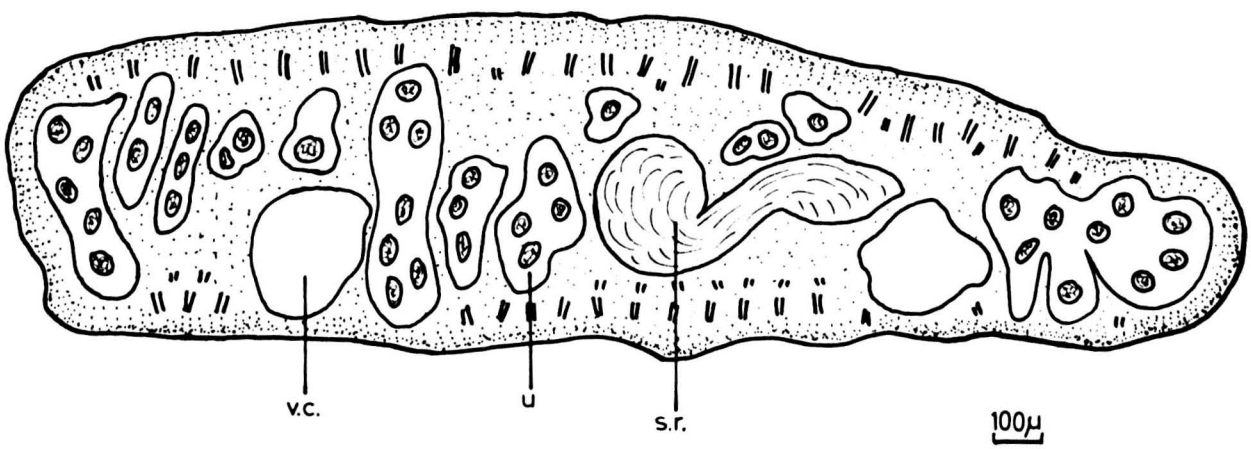
FIG. 2 Posterior of H. diminuta

A. Drawing from a wax section through a posterior proglottis

B. Diagram to show canals in the living worm

Key: As Fig. 1.

A



B

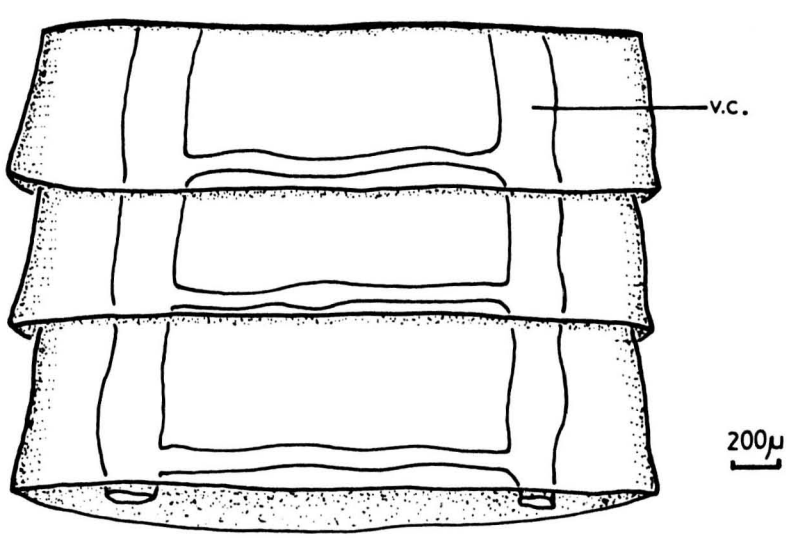


FIG.2. Posterior

FIG. 3 Scolex of H. diminuta
Drawn from living specimens

Key: dc dorsal; rv ring vessel;
vc ventral canal

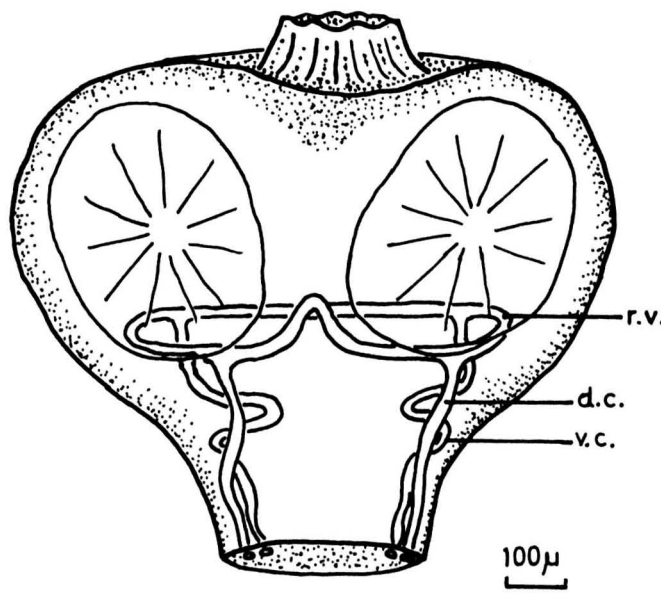


FIG.3. Scolex.

Electron microscopy was used to study the dorsal and ventral canals and also the tubules leading from the flame cells. The dorsal canals are bounded by a wall of cytoplasm having no apparent lateral plasma membranes or desmosomes, suggesting that it is a syncytium (Plate 2). Beneath the canal wall is a thick basal lamella below which the cell bodies of the wall are found. Cytoplasmic channels connect the cell bodies to the wall syncytium. The canal lumen is lined by numerous, closely-packed microvilli. The wall cytoplasm contains granules, ribosomes, mitochondria, endoplasmic reticula and possibly some Golgi apparatus. The cytoplasmic channels contain lamellae and ribosomes. The perinuclear cytoplasm of the cell body contains densely packed ribosomes, some mitochondria and some lamellae. The basal lamella consists of granules and fibrils; these are present in the invaginations of the canal wall (Plate 5A illustrates this).

The ventral canals are similar morphologically to the dorsal canals. The wall cytoplasm is a syncytium; its cell bodies are again below the basal lamella. Within the basal lamella and between the cytoplasmic channels are blocks of muscle tissue running parallel with the axis of the canal. (Plate 4). The canal lumen is lined with microvilli and the opposite surface of the wall has many basal invaginations (Plate 5A). Plate 5B shows the bead-like appearance of the microvilli; they are bounded by a plasma membrane continuous with that of the wall. Many lamellate structures can also be seen in Plate 5B; these are probably parts of the basal invaginations. In the posterior part of the worm, the wall cytoplasm is much thinner, measuring 0.3 μ across instead of 0.6 μ, as in the anterior. The parenchyma tissue

below the canal wall is very much flattened, probably because of pressure exerted by the enlarging uterus.

The flame cell tubules are found undulating through the parenchyma, emptying into either the dorsal or the ventral canal. Plate 7, A & B, show the luminal surface of the tubule walls to be lined with numerous closely-packed microvilli. The wall cytoplasm is electron dense and contains granules, ribosomes and lamellae. In cross section (Plate 7B), the tubule wall appears to be continuous. No desmosomes are apparent, suggesting that the tubule lumen is intracellular. The tubule is connected to a main canal by a desmosome (Plate 7C). This ensures a tight junction and also maintains the fluid in the lumina of the tubule and canal discrete from interstitial fluid.

Few sections of flame cells were obtained and those seen were poor. Sufficient of their structure was present for them to be considered similar to flame cells described in other Platyhelminthes. Approximately 58 cilia form the "flame". The cilia arise from one surface of the flame cell cap and have striated rootlets extending into its cytoplasm. The barrel in which the flame beats is formed by interdigitating processes from the cap and from the tubule. The barrel is 3 μ diameter; the flame cell is 10 μ total length, half of this consisting of the nucleate cap. Leptotrichs were seen within the barrel lumen, surrounding the ciliary tuft, but none were seen exterior to the barrel.

DISCUSSION

The tubules and canals of H. diminuta are similar in structure to those of M. expansa (Howells, 1969). Neither

worm has desmosomes in the tubule walls, so the tubule lumina are probably intracellular. Howells suggested them to be "intercellular" but this is thought to be a misunderstanding of the word, rather than a contrast of opinion. No differences were found between the dorsal and ventral canal walls in M. expansa. H. diminuta, however, has muscle blocks in the region around the ventral canal wall but not around the dorsal canal, which is many times smaller. Howells described the canal walls as "intercellular" but, again, the word appears to have been misused; his micrographs show similar syncytial structure to the canal walls of H. diminuta.

The possible intracellular lumen of the flame cell tubules is different from the structure found in trematode tubules. The latter are formed from a single row of cells, the flattened ends of which encircle until they meet. At their junction is a distinct desmosome (Wilson, 1969). The tubule lumina are thus extracellular (i.e. truly intercellular). Microvilli are not usually present in trematode tubules. Irregular projections from the walls were observed but distinct microvilli were only seen in a small region near the excretory pore of the miracidium of Fasciola hepatica (Wilson, 1969). The distinct bead-like microvilli observed in H. diminuta and M. expansa seem peculiar to cestodes. No physiological work has been done on trematode protonephridia directly, but it would be interesting to know whether absorption occurs in their tubules and canals, as it is now known to occur in H. diminuta. Absorption is normally associated with microvilli or similar structures but it need not necessarily occur only in their presence.

SUMMARY

1. The dorsal canals of the protonephridial system of H. diminuta are present in the anterior and middle regions of the worm but could not be found in the posterior. Their walls are syncytial; the cell bodies lie below the basal lamella and are connected to the syncytium by cytoplasmic channels. The lumina of the canals are lined by microvilli.

2. The ventral canals are present throughout the length of the worm. They are connected by transverse canals at the posterior of each proglottis. The ventral canal walls are similar to the dorsal canal walls but have, in addition, muscle blocks around them. Ventral and dorsal canals are linked in the scolex by a ring vessel.

3. The flame cell tubules are lined by closely packed microvilli. There are no apparent desmosomes in their walls, suggesting intracellular lumina. The tubules open into either dorsal or ventral canals, a desmosome sealing the junction and maintaining the lumina discrete from interstitial fluid. Flame cells are probably similar to those of other Platyhelminthes.

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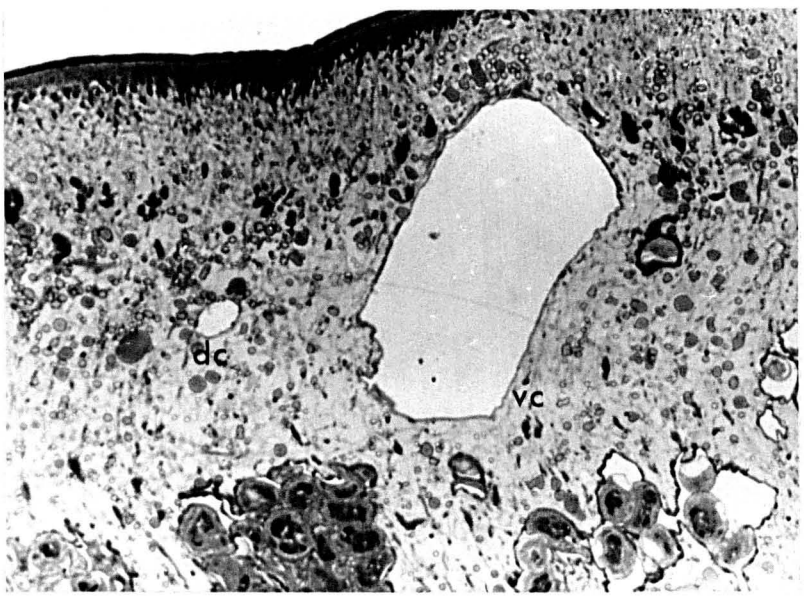
PLATE 1. Transverse section through middle region of a worm embedded in araldite. (x 140)

dc dorsal canal; vc ventral canal

PLATE 1

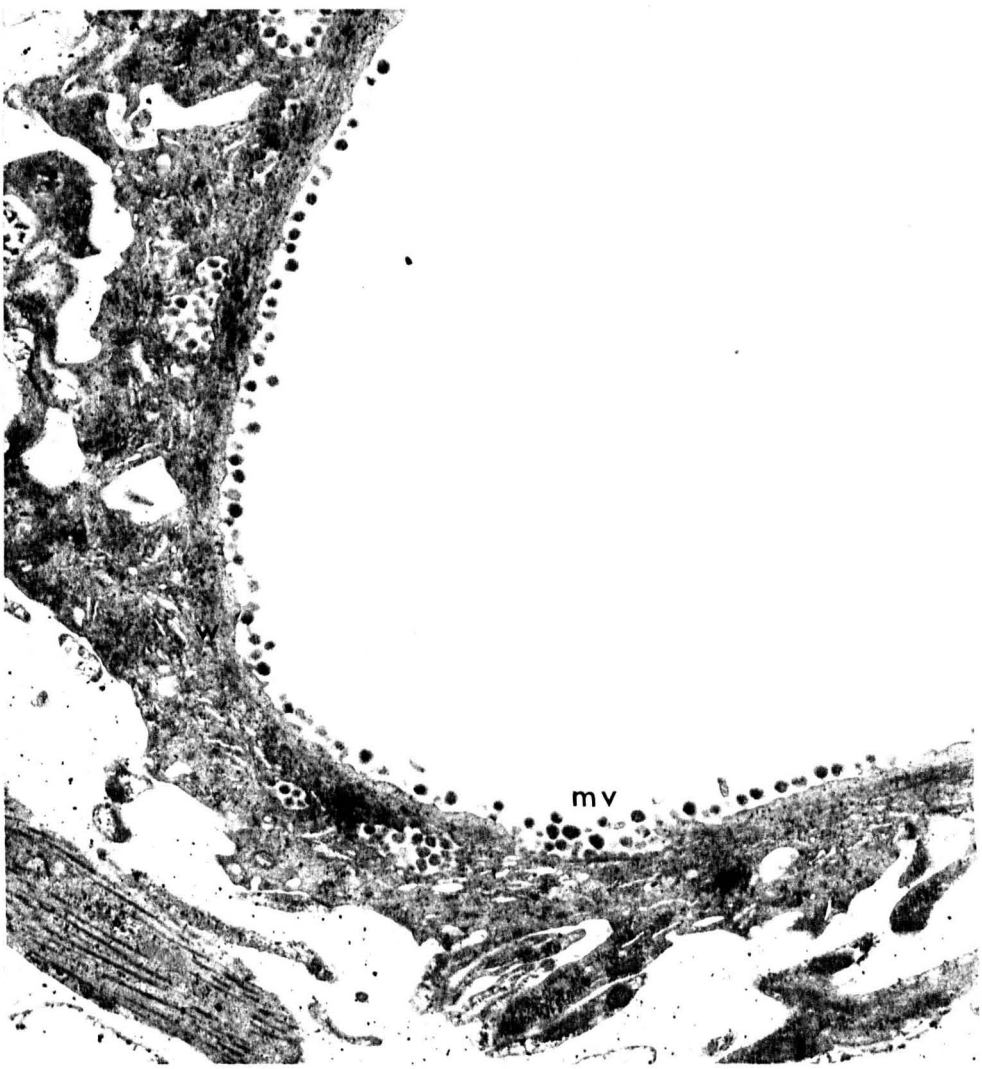


100 μ

PLATE 2. Transverse section through dorsal canal in anterior of worm (araldite) (x 18,000)

mv microvilli; w canal wall

PLATE 2

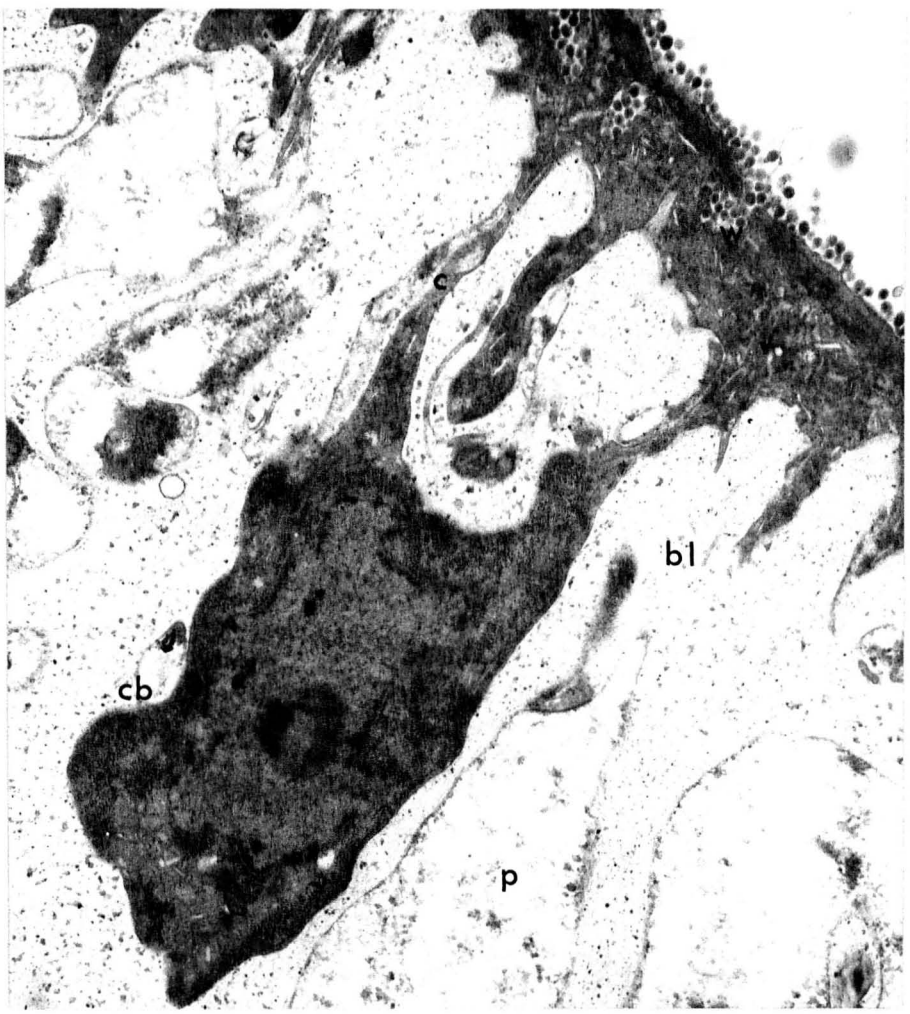


1 μ

PLATE 3. Cell body of dorsal canal wall in anterior of worm (araldite) (x 14,250)

bl basal lamella; c cytoplasmic channels; cb cell body; p parenchyma; w canal wall

PLATE 3

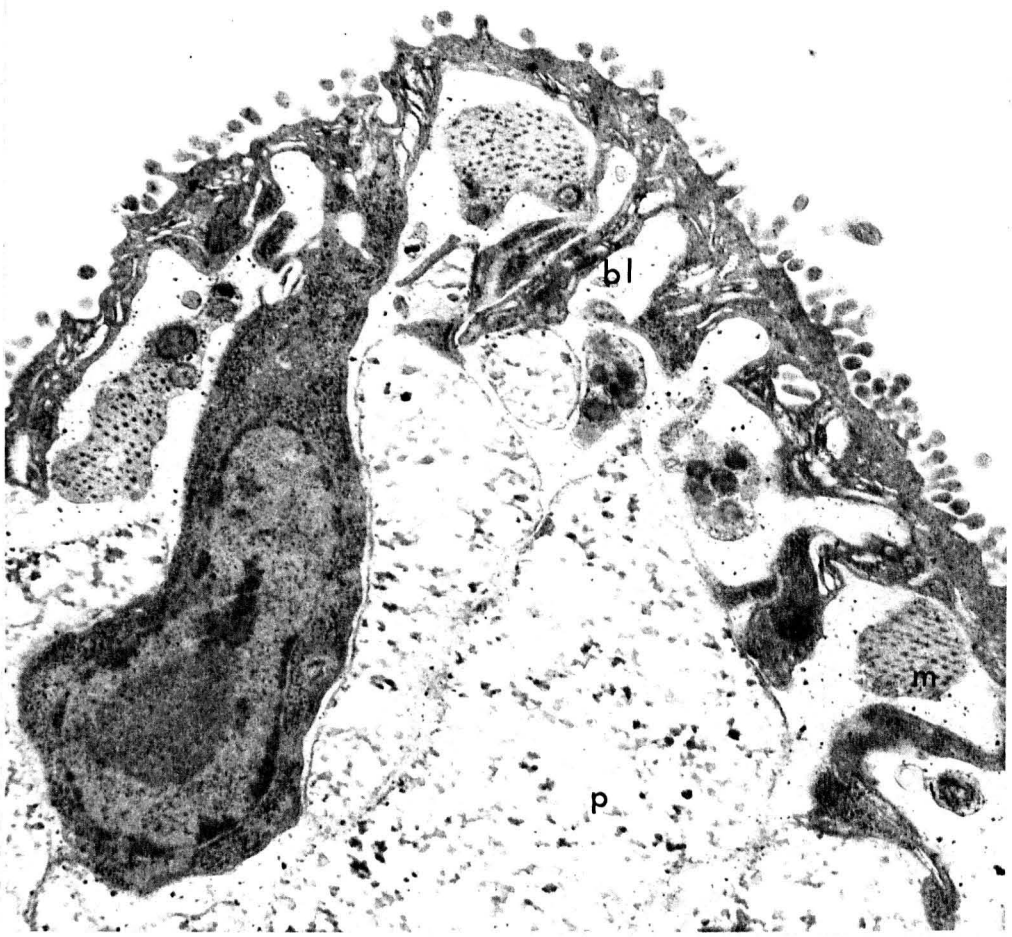


1 μ

PLATE 4. Ventral canal wall in anterior of worm (araldite) (x 22,000)

bl basal lamella; m muscle;
p parenchyma

PLATE 4



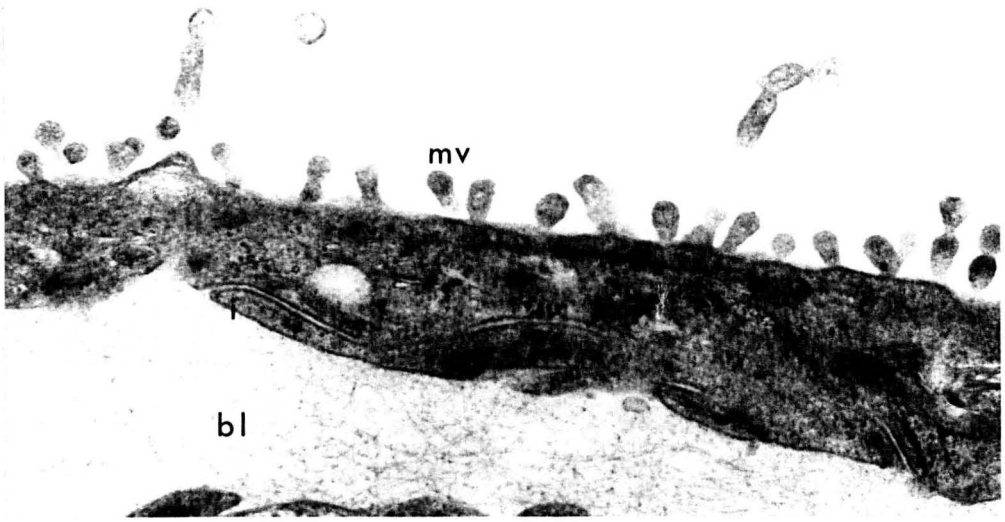
1 μ

PLATE 5. A. Ventral canal wall in anterior of worm (araldite) (x 34,000)

B. Microvilli, to show bounding membrane (x 115,900)

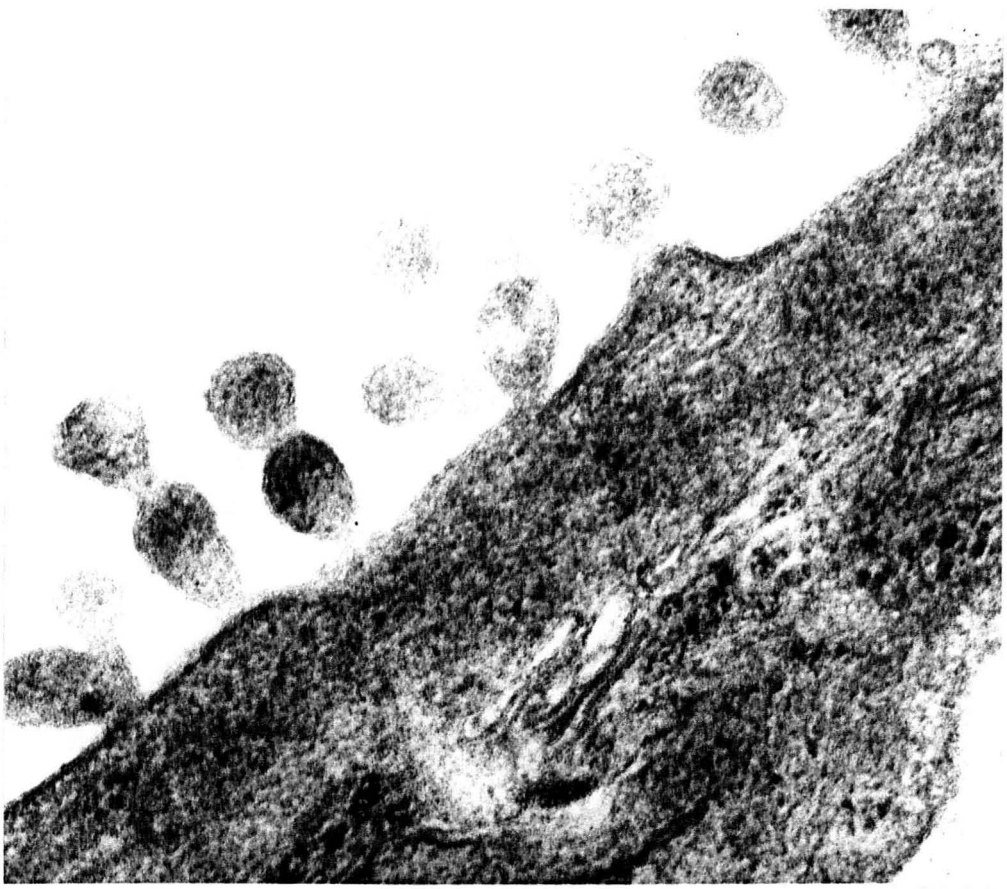
bl basal lamella; i basal invaginations; mv microvilli

PLATE 5



A

0·5 μ



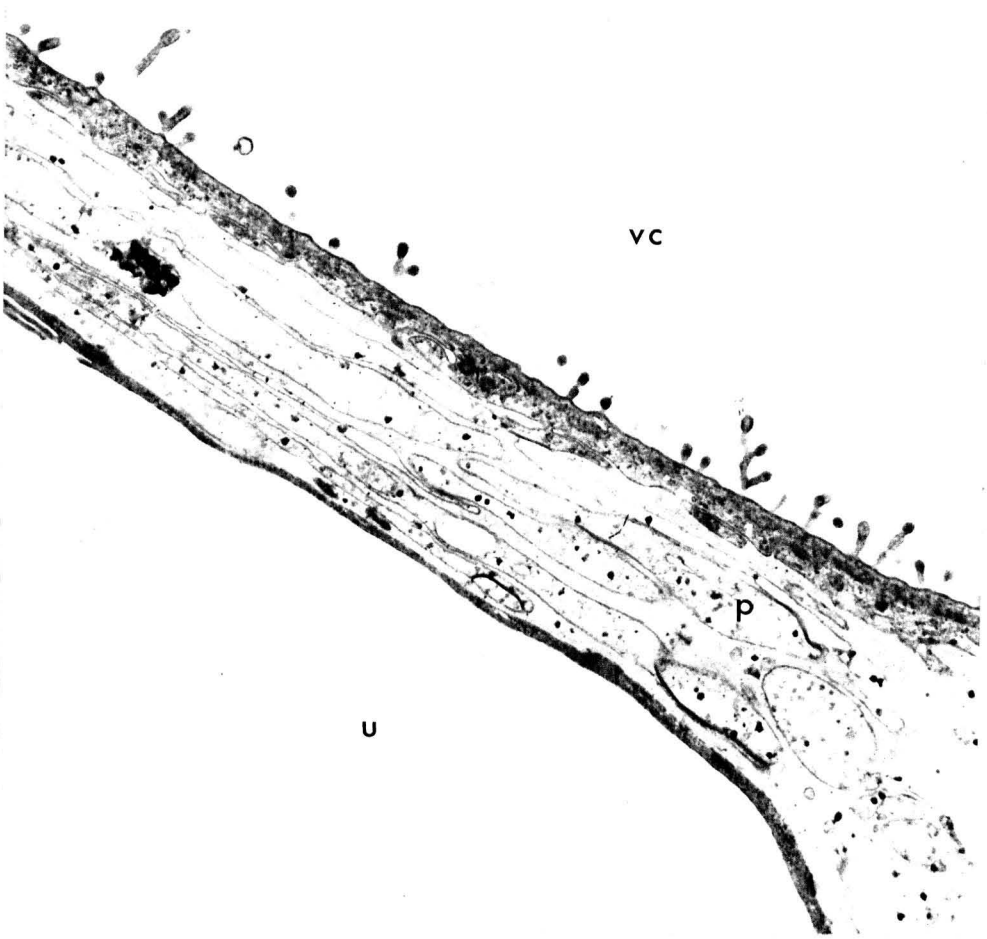
B

0·2 μ

PLATE 6. Ventral canal wall in posterior of worm (araldite) (x 14,250)

p parenchyma; u uterus;
vc ventral canal

PLATE 6



1μ

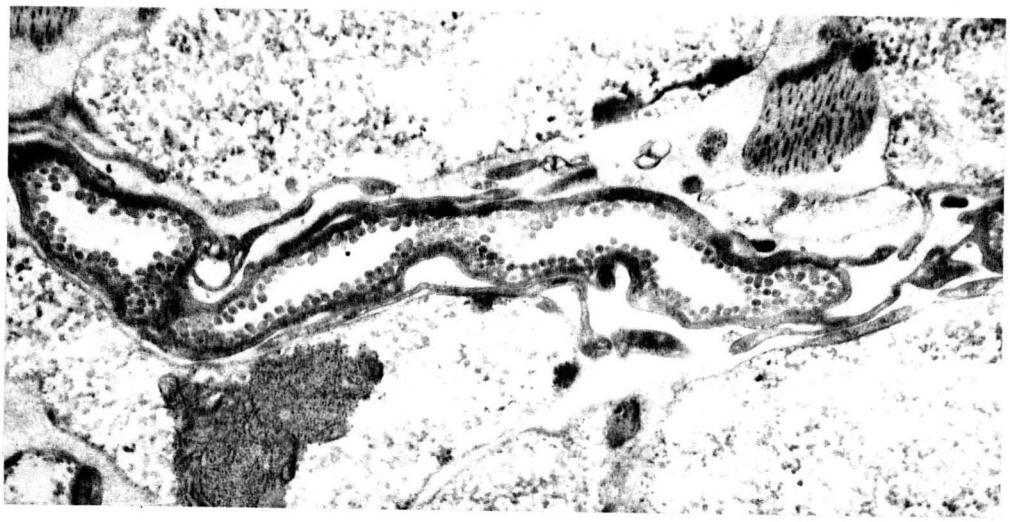
PLATE 7. A. Longitudinal section through a flame cell tubule (araldite) (x 13,000)

B. Transverse section through tubule. No desmosome present (araldite) (x 24,000)

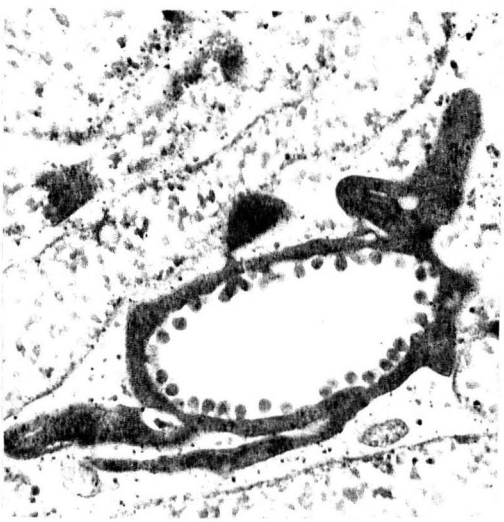
C. Junction of tubule with dorsal canal (araldite) (x 22,500)

d desmosome; dc dorsal canal; t tubule

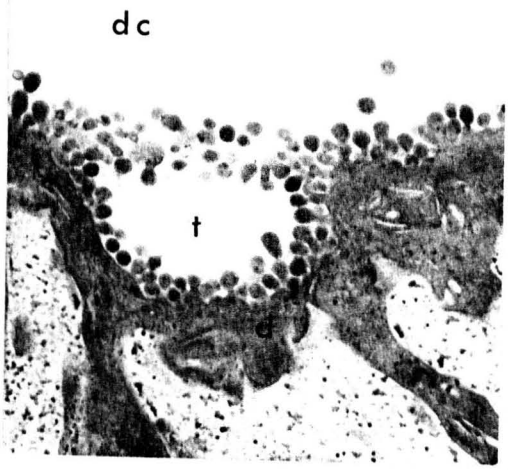
PLATE 7



A 1 μ



B 1 μ



C 1 μ