ELECTROPHYSIOLOGY OF THE PERIPHERAL NERVE NET IN THE POLYCLAD FLATWORM *FREEMANIA LITORICOLA*

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SUMMARY

1. A diffuse-conducting system close to the dorsal epithelium of the polyclad flatworm *Freemania litoricola* is described. Tactile stimuli elicit small action potentials which can be conducted around lesions through the body wall. The potentials can occur in bursts or barrages.

2. This conducting system appears to be insensitive to Mg^{2+} ions.

3. Conduction velocities (0·26–71 m/sec) vary over the animal. Conduction spread in the anterior half of the animal appears to be greater than that in the posterior portion.

4. Response decrement to repeated stimulation can be recorded in the peripheral system but it is not clear if this is due to habituation or fatigue.

5. Conduction from the peripheral net to the brain occurs. Some central units appear to pick up information only, or mainly, through the anterior nerves, while other units can respond to information conducted through the network to nerves of the contralateral side.

6. Different possibilities to account for this system are discussed, and it is suggested that the animals either possess a unique Mg^{2+} insensitive synaptic nerve-net or else the network is electrically coupled.

INTRODUCTION

Polyclad flatworms possess plexiform nervous systems connected to an anterior brain. Besides these plexes, which occupy a submuscular position, the animals also possess a subepithelial feltwork of nerve fibres which lie between the basement membrane and the muscle layers. Although attempts have been made to analyse nerve-net properties within these nervous systems, these have to a large extent been unsuccessful (Gruber & Ewer, 1962; Koopowitz, 1974). Using behavioural criteria it was possible to demonstrate a sensory nerve-net concerned with tactile stimulation and locomotion in the polyclad *Notoplana acticola* (Koopowitz, 1973). Recently it has become possible to isolate this tactile sensory net physiologically and to study some of its properties. A rather surprising feature of the net-work is its Mg^{2+} insensitivity, which suggests that flatworm nerve-nets may possess some novel and unexpected features. Some other properties of the tactile response, especially those concerned with habituation and integration within the brain of *Freemania*, are described in a following paper.

METHODS

Methods of handling these preparations were described in some detail elsewhere (Koopowitz, 1975). Briefly, recordings were made employing polyethylene suction electrodes directly on the epithelium and using conventional a.c.-coupled amplification. The preparation consisted of entire or anterior halves of animals pinned out on a dish containing Sylgard resin. Most experiments were carried out in sea water containing 30% by volume of isotonic $MgCl_2$ (0·36 M). In some experiments the brain was exposed by dissection so that electrodes could be attached directly to it. Although the net can be stimulated electrically, in these experiments animals were stimulated by gentle tapping with a plastic probe (tip = 0·5 mm diameter). Touching the animal anywhere will usually produce one or two spikes irrespective of the recording site, but stimulating certain places close to the margin may produce a barrage of spikes. Barrage producing areas were sought out and used for most experiments.

RESULTS

If an electrode is attached anywhere on the dorsal epithelium of a polyclad which has been anaesthetized with $MgCl_2$, it is possible to record one to several action potentials in response to a light touch administered anywhere on the animal's body. A typical response consists of a number of spikes which might vary in amplitude from 10 to 50 V (Fig. 1). Responses can be obtained from preparations for up to 12 h in 30% isotonic $MgCl_2$; longer immersions cause the preparation to start disintegrating. The results were obtained after 11 h in Mg^{2+} (Fig. 1). These results raised two important questions: (1) was Mg^{2+} getting into the preparation, and (2) were the spikes actually action potentials generated by nerves? Observations on the brain of *Freemania* (Koopowitz, 1975) suggest that Mg^{2+} was able to penetrate the brain sheath and silence responses of second-order cells. A 10% isotonic $MgCl_2$ acts as an anaesthetic, rapidly relaxing the animals. Most nervous activity is abolished within 25 sec. Potentials can be measured from the epithelium of unanaesthetized worms (Fig. 2a), but these tend to be obscured by other electrical activity. The very large fluctuations in potential may be due to muscle but one is probably also recording from a variety of neural units. Mg^{2+} abolishes most potentials, leaving only one kind of activity (Fig. 2b). Therefore Mg^{2+} does get into the animal and blocks most neuronal activity. Arguments that these spikes actually represent neuronal rather than epithelial potentials are based on indirect evidence which will be discussed later.

The action potentials elicited by a touch stimulus are not all of equal amplitude, which suggests that the electrode is picking up signals from a number of discrete units. Recording from the margin on one side of a preparation while stimulating at a variety of sites (Fig. 3a–d) usually elicits activity in at least two different units (Fig. 3a). Often there are sites on an animal's body where a light touch will initiate a barrage of action potentials, sometimes lasting 1 or 2 sec. In these cases there is an initial burst of spikes followed by a sustained discharge at a somewhat lower frequency (Fig. 3d). Usually there is a distinct pause between the initial and following discharge (Fig. 3d). The follower barrage usually occurs at a steady frequency
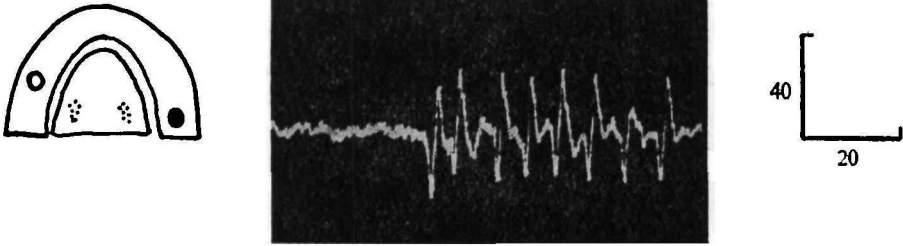
Fig. 1. Action potentials recorded from a strip of margin following tactile stimulation. The position of the recording electrode is denoted in this and succeeding figures by a solid circle and stimulation site as an open circle. This response was recorded in 30 % isotonic $MgCl_2$:70 % sea water, following 11 h of incubation in the medium. The vertical scale is in microvolts and the horizontal in msec.
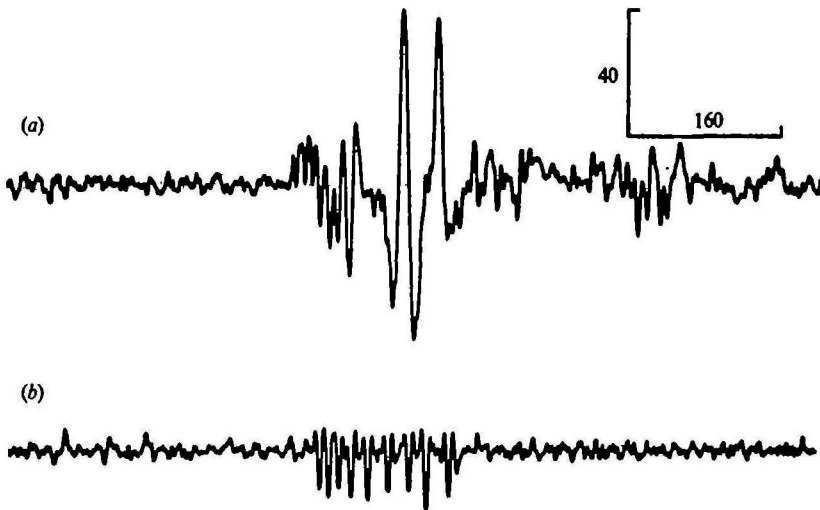

Fig. 2. Potentials recorded before and after the application of $MgCl_2$. Traces have been taken from chart recordings and amplitudes should not be read as absolute values. Both traces were measured from the same site on the body and stimulated at the same place. (a) Response in normal sea water and (b) the response $4\frac{1}{2}$ min after the addition of 10 % isotonic $MgCl_2$. The vertical scale is in microvolts and the horizontal scale is in milliseconds.

(Fig. 3d) but may sometimes be irregular. Both Figs. 3(d) were elicited from the same site, but the response recorded appears to be rather different. Repeated stimulation at barrage-producing sites results in response decrement. With sufficiently long inter-stimulus intervals (at least 5 min) the response recovers. Whether or not the change in responsiveness is due to fatigue or refractoriness at the receptor site, or to an integrative process such as habituation, has yet to be determined.

A similar spike-producing system occurs on the ventral surface of *Freemania* but barrage-producing sites have not been found. Mg^{2+}-insensitive conduction systems have also been found in two other polyclad species, *Notoplana acticola* and *Alloioplana californica*, which suggests that these Mg^{2+}-insensitive systems may be of general occurrence within this group of animals.
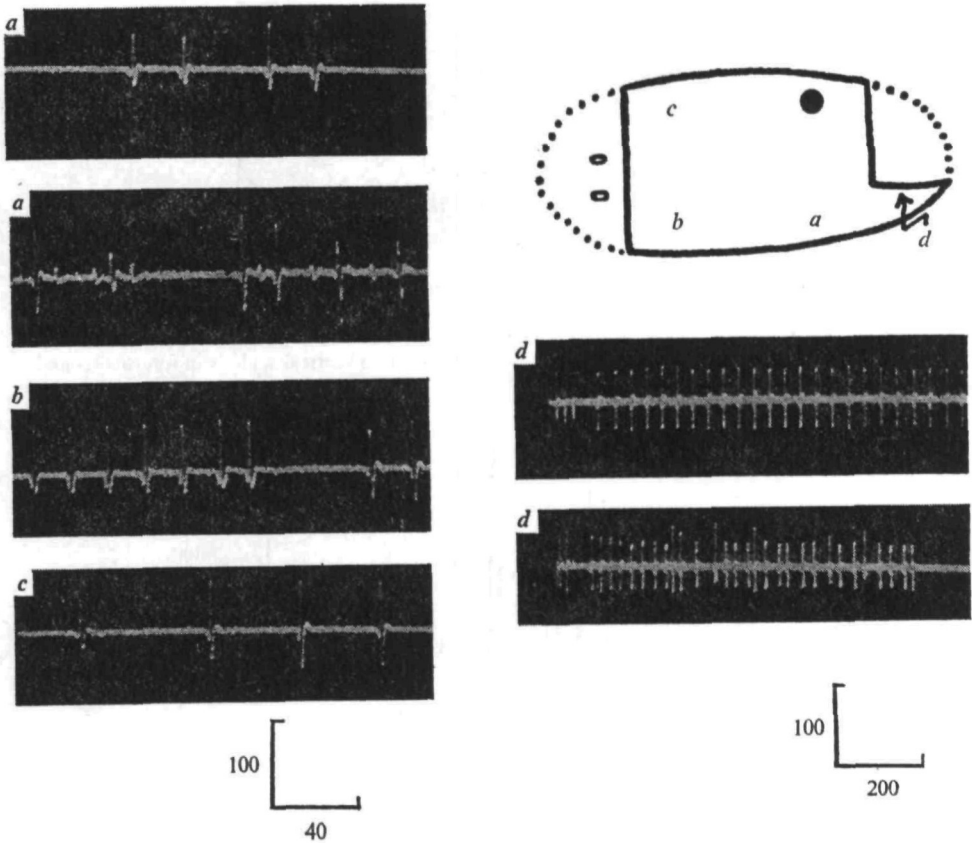
Fig. 3. The variety of potentials measured at one position. The letters above each trace refer to the stimulation sites in the diagram. Cross-hatched parts of the worm had been cut away for this experiment. Preparation had been maintained in 30% $MgCl_2$:70% sea water for between 4 and 5 h for this experiment. Note that the time scale in the (d) traces differs from the others.

Diffuse conduction

The experiments reported above suggest that the system might be diffuse-conducting as would be expected from a nerve-net. The possibility also exists that conduction could be accounted for by a series of individual nerve cells directly connected to various points over the worm's body. The latter type of system has been invoked to explain certain aspects of superficial conduction in sea urchins (Bullock, 1965) and the coordination between epithelial papillae in *Thysanozoon*, a polyclad (Koopowitz, 1974).

The diffuse-conducting properties of the nerve-net can best be demonstrated with the following kind of experiment. A barrage-producing site was determined on the margin on one side of the animal. A pair of suction electrodes were applied, 5 mm apart, on the other side of the animal (Fig. 4A). Tactile stimulation produced activity at both recording sites (Fig. 4B). The spikes making up the bursts did not correspond exactly at the two recording sites. This is important because it suggests that one is recording from nerves rather than the epithelium itself, since if this were the case, one would expect exactly similar responses at the two sites. A T-shaped cut right

through the body (Fig. 4C) still allows one to record the response (Fig. 4D), which apparently indicates that the information is conducted around cuts. It is possible, however, that conduction takes place along the periphery of the margin. That this cannot be the case is shown in Figs. 4E and 4F, where a cut across the margin still does not interfere with conduction. Finally, if the dorsal body wall alone is cut across the gap (Fig. 4G), the conduction ceases (Fig. 4H). In other experiments it was possible to isolate the recording electrode from the nerve net by carefully cutting through the dorsal body wall to form a small island of skin attached to the electrode. Tactile stimuli outside the island gave no response although stimuli within produced the usual potentials. A gap of 0·75 mm is sufficient to allow conduction across. As yet, no attempts have been made to find the smallest gap which still allows conduction.

Conduction velocities

Conduction velocities were determined by using pairs of recording electrodes spaced at known distances and administering a tactile stimulus in line with the two electrodes but not between them. Velocities were calculated from the time it took the response to travel between the two electrodes. Values calculated should be taken as minimum velocities as they do not take into account possible convolutions along the conducting tract nor the extensibility of the animals. The distance between the recording electrodes was taken with the animal at rest but an active animal can sometimes stretch to twice this length. Lateral and anterior–posterior conduction rates are given for four animals (Fig. 5). The range in conduction velocities measured varied from 0·26 to 0·71 m/sec. Velocities depended not only on electrode position but also on the direction of conduction. A number of observations made during the course of these experiments provide some insight into the system. There was not always correspondence between the patterning of spikes measured at the two sites. Sometimes potentials were only measured at the nearest electrode; this was particularly noticeable across the posterior part of the animal. It appeared to be easier to elicit spikes travelling from posterior to anterior end rather than vice versa. Spikes produced in the anterior half of the animal appeared to be through-conducting in that region, i.e. one did not record conduction failures at the second electrode in this region.

Habituation

The presence of peripheral habituation is of some interest as behavioural habituation has been described in the rhabdocoel *Stenostomum* (Applewhite, 1971). Repeated tactile stimuli to posterior halves of those flatworms resulted in decreasing responsiveness. In *Freemania*, response decrement also occurs with repeated tactile stimuli. Barrage-producing sites will usually only fire if stimulated at frequencies of less than once every 5 min. Sites producing short bursts of spikes also appear to show decrement with repeated stimulation (Fig. 6). Whether this is really habituation or merely reflects fatigue or a long-lasting refractoriness in the sensory part of the system is yet to be determined. Failure to respond appears to be associated with the site rather than the general net, as barrages can often be elicited within 2 mm of a site which has become temporarily silenced. Adjacent sites do not appear to dishabituate each other, and other modalities which might dishabituate the tactile
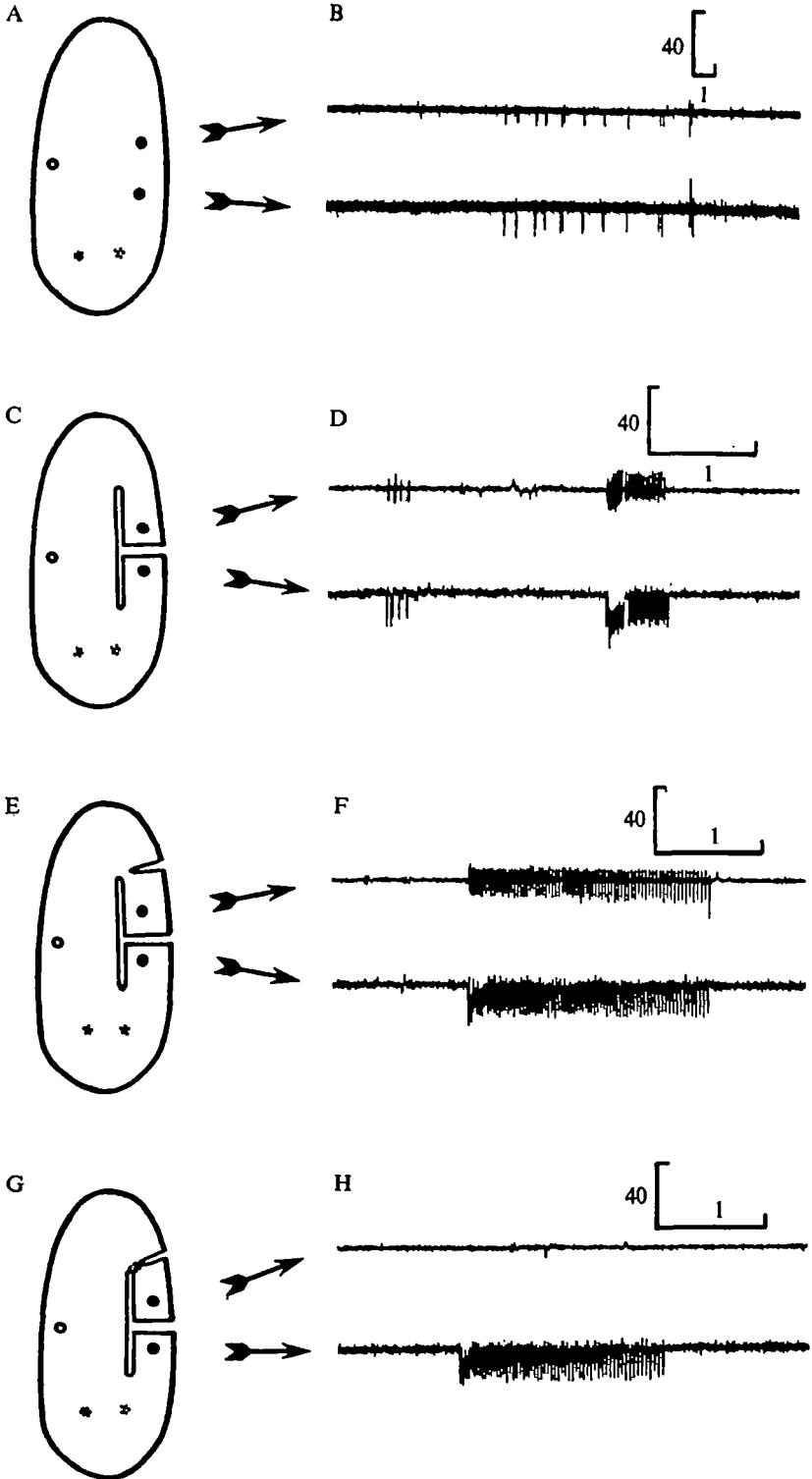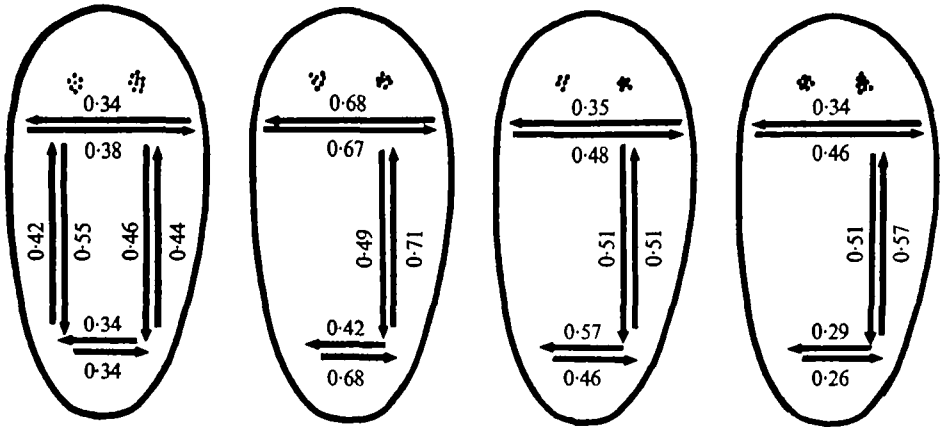
Fig. 4. For legend see facing page.

Fig. 5. Conduction velocity maps from four preparations. Arrows indicate the direction of conduction and the numbers associated with them, the velocity in m/sec. Measurements were made in Mg²⁺ anaesthetized preparations.
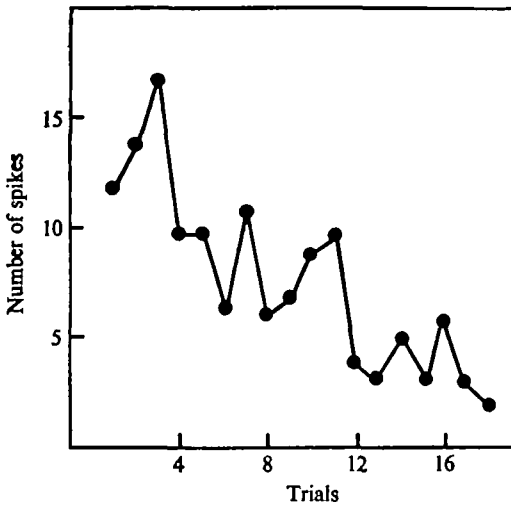

Fig. 6. Waning responsiveness to repeated tactile stimuli. Measurements were made in an Mg²⁺ anaesthetized preparation. Axis is the number of spikes evoked and the abscissa the trial number. Stimuli were administered at 2 sec intervals. The recording was taken from the margin at the level of the eyes and the stimulation site was near the rear of the animal.

Fig. 4. Conduction around lesions. Recording and stimulation sites and extent of lesions are shown next to each pair of recordings. Note that in B the time scale is slower and the responses are to a number of tactile stimuli; there was no prolonged discharge. In D two stimuli were administered initially, but only a few spikes were obtained from the first stimulus while a barrage of activity followed the second stimulus. In this preparation barrages were exceptionally long lasting. That in D is most typical. In G the dorsal body wall was cut between the two lesions. All recordings were made from the same preparation and while it was under Mg²⁺ anaesthesia. The vertical scale is in microvolts and the time scale is 1 sec.
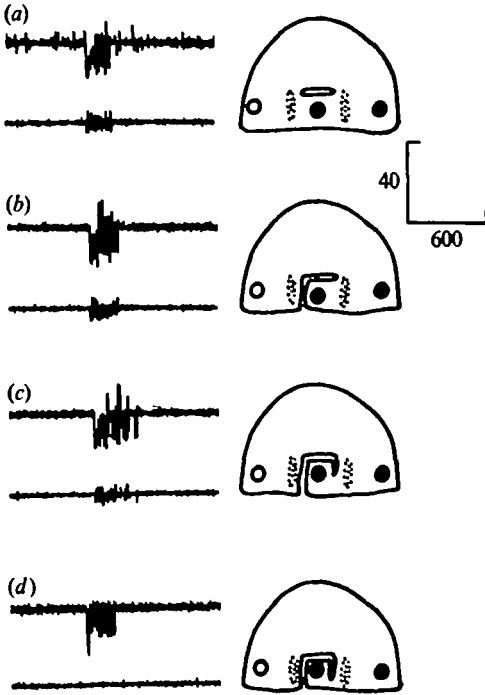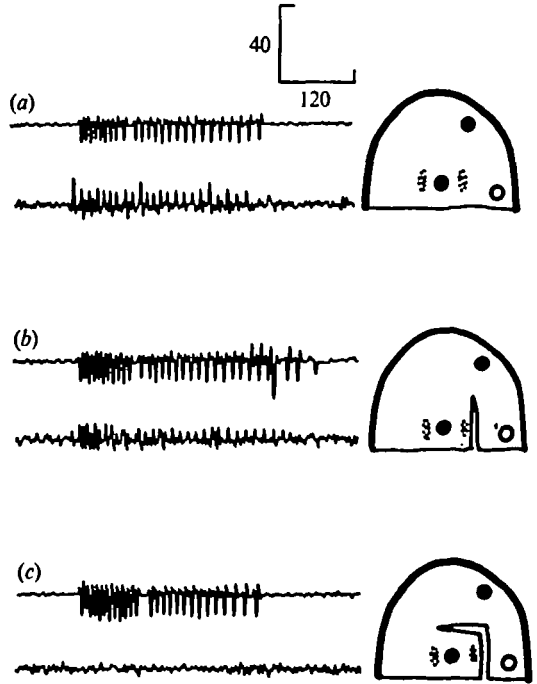
Fig. 7   Fig. 8

Fig. 7. Conduction around to the contralateral side of the brain. In each pair of recordings the upper trace is from the epithelium and the lower trace from a posterior dorsal section of the brain. Only the anterior third of the animal was used. Lesions, recordings and stimulating sites are shown in the diagrams alongside each pair of tracings. All tracings are from the same preparation. Vertical scale is in microvolts and horizontal scale is in milliseconds.

Fig. 8. Conduction to the brain through anterior and ipsilateral tracts. In each pair of tracings the upper tract is recorded from the epithelium and the lower tract recorded from a posterior dorsal site on the brain. Lesions, recording and stimulating sites are shown on the drawings alongside each pair of recordings. It should be noted that the brain recordings have been inverted to facilitate comparison with the epithelial records. All traces are from the same preparation. The vertical scale is in microvolts and the time scale in milliseconds.

system have not yet been discovered. It should be noted that evoked potentials recorded from the brain and elicited by peripheral tactile stimuli also show response decrement with repeated stimulation (Koopowitz, 1975), but this only occurs if the preparation is in normal sea water. The presence of increased Mg^{2+} appears to abolish central habituation. This suggests that, in the brain at least, the usual Mg^{2+}-sensitive synaptic mechanisms occur.

Pathways from the peripheral net to the brain

In the polyclad *Notoplana* there appeared to be two kinds of information which could be elicited by peripheral tactile stimulation. One was the diffuse-conducting system, the other a system bearing positional information (Koopowitz, 1973). It has been possible to demonstrate these systems recording from both the brain and the epithelium of *Freemania*. Fig. 7(a) shows the burst of activity recorded from these two sites following a peripheral stimulus. In this case the two anterior pairs of nerves leading off the brain had been severed, close to where they emerged from the brain.

A lateral incision severing all of the nerves on the stimulated side of the preparation had little effect on the activity recorded from the brain (Fig. 7b). This means that the brain recordings were probably those elicited by non-specific information, conducted through the nerve-net surrounding the cut to the contralateral side of the brain. Further cutting of some of the remaining contralateral nerves reduced the amount of activity recorded from the brain (Fig. 7c). It appeared, therefore, that there were a number of indirect routes into the brain. Severing most of the contra-lateral nerves abolished all activity recorded from the brain (Fig. 7d). The results described above were obtained from three out of five preparations. In the other two, the units in the brain from which the recordings were made appeared to obtain their input from the two anterior pairs of nerves. Cutting all the ipsilateral nerves on one side hardly reduced the activity recorded from the brain (Figs. 8a, b). Severing the anterior pairs of nerves, however, abolished all the activity recorded from these brain units (Fig. 8c) even though all the contralateral nerves had been left intact. It seems that these units only received input from the ipsilateral side.

DISCUSSION

A diffuse-conducting system such as that described in *Freemania* would normally be ascribed to a nerve-net; however, the Mg^{2+}-insensitivity of the system also suggests that perhaps one might be dealing with conduction in muscle sheets or through the epithelium.

A previous study on the ultrastructure of *Notoplana* muscle (Chien & Koopowitz, 1972) reported the presence of tight junctions between adjacent muscle cells. There are two major reasons for rejecting muscle–muscle propagation as the source of the spikes. In non-anaesthetized preparations, large potentials can be measured (Fig. 2a), and it seems much more likely that these could be due to muscle activity. The other evidence from Mg^{2+}-anaesthetized preparations suggests that there is no correspondence between muscle contraction and the Mg^{2+}-insensitive spikes recorded. Muscles can be activated by mechanical stimuli if the pressure is great enough. During the refractory period of a barrage-producing site, it is possible to make that part of the body contract if the probe is pushed hard enough but contraction does not elicit spikes at the recording site. Furthermore, spikes are usually elicited in the absence of muscle contraction in anaesthetized preparations.

It is more difficult to rule out epithelial conduction as a source of these potentials. Nerve-free epithelial conducting systems have now been described from a variety of phyla. Neuroid spikes tend to be slowly conducted, ranging from 7·7 cm/sec in amphibian tadpoles (Roberts & Stirling, 1971) to 35 cm/sec in Hydromedusae (Mackie & Passano, 1968), but otherwise appear similar to potentials recorded from nerve cells. The conduction velocities recorded in *Freemania* are similar to those of other epithelial conducting systems, but velocities are also like those recorded from small unmyelinated nerve fibres of vertebrates (Erlanger & Gasser, 1937). Evidence against the possibility of an epithelial conducting system comes from examination of indi-vidual records. It is quite apparent that a single electrode can pick up activity from a wide variety of spikes (Fig. 3). This is not what one would expect from an epithelial system. Differences in conduction velocities in different directions also have to be

accounted for, more easily done in terms of axon diameter than epithelial conduction. If the units are neuroid then the epithelium must be made up of a mosaic of conducting units. A simpler explanation would conclude that the spikes were being generated in a nerve-net.

If, indeed, the system is a nerve-net then one has to explain the Mg^{2+} insensitivity of the system. At least four possible explanations exist to account for these responses. (1) The net might be mediated by Mg^{2+}-insensitive chemical synapses. If this turns out to be the case then a completely new form of chemical transmission may be involved in this phylum. Although a wide variety of chemical synapses can be found in the peripheral submuscular plexus of *Notoplana* (Koopowitz & Chien, 1975), actual synapses have not been found in the subepithelial nervous system. Synaptic vesicles, however, both dense cored and clear, have been found (rarely) in the subepithelial system, so synapses probably do exist there. Whether or not these are Mg^{2+}-sensitive is not yet known. (2) Normal chemical transmission might occur but the synapses could be protected in such a way that they were not exposed to the Mg^{2+} ions. The vast majority of synaptic interactions and the neuromuscular junctions are unprotected. Mg^{2+} diffuses readily through the rather tough brain sheath the animals possess and knocks out central interactions. The reason why the tactile network should be afforded this protection is not clear, particularly as central processing of the tactile information is abolished by Mg^{2+} (Koopowitz, 1975). At an ultrastructural level this hypothesis has to be rejected as enveloping glia does not occur in either the subepithelial nervous system or around the synapses in the submuscular plexuses (Koopowitz & Chien, in preparation). (3) The third possibility invokes a syncytial nerve-network. A syncytial system has been described for *Velella*, a coelenterate, on the basis of investigations using light microscopy (Mackie, 1960). This kind of system would be extremely difficult to prove or disprove without tedious detailed reconstructions from ultrastructural sections. Observations by Lentz (1968) indicate that in the planarian *Procotyla* the subepithelial network is made up from discrete units. Unless there are multiple syncytia one must reject this hypothesis for the same reasons that epithelial-conducting systems should be discounted. (4) The last and possibly most intriguing possibility is of a nerve-network composed of electrically coupled units. As long as some heterogeneity in coupling resistances and perhaps some rectifying synapses occur, then it should be possible to account for all of the observations made. A preponderance of low-resistance electrical synapses in the anterior half of the animal could explain the through-conduction recorded from that point. One could record from different units and one might expect individual cells to respond to different thresholds and possibly be responsible for different firing rates and amplitudes; hence one might record slightly different patterns of activity between two recording electrodes even if they were fairly close together. Electrically coupled conducting systems in the invertebrates often appear to be concerned with rapid through-conduction and/or are involved with alerting systems, e.g. lateral giant fibre systems in the earthworm and crayfish (Bullock & Horridge, 1965). One of the main functions of the tactile system in *Freemania* probably has to do with alerting the animal and dishabituating other sensory modalities (Koopowitz, 1975). Whether or not this system is more rapid than other conducting systems in (these worms) remains to be demonstrated.

The morphological basis of this conducting system will presumably give a clue as to the mechanisms underlying transmission and it is expected that future experiments using and examining isolated pieces of epithelium will help to elucidate the problem.

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