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THE IONIC BASIS OF AXONAL CONDUCTION IN THE CENTRAL NERVOUS SYSTEM OF *ANODONTA CYGNEA* (MOLLUSCA: EULAMELLIBRANCHIA)

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INTRODUCTION

The bivalve mollusc *Anodonta cygnea* appears to be unique in possessing the most dilute body fluids so far described in the Animal Kingdom. The osmotic concentration of the blood of this species has been found to amount to only about 44·0 m-osmole, with a sodium concentration which averages 15·6 mM/kg. (Potts, 1954). It is difficult to explain the ionic basis of electrical activity of excitable tissues with such specialized body fluids. In particular, the relatively low sodium concentration of the blood presents difficulties in the interpretation of neuronal activity in terms of the conventional membrane theory, for the propagation of the action potential requires a steep concentration gradient of sodium ions across the nerve cell membrane.

The condition of a specialized blood composition with very low concentrations of sodium ions is, however, not confined to freshwater molluscs. A somewhat similar situation is encountered in several phytophagous insect species, although in these arthropods the total osmotic concentration of the blood does not approach the extreme condition found in *Anodonta*. Recent investigations on axonal function in the stick insect, *Carausius morosus*, which has a blood containing only about 15·0 mM/l. of sodium, have indicated that electrical activity is maintained by a local regulation of the extra-axonal sodium concentration (Treherne & Maddrell, 1967*b*; Treherne, 1967). In this latter species it appears most likely that the regulation in the sodium concentration in the fluid immediately surrounding the axons is achieved by the activity of the glial cells. It is envisaged that there might be an extrusion of sodium ions into the narrow extracellular channels formed at the axon surfaces by the closely applied glial cell membranes. The postulation of a glial involvement in the extra-axonal sodium regulation is in accord with electron microscopic study of the central nervous tissues of this insect, which shows a very close glial-axon association in which each axon is surrounded by an appreciable number of glial folds (Treherne & Maddrell, 1967*b*).

The organization of the central nervous tissues in the insect species described above contrasts strikingly with that observed in the central nervous connectives of *Anodonta cygnea* (Gupta, Mellon & Treherne, 1969). In this mollusc the central nervous tissues contain extensive extracellular spaces surrounding the axons, with only very sparsely

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distributed glial elements. The extracellular space appears to be freely accessible, for apart from the neural lamella no other visible structures are interposed between it and the fluid surrounding the connectives. These structural findings suggest that different physiological mechanisms might be involved in the maintenance of electrical activity in the neurones in a nervous system bathed with extremely dilute blood. For this reason the present study was carried out in an attempt to elucidate the problems associated with the ionic basis of axonal conduction in *Anodonta*. Unfortunately, the extremely small size of the central axons in *Anodonta* (cf. Gupta *et al.* 1969) precluded the study of their electrical properties using intracellular micro-electrodes. Similarly, little success was obtained in attempts to impale the relatively small cell bodies with glass micro-electrodes. For these reasons the present investigation was confined to extracellular recording techniques.

METHODS

Pieces of cerebro-visceral connective, up to 5 cm. long, were dissected free from the animals and were arranged in an experimental chamber. In preliminary experiments the latter consisted of three adjacent compartments, separated from each other by petroleum jelly (Vaseline) walls. The nerve was arranged so that part of it passed through each of the three compartments. The compartments at either end were filled with *Anodonta* blood or Ringer solution and were used for stimulation and recording respectively. The middle compartment contained the experimental solution; its fluid level was always kept at a higher level than that of the flanking ones so as to minimize the possibility of leakage of Ringer or blood from the stimulating or recording compartments. Brief stimuli (1·0–5·0 msec. pulses) were delivered to the nerve by paired platinum electrodes connected to an isolation transformer. Records of compound action potentials were obtained between an active electrode, over which one end of the nerve was draped, and an indifferent lead placed in the grounded pool of Ringer solution. In later experiments isolated connectives were placed in an experimental chamber containing five compartments. This consisted of a Perspex trough, the connective crossing each partition through Vaseline seals. Silver stimulating electrodes were placed into two adjoining compartments at one end of the chamber, while platinum recording electrodes projected into the two blood-filled compartments at the opposite end. The central compartment contained the experimental solution. To change experimental solutions the central compartment was emptied by pipette, rinsed with fresh solution and refilled. The latter operation was accomplished in approximately 10 sec. Signals were led to a capacity-coupled high-gain pre-amplifier and were displayed and photographed by conventional oscillographic techniques.

Connectives were maintained in either filtered *Anodonta* blood or in *Anodonta* heart-muscle Ringer (Potts, 1954). This consisted of 14 mM-NaCl, 0·5 mM-KCl, 5 mM-$CaCl_2$, 0·25 mM-Na_2HPO_4 and 1 mM glucose. The pH was adjusted to 7·5 by addition of dilute NaOH.

The distribution of axon diameters in the connective was estimated using representative fields from electronmicrographs (× 10,000 to × 30,000) obtained during a previous investigation carried out in this laboratory (Gupta *et al.* 1969). The dimensions of the connective and the number and distributions of the larger axons were calculated from measurements made using light micrographs (× 240).

RESULTS

The form of the compound action potential. The compound action potentials recorded in these preparations showed a large homogeneous slow component, with a conduction velocity of between 0·03 and 0·04 m.sec.$^{-1}$, together with a rather more variable rapidly conducting component (Fig. 1). The conduction velocity of the latter was between 0·4 and 0·5 m.sec.$^{-1}$ for the most rapidly conducting fibres.

Figure 1 represents an attempt to relate the form of the compound action potential

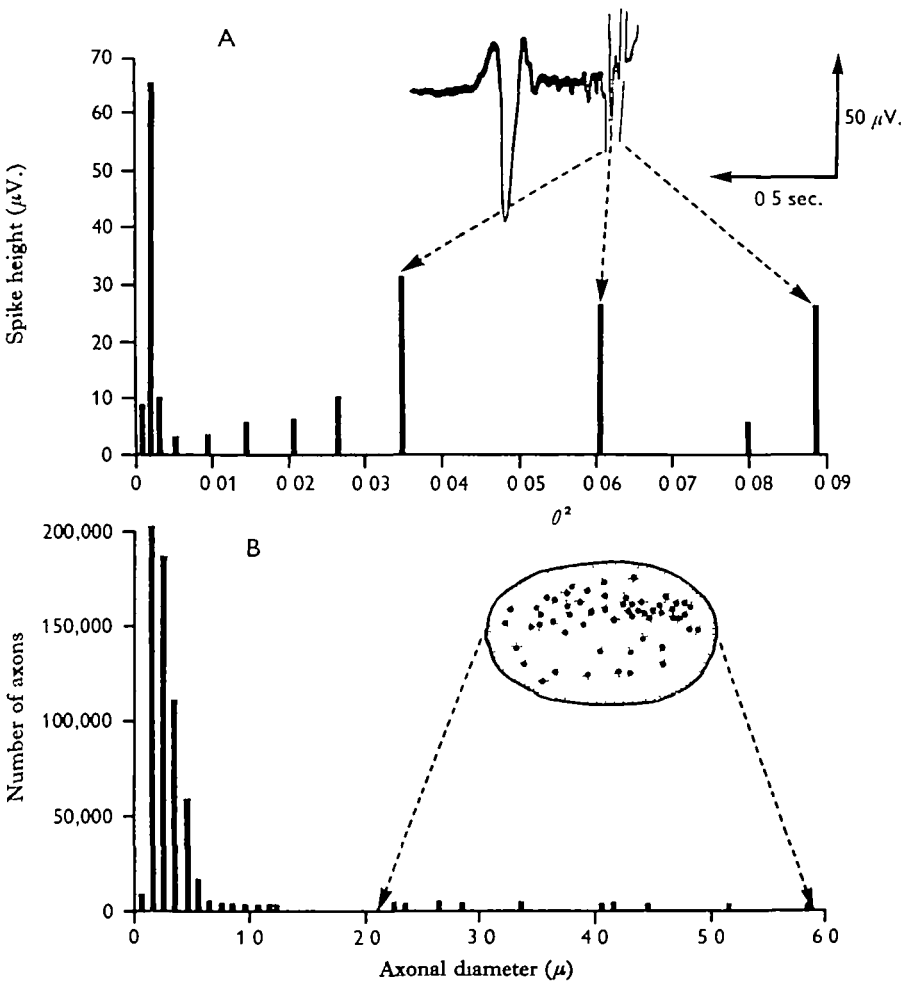

Fig. 1. A. The square of the conduction velocity (θ^2) of the various components of the compound action potential, recorded from a cerebro-visceral connective, plotted against spike height. θ^2 is proportional to axon diameter (Rushton, 1951) and is, thus, a measure of the dimensions of axons associated with different components of the compound action potential. The broken arrows indicate the values of θ^2 associated with the rapid initial components of the action potential.

B. The distribution of axon diameters estimated, from light and electron micrographs, in the cerebro-visceral connective of *Anodonta*. The inset diagram illustrates the distribution of the larger axons in the connective as revealed by light microscopy. Because of the small number of the larger axons (2·0–6·0 μ) the values have been multiplied by × 1000.

to the distribution of fibre size in the cerebro-visceral connective. In Fig. 1A the extent of the various components of the compound action potential have been plotted against the square of the conduction velocity (θ^2). As conduction velocity in non-myelinated fibres is proportional to the axon diameter (Rushton, 1951) it follows that θ^2 represents a function of axonal diameter. The latter data is compared with the distribution of axon diameters in a cerebro-visceral connective, estimated from counts made using light micrographs and electron micrographs (Fig. 1B). It will be seen that there is relation between the very large population of small axons, in the range of 0·1 and 0·3 μ in diameter, and the large slowly conducting component of the compound action potential. Comparison of θ^2 with the distribution of fibre size also shows that the initial, rapidly conducting, components of the action potential can be related to the relatively large axons in the range of 2·0–6·0 μ in diameter. Counts made on light micrographs (at a magnification of $\times$ 240) showed that there were approximately 70 axons, in the range of 2·0–6·0 μ, in a cerebro-visceral connective.

It is of interest to note that the ability of the isolated connectives to conduct action potentials persisted for relatively long periods in preparations bathed in blood or Ringer solution. *In situ* the cerebro-visceral connectives are situated in a blood space which is flanked by the epithelial walls of the proximal wall of the kidney (cf. Gupta *et al.* 1969). In view of the undoubted involvement of the kidney epithelium in ion secretion into the blood space surrounding the connectives (cf. Picken, 1937; Potts, 1967) it seemed possible that electrical activity might be maintained by a local elevated ionic environment in the fluid bathing the surfaces of the connectives. The fact that there was no apparent alteration in the form of the compound action potentials recorded from connectives bathed in blood or Ringer solution for relatively long periods suggests that axonal activity is not sustained by any specialised ionic environment maintained in the fluid bathing the surface of the connectives.

Effects of isotonic sucrose solutions on conduction processes. Bathing the connectives with isotonic sucrose solutions was found to result in a rapid decline in the large slowly conducting component of the compound action potential (Fig. 2). This effect was readily reversed and within 30 sec. after return to normal Ringer solution the slowly conducting fibres showed a return to full activity.

The effects of isotonic sucrose solution on the rapidly conducting fibres is illustrated in Fig. 3. This shows a recording of the fast component of a compound action potential at a rapid sweep speed. It will be seen that, unlike the small axons, the electrical activity of the large ones persisted for appreciable periods in preparations bathed in isotonic sucrose solutions. It should also be noted that there was a significant fall in the conduction velocity of the action potentials recorded from the large axons in these experiments. These action potentials also persisted in connectives which were bathed in sucrose solution for the whole of their lengths, indicating that their activity could not be attributed to a diffusion of inorganic ions from the recording or stimulating compartments. In these experiments action potentials were recorded by lifting the connectives from the solution on the recording and stimulating electrodes.

Figure 4 shows the time course of the change in conduction velocity in the rapidly conducting axons in preparations maintained in isotonic sucrose solution. This effect occurred relatively rapidly, there being a halving of the conduction velocity within 30–60 sec. and a slower reduction to about one-third of the initial value after 360 sec.

The above effects did not appear to result from alteration in the resistance of the fluid bathing the surface of the connectives. It was found, for example, that replacement of the normal Ringer solution with mineral oil did not cause any appreciable reduction in conduction velocity (Fig. 5).

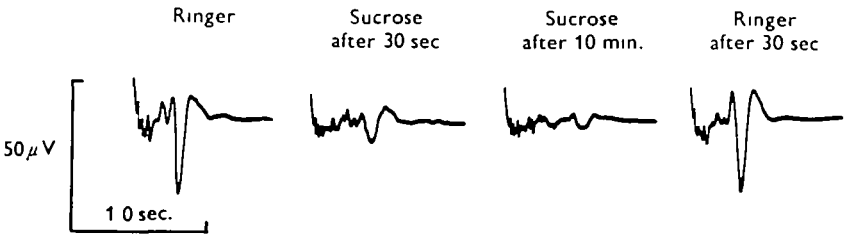

Fig. 2. The effects of isotonic sucrose solution on compound action potentials in cerebro-visceral connectives.

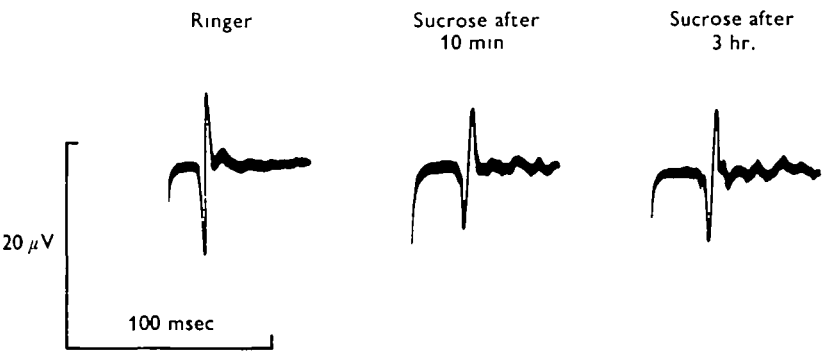

Fig. 3. The effects of isotonic sucrose solution on the fast action potentials.

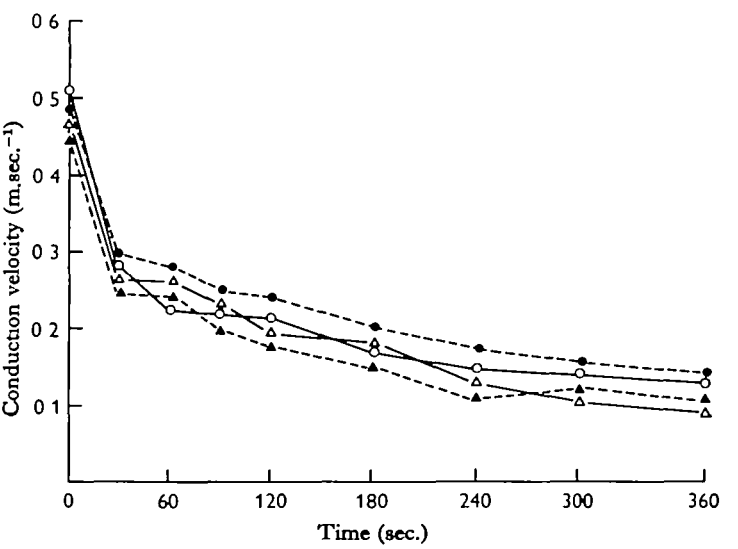

Fig. 4. The effects of isotonic sucrose solution on the conduction velocity of the action potentials in fast axons. This diagram summarizes the data obtained from four different preparations.

Effects of distilled water and dextran solutions. Figure 6 illustrates the effects of these substances on the rapidly conducting axons. Contact with distilled water resulted in rapid development of a conduction block. When the connectives were bathed with a 42 mM solution of dextran (M.W. 10,000) there was a rapid return of the fast action potentials, despite the fact that the preparation had no further access to solutions containing inorganic ions. As will be seen from Fig. 6 the conduction velocity of these fast action potentials was slightly increased by exposure of the connectives to the dextran solution. In isotonic sucrose the action potentials were maintained, but with significant reduction in conduction velocity. Finally, on return to *Anodonta* blood the conduction velocity increased to a value similar to that obtained at the commencement of the experiment.

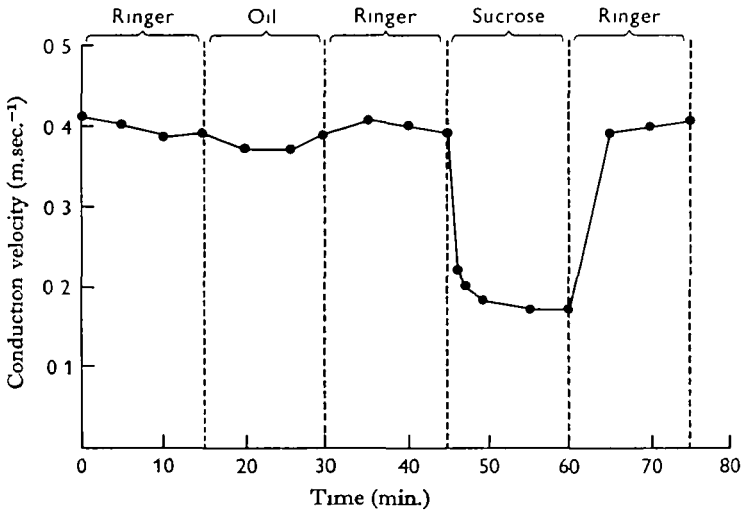

Fig. 5. The effects of mineral oil and sucrose solution on the conduction velocity of action potentials in the fast axons of the cerebro-visceral connective.

Effects of tetrodotoxin on the rapidly conducting fibres. Addition of dilute tetrodotoxin to the bathing fluid resulted in the development of a conduction block for the large axons within 30 sec. On return to normal Ringer solution there was an equivalent rapid return of activity in these axons (Fig. 7).

Effects of inhibitors on the electrical activity of the fast axons. Addition of 5·0 mM 2:4 dinitrophenol to the bathing solution was found to result in a decline in the amplitude of the action potentials and finally to produce a complete conduction block (Fig. 8). Essentially similar results were also obtained in preparations bathed with Ringer containing 1·0 mM ouabain. It will be seen from Fig. 8 that the substitution of a solution containing 100 mM-NaCl, together with the same concentration of 5·0 mM DNP, resulted in an extremely rapid return of the fast action potentials despite the continued presence of the poison molecules. The renewed activity of the rapidly conducting fibres was, however, not maintained in the latter solution and the action potentials began to show a decline in amplitude after approximately 15 min.

Figure 8 also shows the effect of addition of 66 mM-$CaCl_2$, in the presence of DNP, to a poisoned preparation. Unlike the results obtained with elevated sodium concentration there was no return in activity of the large axons.

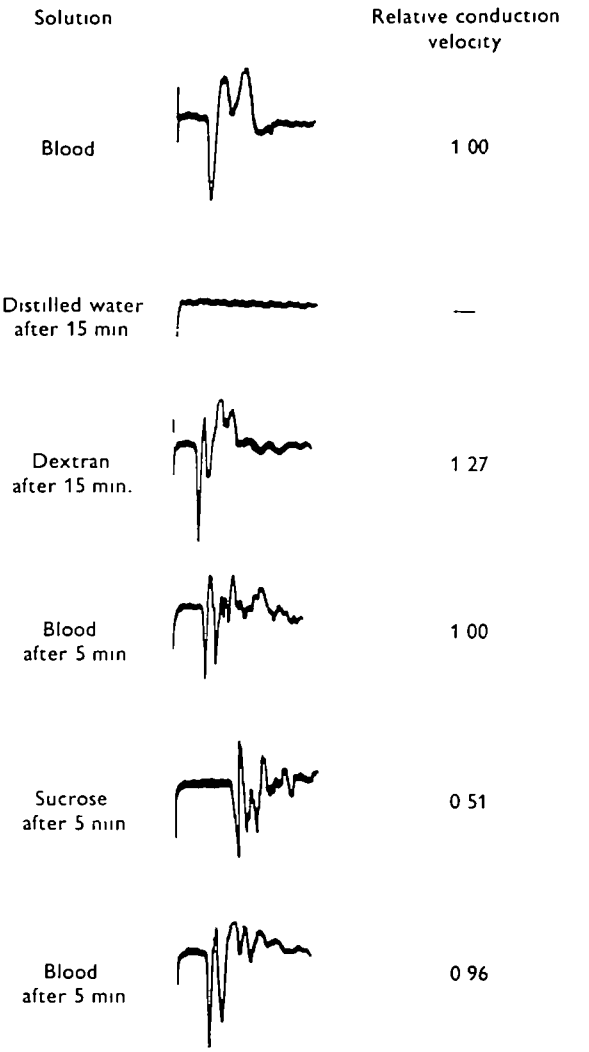

Fig. 6. The effects of distilled water, isotonic dextran and sucrose solutions on the fast action potentials.

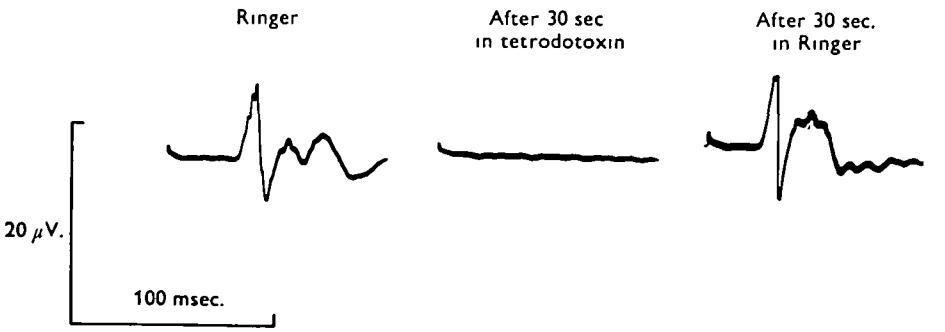

Fig. 7. The effects of 5×10^{-7} M tetrodotoxin on the production of action potentials in the fast axons.

DISCUSSION

The form of the compound action potential recorded in the cerebro-visceral connectives of *Anodonta* is essentially similar to those obtained in *Cristaria* (Nakajima, 1961). In this latter bivalve the compound action potential showed a large component with a conduction velocity in the range 0·03–0·04 m.sec.$^{-1}$ with a smaller fast component in the range 0·3–0·4 m.sec.$^{-1}$. These correspond to the values for the slow and fast components obtained in the *Anodonta* connective. As in *Cristaria* (Nakajima,

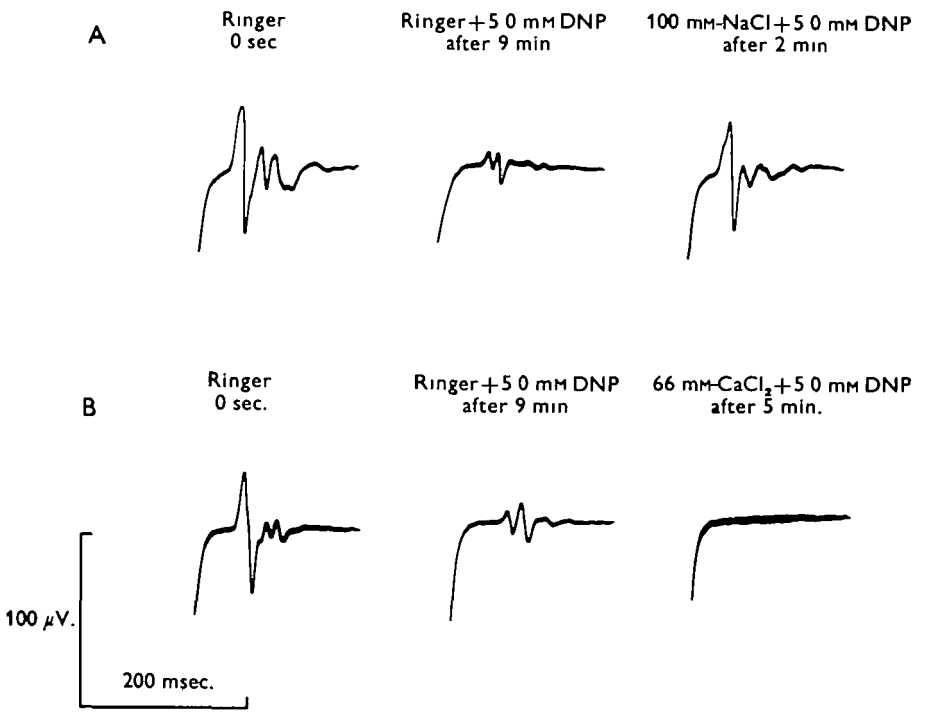

Fig. 8. The effects of 2:4-dinitrophenol on the fast components of the compound action potentials and the subsequent effects of elevated concentrations of NaCl and $CaCl_2$.

1961) the form of the compound action potential can be related to the size distribution of axons in the connective of *Anodonta*, where the majority of axons are below 2 μ in diameter with only a sparse distribution of larger axons of up to 2–6 μ in diameter (see also Gupta *et al.* 1969).

The results show that isolated cerebro-visceral connectives were able to conduct action potentials for appreciable periods when bathed in blood or Ringer solutions. This suggests that the fluid contained in the compartment formed by the epithelium of the proximal wall of the kidney does not produce a specialized ionic environment which is essential for the maintenance of axonal function. *Anodonta* appears to be similar in this respect to the phytophagous insect *Carausius morosus*, which also has a very low blood sodium level. In this latter species, which also has an additional fluid compartment surrounding the nerve cord formed by the neural fat-body sheath

(Maddrell & Treherne, 1966), electrical activity of the connectives continued in the absence of the fat-body sheath when bathed in *Carausius* Ringer (Treherne & Maddrell, 1967*a*, *b*).

The evidence presented above would seem to indicate that axonal function is achieved by sodium-dependent mechanisms. This is particularly evident in the case of the slowly conducting fibres in which it was shown that there was a relatively rapid decline in amplitude of the compound action potentials in sodium-free solution. The time course of this effect approximated to that observed in connectives of the leech, *Hirudo medicinalis*, in which it has been shown that there is a ready movement of sodium ions away from the axon surfaces in preparations bathed in sodium-free solutions (Nicholls & Kuffler, 1964).

An unexpected feature of these results was the observation that the rapidly conducting fibres continued to function for extended periods in preparations bathed with isotonic sucrose solutions. The available evidence would seem to indicate, however, that these fibres also function by sodium-dependent mechanisms. Thus, for example, a conduction block can be produced by addition of dinitrophenol or ouabain to the bathing medium. This state of affairs contrasts with that observed in squid giant axons which function for appreciable periods in the presence of ouabain in the bathing medium (Caldwell & Keynes, 1950). The very much more rapid effect obtained with the *Anodonta* preparation can presumably be correlated with the relatively large surface/volume ratio of these small axons in which it might be reasonably supposed that supression of the sodium pump would result in a more rapid abolition of the sodium gradient across the axon membranes. The important point in the present experiments is that elevation of the external sodium concentration caused a temporary return of axonal activity even though the inhibitor molecules were still present in the bathing medium. This result is most easily explained in terms of a temporary re-establishment of a gradient of sodium ions across the axon membranes at the higher external concentration of this cation. Equivalent experiments using elevated concentrations of calcium ions did not, however, result in a return of the action potentials which suggests that this divalent cation is not involved in the production of the action current in this species. The latter experiment also showed that increased osmotic pressure of the bathing medium, in 100 mM-Na, was not an important factor in re-establishing conduction. The presence of a sodium-dependent mechanism in action-potential production can also be inferred from the effects of dilute concentrations of tetrodotoxin on the rapidly conducting fibres. This compound is known to affect the production of action potentials by its specific action on the sodium conductance channels of the axon membrane (cf. Narahashi, Moore & Scott, 1964).

The conclusion that calcium ions do not appear to carry the inward component of the action current in the fast axons in the connective of *Anodonta* contrasts with the suggestions which have been made in regard to some molluscan giant neurones. In some gasteropod neurones, for example, it has been suggested that the inward current may be wholly or partly carried by calcium ions (Gerasimov, Kostyuk & Maiskii, 1965; Kerkut & Gardner, 1967). However, more recent investigations have lent support to the idea that, despite their ability to function in sodium-free solutions, the gasteropod giant neurones may utilize sodium ions as the major component in carrying the inward current associated with the action potential (Chamberlain & Kerkut, 1967;

Moreton, 1968). The present data does not, therefore, present any major differences when compared with the later results obtained with gasteropod giant neurones.

The very rapid return of the fast action potentials, on application of high sodium concentrations to poisoned preparations in the present study, suggests that, as with the small fibres, there is a rapid access of this cation to the axon surfaces. Similarly, the rapid and reversible effects of tetrodotoxin also suggest a ready access to the surfaces of the rapidly conducting fibres.

The postulation of a relatively free access of ions and molecules to the axon surfaces is in accord with fine structural organisation of the connectives of *Anodonta*. A recent electron microscopic investigation (Gupta *et al.* 1969) has shown that there is no specialized cellular layer underlying the connective tissue sheath comparable to that described in insects (cf. Smith & Treherne, 1963). There is also no evidence of any cell junctions in the thin layer of glial processes which separate the axon bundles from the connective tissue sheath. The only type of junction observed between the glial processes in the connectives is of the *macula adherens* type of desmosomal junction which do not apparently restrict the movements of small water-soluble ions and molecules (Farquhar & Palade, 1963). It was thus concluded that there are no visible structures which are likely to restrict the intercellular movements of inorganic ions between the bathing medium and the axon surfaces (Gupta *et al.* 1969).

The above structural findings are, however, difficult to reconcile with the present observation that the rapidly conducting fibres are able to function for extended periods in connectives bathed with isotonic sucrose solutions. Furthermore, the very sparse distribution of glial elements in these connectives (Gupta *et al.* 1969) does not allow the postulation of a dynamic extra-axonal sodium regulation by the glial cells such as has been suggested in the central nervous system of the insect, *Carausius morosus* (Treherne & Maddrell, 1967*b*; Treherne, 1967).

It is now relevant to consider the changes in conduction velocity of fast action potentials in preparations maintained in sucrose or dextran solutions. The experiments in which the conduction velocity was measured in connectives maintained in mineral oil show that this effect did not appear to result from the changes in the conductivity of the bathing medium. It follows from this that the alterations in conduction velocity in these non-electrolyte solutions must have resulted from alteration in some physiological parameters within the central nervous tissues. Now in a conventional system in which the volume of the conducting fluid is small, as would be the case with the extracellular fluid in the connectives, the conduction velocity would be expected to be proportional to $(r_i+r_e)^{-\frac{1}{2}}$ (Hodgkin, 1954) where r_i is the axo-plasmic resistance and r_e the resistance of the extracellular fluid per unit length. It would thus be possible to explain the decrease in conduction velocity in the isotonic sucrose solution, which produces an uptake of water from the connectives (Carlson & Treherne, 1969), as being due to an increase of r_e resulting from the dilution of extracellular ions. Similarly, the high conduction velocity in dextran can be attributed to the decrease in r_e resulting from a concentration of extracellular ions produced by a withdrawal of water from the central nervous tissues (Carlson & Treherne, 1969).

The problem still remains, however, as to the precise mechanism by which action potentials are maintained in the fast fibres with sodium-free solutions in view of the apparent accessibility of the axon surfaces to the external medium. As has already

been pointed out this difficulty is increased by the paucity of glial elements in the connectives which would seem to preclude the possibility of postulating any dynamic local glial regulation of the extra-axonal sodium. There are several possibilities which could be advanced to explain the remarkable ability of the fast axons to function in the absence of sodium ions in the external medium. It could be argued, for example, that a relatively high extracellular sodium level is maintained by Donnan forces due to the presence of large organic anions in the extracellular fluid. In such a system, however, there would be some reduction in the activity coefficient of the sodium ions maintained by Donnan forces in preparations bathed in sucrose solutions (cf. Treherne, 1967) which might militate against the acceptance of this postulated physiological mechanism. Alternatively, it could be argued that in sodium-free solutions the action current switches from a mechanism involving the conventional influx of sodium ions to one involving an efflux of intracellular anions. These and other possibilities are being actively pursued in this laboratory and it is hoped that further work will throw some light on the possible physiological mechanisms involved.

SUMMARY

1. The compound action potentials recorded in cerebro-visceral connectives consisted of a large homogeneous slow component, with a conduction velocity of between 0·03 and 0·04 m.sec^{-1}, together with a variable rapidly conducting component, showing spikes with maximum velocities in the region of 0·4–0·5 m.sec^{-1}.

2. Comparison of the square of the conduction velocity, for the various components of the compound action potential, with distribution of axon diameters in the connective showed that the small axons (0·1–0·3 μ in diameter) contributed to the slow component, the larger axons (2·0–6·0 μ in diameter) forming the initial rapidly conducting spikes.

3. The small axons showed a rapid loss of function in preparations bathed in isotonic sucrose solutions. The larger axons, however, continued to function for appreciable periods in isotonic solutions of non-electrolytes.

4. The larger axons were rapidly and reversibly blocked by dilute tetrodotoxin. Additional evidence is also presented which suggests that the action potentials associated with the axons are sodium-dependent and do not depend upon any appreciable involvement of calcium ions in carrying the action current.

5. Both the large and the small axons appear to be relatively accessible to small ions and molecules in the bathing medium. The results are discussed in relation to the possible physiological mechanisms involved in the function of the larger axons in sodium-free solutions.

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