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THE PERMEABILITY TO IONS OF THE NEURAL LAMELLA AND THE EXTRACELLULAR SPACES IN THE C.N.S. OF *ANODONTA CYGNEA*

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SUMMARY

Rapid axonal depolarization follows the elevation of $[K^+]_o$ in the Ringer bathing the surfaces of the connectives of both *intact* and *desheathed* cerebro-visceral connectives of *Anodonta cygnea*. Potassium movements between the blood or medium bathing the surface of the connectives and the axonal membranes can be accounted for in terms of a first-order diffusion process. No visible structural barriers to prevent free movements of materials through the neural lamella and the extracellular spaces can be detected in electron micrographs of the cerebro-visceral connectives. In *desheathed* preparations fast action potentials are conducted in sodium-free (dextran) Ringer. Mechanisms of axonal function and ionic regulation in this and other invertebrate central nervous tissues are discussed in the light of these observations.

INTRODUCTION

Studies on the accessibility of the extracellular spaces within the central nervous connectives of *Anodonta cygnea* (L.) have produced a number of apparently conflicting results. It has been shown that the bulk of the axons (0·1–0·3 μm diam.) in the cerebro-visceral connectives are accessible to sodium ions in the bathing medium (Treherne, Mellon & Carlson, 1969; Mellon & Treherne, 1969). Nevertheless, a small population of axons (2·0–6·0 μm diam.) are able to conduct sodium-dependent action potentials when the connectives are maintained for several hours in sodium-free solutions (Treherne, Mellon and Carlson, 1969; Carlson & Treherne, 1969). A number of possible explanations of this phenomenon have been discussed. On the basis of the experimental evidence to date two of these possible theories deserve further consideration.

First, based on electrophysiological experiments, exchanges of radiolabelled sodium, and electronmicroscopical studies a sodium-store in close proximity to the large axons is postulated to account for the maintenance of conduction under sodium-free conditions (Carlson & Treherne, 1969; Mellon & Treherne, 1969; Treherne, Carlson & Gupta, 1969; Gupta, Mellon & Treherne, 1969). These studies also indicate the rapid access of sodium ions in the bathing medium to the surfaces of most of the axons in the connective. Such a finding is in good agreement with the apparent absence of any features of the ultrastructure of the central nervous connectives which might either

physically occlude or in other ways regulate the permeability of the extracellular spaces to ions (Treherne, Carlson & Gupta, 1969; Gupta *et al.* 1969). Also, the demonstration that the exogenous tracer substance horseradish peroxidase (M.W. 40 000) penetrates the neural lamella and extracellular spaces of the cerebro-visceral connectives, indicates that the axonal surfaces should be readily accessible to ions and small water soluble molecules in the blood or bathing medium (Lane & Treherne, 1972).

Secondly, based on experiments in which the nerve sheath of the *Anodonta* connective is surgically removed, thereby abolishing conduction of fast axons in sodium-free Ringer, it has been suggested that the role of the nerve sheath in the regulation of sodium ions in the extracellular environment of axons should be reconsidered (Twarog & Hidaka, 1972). These authors concur with the results of previous workers that the sheath does not constitute an absolute barrier to ion diffusion but suggest the following three possible mechanisms by means of which the sheath could be implicated in sodium regulation: (*a*) active regulation of ion concentrations by the nerve sheath, (*b*) mechanical limitation of swelling by elastic properties of the sheath, thereby restricting ion loss and limiting variations in extracellular space, and (*c*) protection by the sheath of the structural integrity of the glia which have a special regulatory function.

Electrophysiological studies to date have primarily involved the use of extracellular recordings of axonal spikes. Since both fast and slow axons appear to be strongly dependent on sodium ions for the generation of the action current, changes in spike amplitude and relative conduction velocity have been used to indicate the changes in the concentration of sodium ions in the extracellular fluid bathing the axon surfaces. We have determined the level of polarization of the connective at various external potassium concentrations which coupled with estimates of the time course of potassium depolarization in *intact* and *desheathed* connectives reveals details of the kinetics of potassium movements through the cerebro-visceral connectives. The ability of the fast action potentials to conduct in sodium-free Ringer is examined in *intact* and *desheathed* connectives. The results of electrophysiological experiments are considered in the light of an ultrastructural investigation of the cerebro-visceral connectives. Our findings are discussed both in terms of current models for ionic regulation in the connectives of *Anodonta* and in the general context of the accessibility of neuronal elements to ions and small molecules in the fluid bathing the central nervous tissues of molluscs and other invertebrate animals.

MATERIAL AND METHODS

A. *Electron microscopy*

The cerebro-visceral connectives of *Anodonta cygnea* were prepared for electron microscopy by perfusing the sinus venosus with fixative for 20 min (McLaughlin & Howes, 1973). The fixative used was glutaraldehyde (Fisher Biological Grade, 50%) diluted to 2% with either 0·02 M-KH_2PO_4 or *Anodonta* blood. A portion of the cerebro-visceral connective 1–1·5 cm long which ran through the kidney was dissected free of the surrounding tissue and removed into a 2·5% buffered solution of glutaraldehyde for 1½ h. The buffers used were 0·02 M-KH_2PO_4, as above, or, for those pieces of connective prepared in *Anodonta* blood, 0·05 M-Na cacodylate. The tissue was then

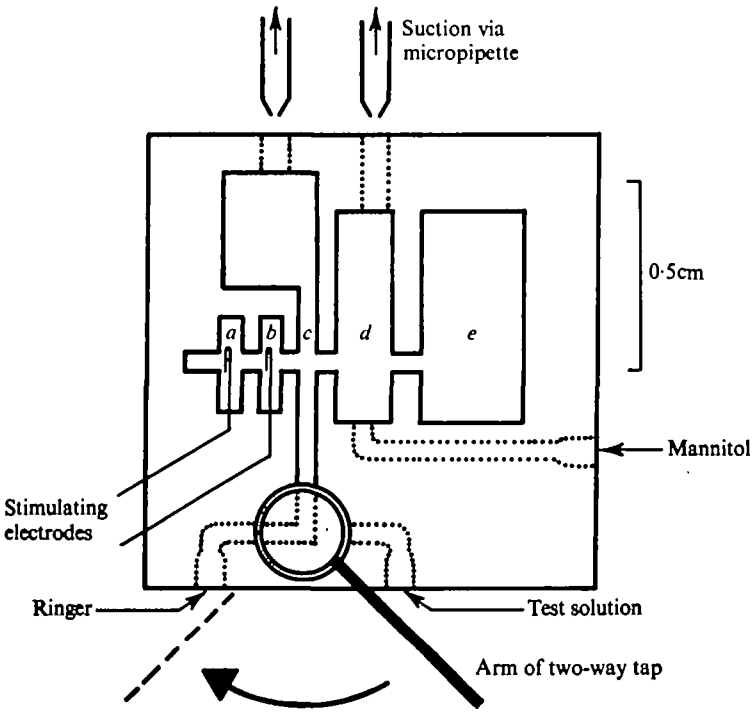

Fig. 1. Plan view of nerve chamber devised to obtain sucrose-gap recordings from cerebro-visceral connectives of *Anodonta cygnea*. Compartments of the chamber are denoted *a–e* and contain: *a*, *b*, stimulating electrodes; *c*, Ringer or test solution; *d*, mannitol; *e*, Ringer. The isolated connective is placed in the channel between *a* and *e*. Solutions in the test compartment *c* are changed by moving the arm of the two-way tap through 90°. Suction via micropipettes maintains constant levels in the perfused compartments *c*, *d*.

rinsed in several changes of buffer, cut into short (3 mm) lengths, post-fixed for 1 h in buffered 1% OsO_4 and dehydrated. After block staining for 1½ h in 1% ethanolic uranyl acetate (Westrum, 1965) the material was taken through propylene oxide and left in 1:1 propylene oxide/Araldite mixture overnight before embedding in Araldite.

Thin transverse and longitudinal sections were cut using glass knives on an LKB Ultrotome III and these were stained with lead citrate (Reynolds, 1963) prior to examination with a Philips EM 300 electron microscope.

B. *Electrophysiology*

Lengths of cerebro-visceral connectives, 2–3 cm, of *Anodonta cygnea* were dissected from the animal and ligatured at each end under normal Ringer. Preparations transferred to an experimental chamber at this stage were referred to as *intact*. In a number of experiments a cylindrical section of the neural lamella (and presumably a portion of the underlying glia and peripheral axons) was removed by microdissection using finely sharpened stainless-steel needles. Connectives treated in this way were referred to as *desheathed*.

Changes in polarization of axons in the connective were studied using a modified sucrose-gap method. The nerve chamber was constructed from a perspex block with

compartments *a–e* as illustrated in Fig. 1. With the connective positioned along the central groove between *a* and *e*, the five compartments were isolated with petroleum-jelly seals, and the preparation covered with normal Ringer. Platinum stimulating electrodes were inserted into *a* and *b*. The test compartment *c* was perfused by normal Ringer or a test solution either of which could be delivered from elevated reservoirs according to the position of the two-way tap. When *desheathed* preparations were used, the *desheathed* portion of the connective was located in compartment *c*.

Compartment *d* was perfused with a mannitol solution (42 mM/l). Constant fluid levels were maintained in compartments *c* and *d* by suction pipettes. Compartment *e* contained normal Ringer. Ringer–agar bridges linked *c* and *e* to Ag–AgCl electrodes by means of which d.c. potentials were recorded. The electrodes were linked to an impedance converter of high input impedance. Electrical signals were displayed using a Smith Servoscribe potentiometric recorder and a Tetronix 565 oscilloscope. With good chloridation a potential of less than 1 mV was recorded between the two Ag–AgCl electrodes. Compound action potentials were recorded from cerebro-visceral connectives of *Anodonta* using techniques described in an earlier study on the central nervous connectives of the prosobranch gastropod *Viviparus contectus* (Sattelle, 1972).

The normal Ringer used in these experiments had the following composition: NaCl 14·0 mM/l; KCl, 0·5 mM/l; $CaCl_2$, 5·0 mM/l; Na_2HPO_4, 0·25 mM/l; glucose, 1·0 mM/l; pH 7·5 (Potts, 1954). High-potassium Ringer solutions were produced by simply replacing NaCl by KCl to maintain isotonicity. Either tris chloride or dextran were used to replace sodium in the sodium-free Ringer solutions.

RESULTS

A. *Fine structure of cerebro-visceral connective*

The two methods of fixation used in this study produce a similar degree of preservation of the tissues; the dimensions of the axons and of the extracellular spaces in each case are the same.

The connectives of *Anodonta cygnea* are approximately 0·25 mm in diameter and are surrounded by an acellular neural lamella some 10 μm thick. In places the width of the neural lamella increases to form wedge-shaped areas which penetrate a short distance (15 μm) into the connective (see Fig. 2*a*). Beneath the neural lamella lies a thin, incomplete ring of glial processes, some of which are elongated and penetrate inward towards the centre of the connective. These processes cluster most thickly and penetrate most deeply beneath the wedge-shaped invaginations of the neural lamella. In addition, there are some glial elements scattered at random throughout the tissues but in the sections studied the glial population never exceeded 12% of the total area enclosed by the neural lamella. The glial processes are all simple structures and there is no glial wrapping around axons.

The axons fall into three categories: small axons (below 1 μm diameter) are the commonest, but a number of larger axons (2–6 μm diameter) are found scattered amongst them, and these large axons are most numerous near the centre of the connective (see Fig. 2*b*). The third axonal type is relatively rare and is found near the periphery of the connective; this type contains a large number of dense-core vesicles and is believed to be neurosecretory in function.

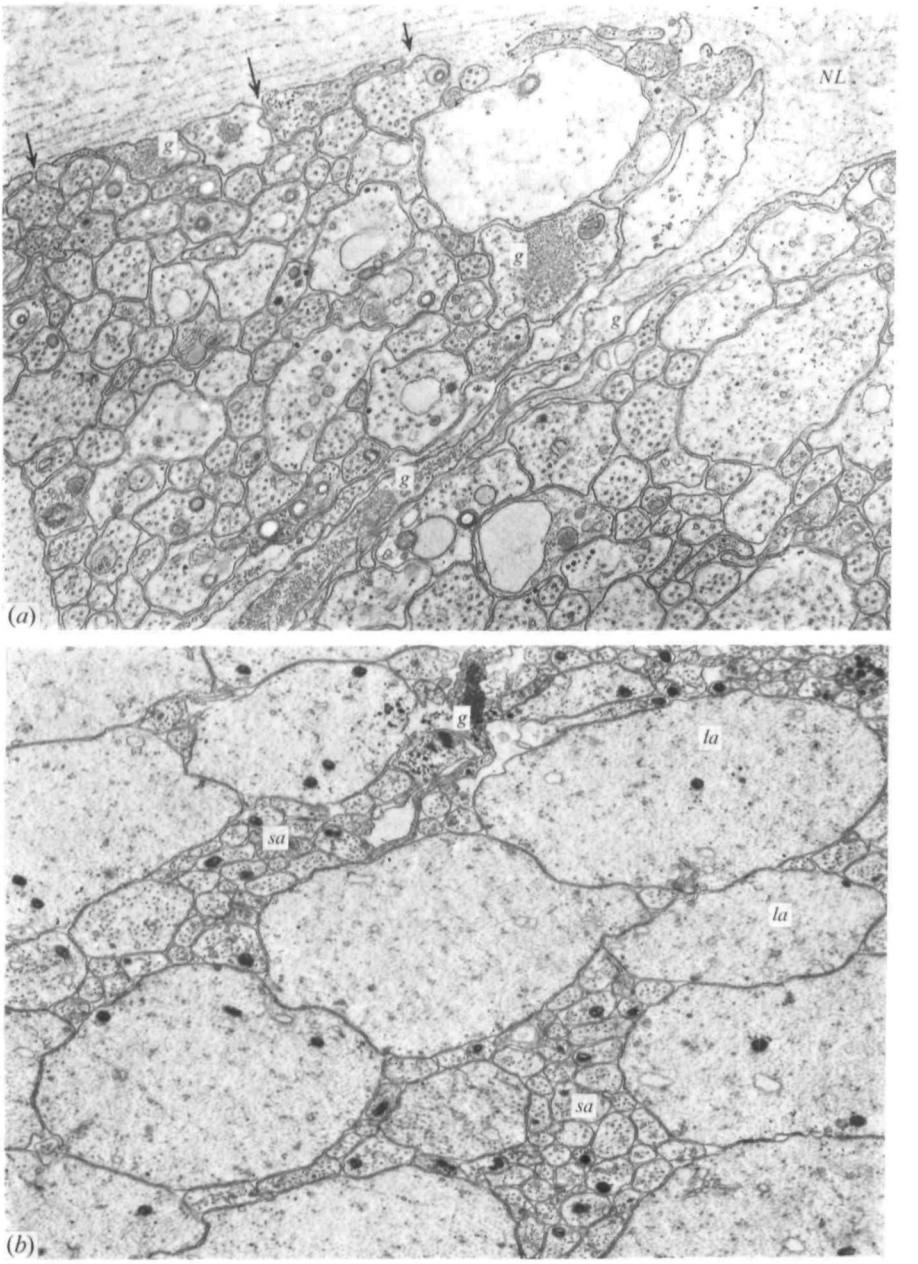

Fig. 2. (*a*) Electron micrograph of a cross-section through the edge of the cerebro-visceral connective of *Anodonta*. On the right of the picture the neural lamella (*NL*) projects a short distance into the connective forming a cleft which is lined by long glial processes (*g*). A number of other glial elements occur at the periphery. The extracellular system appears to open directly on to the surface of the connective (arrows). × 19500.

(*b*) Electron micrograph of a cross-section at the centre of the cerebro-visceral connective of *Anodonta*. A number of large axons (*la*) are interspersed with bundles of small axons (*sa*); glial representation (*g*) is sparse. The extra cellular space is restricted to a narrow channel (150 Å diameter) around the axons except in the region of the glial processes where it forms large spaces (1000 Å diameter). × 13000.

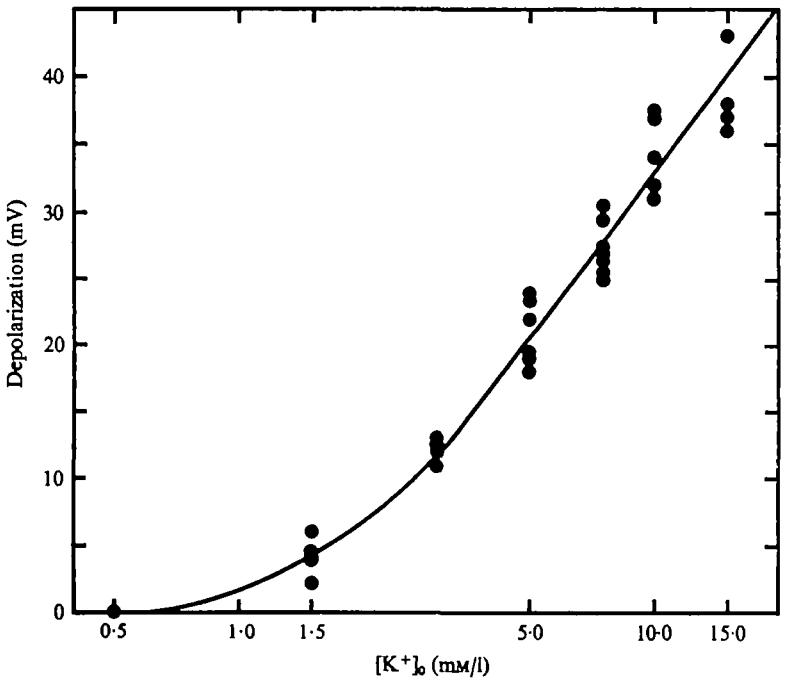

Fig. 3. Graph of depolarization against the logarithm of the external potassium concentration $[K^+]_o$. Data obtained from seven *intact* cerebro-visceral connectives.

The axons are closely packed and the extracellular space is usually restricted to a channel around the axon some 150–250 Å wide. In some areas, particularly around the glial invaginations and in places where a number of axons share a common extracellular channel, the space may be considerably larger (up to 1500 Å). There is no visible structural barrier to prevent free movement of materials through the extracellular system, and it is possible to trace an uninterrupted pathway from one side of the connective to the other through the extracellular spaces.

Any materials passing from the surface of the connective to its centre by way of the extracellular system, must follow a convoluted pathway whose length is 30–40% greater than the radius of the connective.

B. *Electrophysiology*

1. *Sucrose-gap*

Effects of potassium ions on axonal polarization. When the external potassium concentration $[K^+]_o$ of the Ringer solution bathing an *intact* connective was elevated a rapid depolarization ensued which at high concentrations was accompanied by the blocking of sucrose-gap recorded action potentials. The observed depolarization appeared therefore to be axonal in origin rather than deriving from an extra-neuronal locus as was demonstrated in the *intact* connectives of the insect central nervous system (Pichon & Treherne, 1970; Pichon, Moreton & Treherne, 1971; Pichon, Sattelle & Lane, 1972). Seven *intact* connectives were exposed to various levels of $[K^+]_o$ between 0·5–15·0 mM/l, and the level of depolarization noted following

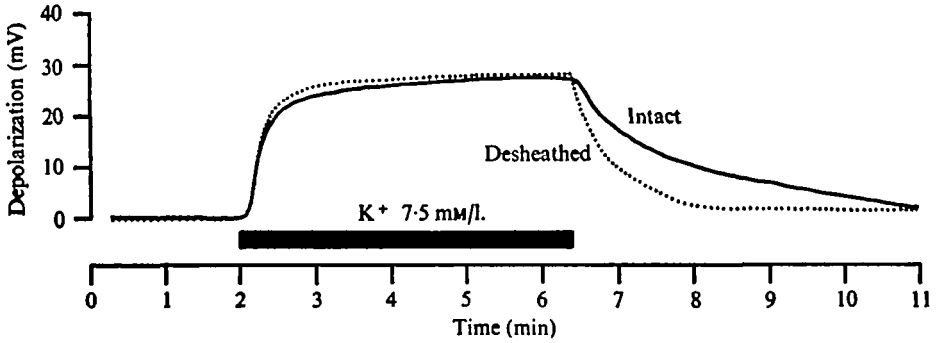

Fig. 4. Time-courses of potassium depolarization for a cerebro-visceral connective in both *intact* and *desheathed* condition. During the period indicated by the black bar, $[K^+]_o$ = 7·5 mM/l. For the remainder of the experiment, $[K^+]_o$ = 0·5 mM/l.

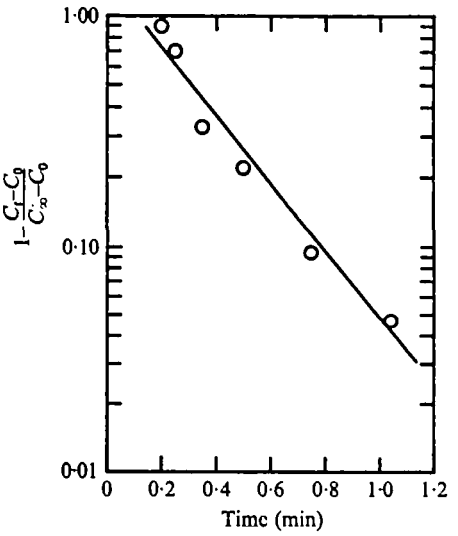

Fig. 5. Rate of change of potassium concentration in the extracellular system of an *intact* cerebro-visceral connective following the elevation of the potassium concentration in the bathing medium from 0·5 to 3·0 mM/l. C_t, estimated concentration of potassium in the extracellular system at time t (min.) following the exposure to high potassium. C_0, initial and C_∞, final concentration of potassium in the extracellular system. It is assumed that at C_0 and C_∞ the concentrations of potassium in the extracellular system are the same as those in the bathing medium.

equilibration to the new level of polarization. The results are summarized in Fig. 3. Over the straight line section of the graph a tenfold change in the potassium concentration corresponded to a 43 mV change in polarization. This departs appreciably from the 58 mV potassium slope that would be predicted for an ideal potassium electrode.

Effects of desheathing on the time-course of depolarization. Rapid depolarization normally followed the exposure of *intact* connectives to an increase in $[K^+]_o$. A number of experiments were performed on *intact* connectives in which $[K^+]_o$ was first increased from 0·5 to 7·5 mM/l and subsequently returned to the initial concentration. Using data from four connectives the mean half-time ($t_{0\cdot5}$) for the depolarization (25–30 mV)

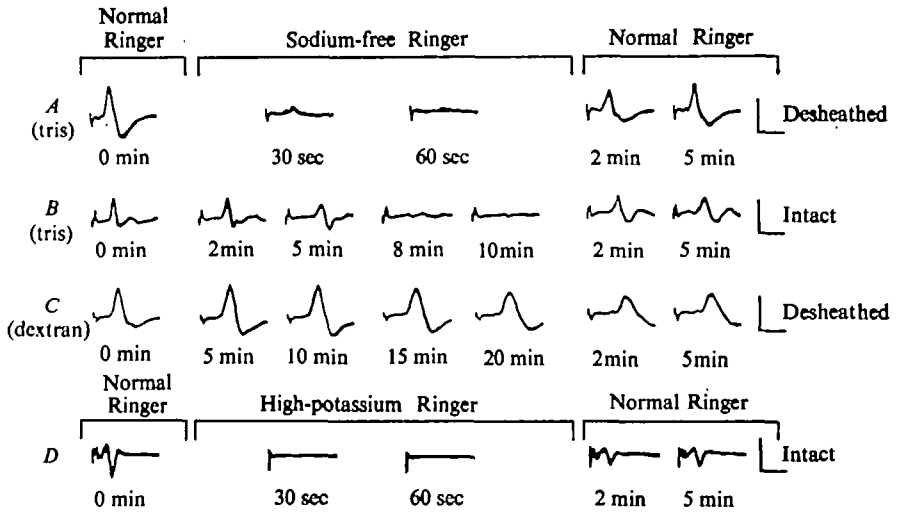

Fig. 6. Extracellular hook-electrode recordings of fast action potentials (*A*, *B*, *C*) and complete compound action potentials (*D*) from *intact* and *desheathed* connectives of *Anodonta*. Scale bars indicate: *A*, 10 μv (vertical), 100 msec (horizontal); *B*, 10 μV, 50 msec; *C*, 10 μV, 50 msec; *D*, 25 μV, 250 msec. Each record is made up of ten superimposed sweeps.

was 16·5 (S.E. ± 6·0) sec, and $t_{0\cdot5}$ for repolarization was 28·0 (S.E. ± 14·5) sec. In two of these experiments following the recovery from a high-potassium pulse, the connective was *desheathed in situ* and a further potassium pulse was applied (Fig. 4). In both cases there appeared to be a very slight decrease in the time taken to attain the new steady level of polarization after *desheathing*.

Kinetics of potassium ion movements in the connective. Using data presented in Fig. 3 to convert the changes in axonal polarization with time during depolarization into changes in concentration with time, it is possible to assess the rate of change of potassium ion concentration at the axonal surfaces. Data are plotted in Fig. 5 from an experiment in which an *intact* connective was exposed to a Ringer containing 3·0 mM/l KCl having previously been bathed in normal Ringer (0·5 mM/l KCl). The straight-line plot indicated that potassium movements between the blood, or medium bathing the surfaces of the connectives and the axonal membranes could be accounted for in terms of a first-order diffusion process.

2. *External electrode recording of compound action potentials*

Effects of high-potassium Ringer. When $[K^+]_o$ in the Ringer solution bathing *intact* connectives was increased from 0·5 to 9·0 mM/l both fast and slow components of the compound action potential were blocked (Fig. 6). Since these correspond to the two populations of respectively larger and smaller diameter axons (Treherne, Mellon & Carlson, 1969), there appears to be no restriction of access of potassium ions to either group of fibres.

Effects of sodium-free Ringer. A number of experiments were performed on the effects of sodium-free solutions on the fast action potentials of *Anodonta*, recorded from *intact* and *desheathed* connectives (Fig. 6). With tris as the substitute for sodium, *desheathing* considerably enhanced the decrease in conduction velocity of these fibres.

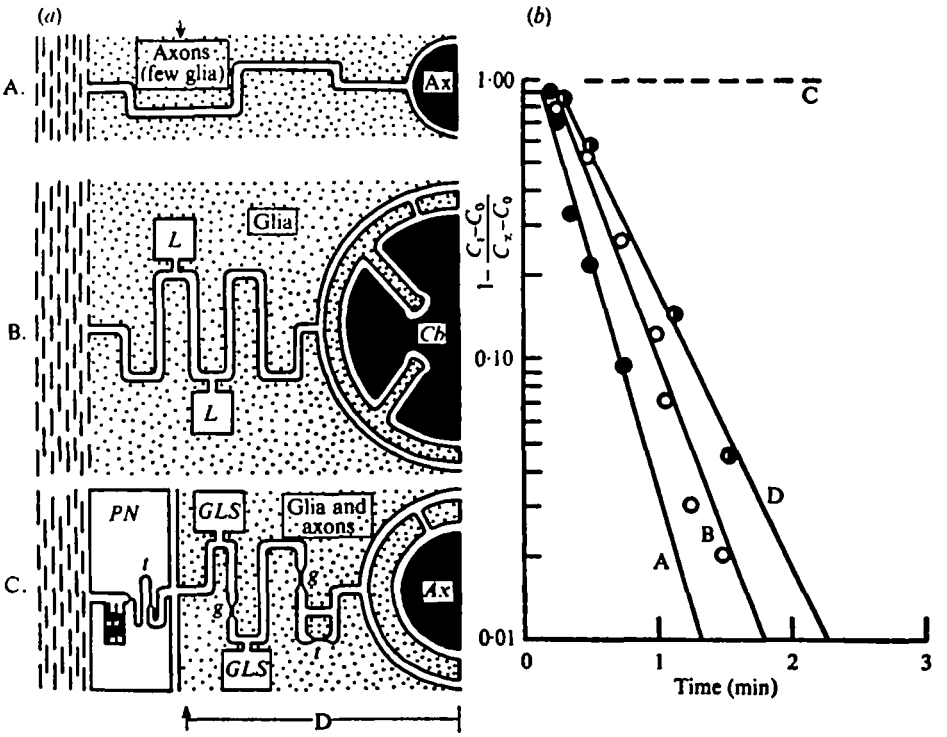

Fig. 7. (*a*) Schematic representation of the main features of the extracellular pathways in the central nervous systems of (A) *Anodonta cygnea* (connective), (B) *Limnaea stagnalis* (ganglion) and (C) *Manduca sexta* (connective). (D) represents the *desheathed* connective of *Manduca*. Tissues to the left of the arrows are removed in *desheathing*. Abbreviations: *Ax*, axon; *Cb*, cell body; *g*, gap junction; *GLS*, glial lacuna system; *L*, lacuna; *PN*, perineurium; *t*, tight junction. These simplified models are based on electron microscopial studies on the central nervous tissues of these invertebrate organisms. In addition to data reported here, information from the following publications has been used: *Limnaea* (Sattelle & Lane, 1972; Sattelle, 1973*b*, 1974) and *Manduca* (Lane, 1972; Sattelle, Pichon & Lane, 1972).

(*b*) Plots of the rate of change of potassium concentration in the extracellular spaces of various invertebrate central nervous tissues: A, *intact* cerebro-visceral connective of *Anodonta cygnea* (data from this report); B, *intact* parietal ganglion of *Limnaea stagnalis* (data from Sattelle, 1973*b*); C, *desheathed* connective of *Manduca sexta* (data from Pichon *et al.* (1972) and Sattelle (unpublished observations)); D, *intact* connective of *Manduca sexta* (inferred from data in Pichon *et al.* 1972).

With dextran as the substitute for sodium, previous authors had noted the persistence of fast action potentials for extended periods in *intact* connectives. We note that in *desheathed* connectives fast action potentials continue for extended periods with only a slight decrease in conduction velocity. Clearly, whatever the mechanism responsible for maintaining action potentials under sodium-free (dextran) conditions it does not demand that the neural lamella and outer glial layers remain *intact*.

DISCUSSION

Depolarization of axons in the cerebro-visceral connectives of *Anodonta cygnea* rapidly follows elevation of the potassium ion concentration in the medium bathing surfaces of both *intact* and *desheathed* preparations. This indicates that the neural

lamella and any peripheral glial and axonal elements removed by the *desheathing* process do not appreciably restrict the access of axonal surfaces to potassium ions in the bathing medium. Consistent with this finding is the calculation of the rate of change of potassium concentration at the axonal surfaces, following an increase in the concentration of this ion in the Ringer bathing an *intact* connective. The results suggest that potassium movements between the blood (bathing medium) and the axonal surface can be accounted for in terms of a first-order diffusion process. Also, observations reported here and in previous studies on the ultrastructure of connectives revealed no structural barriers to the free movements of materials through the extracellular spaces (see also Gupta *et al.* 1969; Treherne, Carlson & Gupta, 1969). It is therefore concluded that potassium ion levels in the extracellular spaces of the cerebro-visceral connectives of *Anodonta cygnea* rapidly follow and equilibrate to changes in the level of this ion in the blood or bathing medium. The pathway for potassium ion exchange appears to be via the extracellular channels and the neural lamella, neither of which substantially restrict the free diffusion of this cation.

These observations are in accord with findings for the central nervous tissues of other molluscan species. The extracellular spaces in the ganglia of the gastropod molluscs *Helix aspersa* (Moreton, 1972), *Limnaea stagnalis* (Sattelle & Lane, 1972; Sattelle, 1973*b*) and *Anisodoris nobilis* (Mirolli & Gorman, 1973) have been shown to be highly permeable to potassium ions. The situation in the *intact* central nervous tissues of these molluscs approximates to that observed in *intact* connectives of the leech *Hirudo medicinalis* (Nicholls & Kuffler, 1964) and in *desheathed* connectives of certain insects. For example, the movements of potassium ions through *desheathed* interganglionic connectives of *Manduca sexta* follow first order diffusion kinetics (see Fig. 7). A slightly more complex situation is noted for the interganglionic connectives of the cockroach *Periplaneta americana*. Here, potassium exchanges between axonal surfaces and the fluid bathing the *desheathed* connective are too rapid to be accounted for by simple diffusion through the extracellular channels (Treherne *et al.* 1970). Clearly in the *desheathed* cockroach connective some additional (possibly intracellular) diffusion pathway has been created by the *desheathing* procedure (Treherne *et al.* 1970; Treherne & Pichon, 1972). The situation obtaining in cockroach connectives which have received less drastic surgery (e.g. stretching or briefly drying previously *intact* connectives) is that the calculated half-times for potassium ion accumulation at the axonal surfaces agree well with the half-time predicted for the free diffusion of this ion through the convoluted extracellular spaces. Fig. 7 attempts to compare the results of accessibility studies on the central nervous tissues of *Anodonta cygnea*, *Limnaea stagnalis* and *Manduca sexta* with a schematized representation of the main features of the extracellular systems of these tissues. Clearly as the complexity of the extracellular pathway increases, the time taken for potassium ions to accumulate at the neuronal surface following an elevation of the potassium level in the bathing medium also increases.

Observations on the effects of sodium-free solutions on the fast action potentials of *Anodonta* do not appear at first sight to be easily reconciled with the demonstration of an extracellular system within the cerebro-visceral connectives that is highly permeable to potassium ions. For example, *desheathing* the connective considerably enhances the decline of the fast action potentials when exposed to sodium-free (tris) Ringer.

The slower decline of the fast action potentials in *intact* connectives under similar conditions may possibly be explained in terms of a source of sodium only available to the fast axons when the integrity of those structures destroyed by the *desheathing* process is maintained. Alternatively, since the slow action potentials decline rapidly in tris Ringer even in *intact* connectives (Treherne, Mellon & Carlson, 1969), such findings may be compared to the differential rate of conduction block in sodium-free Ringer according to axonal diameter noted in vertebrate axons. For example, in the case of myelinated and non-myelinated axons of the spinal roots of the cat, the concentrations of sodium in the bathing medium which just block conduction are lowest for the faster conducting (larger diameter) axons (Nathan & Sears, 1960, 1962). Similar findings are reported for the sciatic peroneal nerves of the toad *Bufo vulgaris*. In this case the sodium concentration in the bathing medium just necessary to maintain conduction of the A fibres is between 2% and 10% of the sodium concentration in normal Ringer (Uehara, 1958, 1960). It may be that exposing *Anodonta* connectives to sodium-free (tris) Ringer and the consequent reversal of the sodium gradient across the axonal membrane results in leakage of sodium ions from the cells. Under these conditions it may take a few minutes to lower the extracellular concentration of sodium to below the threshold concentration for conduction of action potentials.

A more striking observation is the ability of the larger diameter (fast) axons to maintain action potentials in *desheathed* connectives bathed in sodium-free (dextran) Ringer. This finding appears to rule out active regulation of the sodium concentration within the connective by the nerve sheath (cf. Twarog & Hidaka, 1972). Also, since *desheathing* almost certainly damages peripheral glial elements, this result implies that the structural integrity of the glial system is not a prerequisite for the prolonged function of the fast axons in sodium-free (dextran) Ringer (cf. Twarog & Hidaka, 1972). In addition, it is difficult to conceive how the mechanical limitation of swelling by the elastic properties of the sheath contributes significantly to the extra-axonal regulation of sodium (cf. Twarog & Hidaka, 1972) when regulation occurs in *desheathed* preparations. There remains, therefore, a strong possibility that an extra-neuronal sodium store (probably located in the glial cell processes intimately associated with the larger axons) accounts for the behaviour of the fast action potentials in sodium-free (dextran) Ringer (Treherne, Carlson & Gupta, 1969; Gupta *et al.* 1969; Carlson & Treherne, 1969). At this stage, however, a direct effect of non-electrolytes on the permeability properties of cell membranes should not be ruled out as a contributory factor in any attempt to explain the persistence of sodium-dependent action potentials in sodium-free Ringer. In this context the results of a parallel investigation on axonal conduction in the pleural-supraintestinal connectives of the prosobranch gastropod *Viviparus contectus* are of interest (Sattelle, 1972, 1973*a*). It has been noted, for example, that increasing the molecular size of the non-electrolyte present in the sodium-free Ringer, results in a progressive lowering of the threshold sodium concentration which could maintain the fast action potentials. Also, a sucrose-induced leaking of ions from lobster axons has been reported (Pooler & Oxford, 1972). So, although the movements of potassium ions through the central nervous tissues of *Anodonta* can be accounted for by simple diffusion along the extracellular channels, this does not preclude the possible existence of specific mechanisms for regulating the levels of sodium ions in the same extracellular channels.

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