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FUNCTION OF THE NEURAL SHEATH IN MARINE AND FRESHWATER MOLLUSCS. EVIDENCE FOR RESTRICTION OF SODIUM LOSS IN FRESHWATER SPECIES

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INTRODUCTION

The sheaths that invest the nerves of amphibia, insects and freshwater clams differ in structure. Previous studies have shown, though, that the nerves of all these animals have in common the ability to function for extended periods in sodium-free solutions (Krnjević, 1954; Twarog & Roeder, 1956; Carlson & Treherne, 1969). In amphibia and insects, it has been shown that this capacity is lost if the neural sheath is removed (Krnjević, 1954; Twarog & Roeder, 1956). However, it has been claimed that the neural sheath of the freshwater clam – unlike those of insects and amphibia – has no function in the mechanism restricting the sodium loss that the nerve undergoes in a sodium-free medium (Carlson & Treherne, 1969; Treherne, Carlson & Gupta, 1969). This study will show to the contrary that removal of the neural sheath (de-sheathing) rapidly and reversibly sensitizes the nerve in freshwater clams to the reduction of sodium ion.

Since this finding strongly suggests that an intact neural sheath is just as vital to the restriction of sodium loss in the freshwater clam as it is in amphibia and insects, it would seem logical to seek out a common denominator of sheath function. Several possible ion-regulation mechanisms have been put forth, and the evidence for each is briefly examined. Although the mechanism itself is presently a matter of speculation, it seems likely that a deeper knowledge of it could well bring us another step closer to an understanding of the so-called 'blood-brain barrier'.

Structure of the neural sheath

In vertebrates, nerves and groups of nerve fibres are entirely enveloped by a perineural epithelium (Shantha & Bourne, 1968) consisting of squamous cells – often multilayered – that are associated with a basement membrane. The perineural epithelium is surrounded by a layer of dense connective tissue called the perineural connective tissue. The inner cellular layers and the connective tissue layer together constitute what is regarded as the neural sheath. Investing the individual axons are collagen fibres, fibroblasts and blood vessels. The collagen fibres, which bind the axons into groups, constitute a structure known as the endoneurium.

The insect central nervous system is entirely enclosed by a continuous cylinder of

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squamous cells that Scharrer (1939) called the perineurium. Outside this cellular layer is the neural lamella, a homogeneous layer of non-cellular connective tissue. Here, the insect neural sheath will be regarded as comprising the perineurium and the neural lamella.

The neural sheath of the freshwater mollusc is, as noted, structurally different from that of the vertebrate or the insect (Gupta, Mellon & Treherne, 1969). The nerve as a whole is contained by a thin surface layer of glial processes; surrounding this is a non-cellular, fibrous capsule called the neural lamella, which is made up of two zones. The inner zone is of closely packed connective tissue, into which glial processes radiate from the glial process layer. The outer zone contains loosely packed material, presumably mucopolysaccharide and a few collagen fibrils. Outside the neural lamella is a muscular tube that encloses most of the connective, forming a more or less complete cylindrical sleeve about it. In the present study, the term 'neural sheath' will refer to the entire covering of the mollusc's nerve – that is, the innermost layer of glial processes, the neural lamella, and the muscular sleeve – since sheath removal obviously eliminates or severely damages all the structures of the covering. The cerebrovisceral connectives in *Anodonta* lie in a blood space flanked by the epithelial walls of the kidney. These authors suggest that the kidney may regulate, on a long-term basis, the ionic content of the blood space.

METHODS

Cerebrovisceral connectives were dissected from two species of freshwater mussel – *Anodonta cataracta* and *Elliptio complanata* – and one species of marine mussel, *Mytilus edulis*. In each experiment a nerve segment of approximately 3 cm in length was placed across a lucite chamber with three compartments. The segment was so placed that portions of it were submerged in the solution in each compartment. (The very small exposed portions of the segment were covered with a protective coating of petroleum jelly.) In the right compartment one tip of the nerve segment was pulled into a suction electrode, and stimuli were applied between the suction electrode and an electrode in the saline. In the left compartment the other tip of the nerve segment was pulled into a second suction electrode, and the compound action potential was recorded between this suction electrode and an electrode in the saline. Test solutions were applied in the central chamber. Compound action potentials were uninterruptedly evoked by supramaximal stimulation at intervals of 1·3 sec; and these potentials were observed continuously over many hours, first in the sheathed nerve and later in the de-sheathed nerve.

The central-chamber segment was de-sheathed with a fine forceps, as described for insect nerve (Twarog & Roeder, 1956). In partial de-sheathing the sheath was torn open, exposing only the upper portion of the nerve and over a length of at least 5 mm. In complete de-sheathing the sheath was entirely removed from around the nerve over the 5 mm. The physiological saline for the freshwater mussels contained NaCl 15 mM, KCl 0·5 mM, $CaCl_2$ 3·0 mM, $MgCl_2$ 0·1 mM, buffered to pH 7·3 with Tris-HCl 1·0 mM. *Mytilus* saline was that of Twarog (1967*a*). In most of the experiments, sodium-free solutions were made up by replacing the NaCl with Tris-HCl buffer at pH 7·3. In a few experiments, isomolar sucrose was used instead of sodium chloride.

RESULTS

Fig. 1 shows that in the marine species, *Mytilus*, responses to stimulation in both the intact and the de-sheathed nerve were blocked after 2 min in a sodium-deficient solution. Restoration of normal sodium levels reversed the block in 5 min.

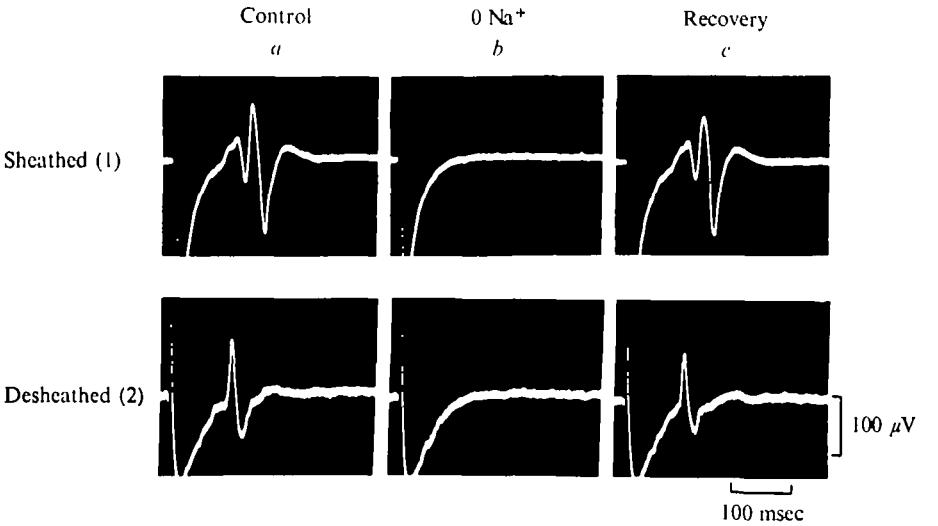

Fig. 1. Compound action potentials recorded from the cerebro-visceral connective of *Mytilus edulis* L. 1*a–c*: sheath intact; 2*a–c*: after de-sheathing. 1*a* and 2*a*: controls; 1*b* and 2*b*: after 2 min in 0 sodium saline; 1*c* and 2*c*: recovery, after 5 min. Calibrations: 100 μV, 100 msec.

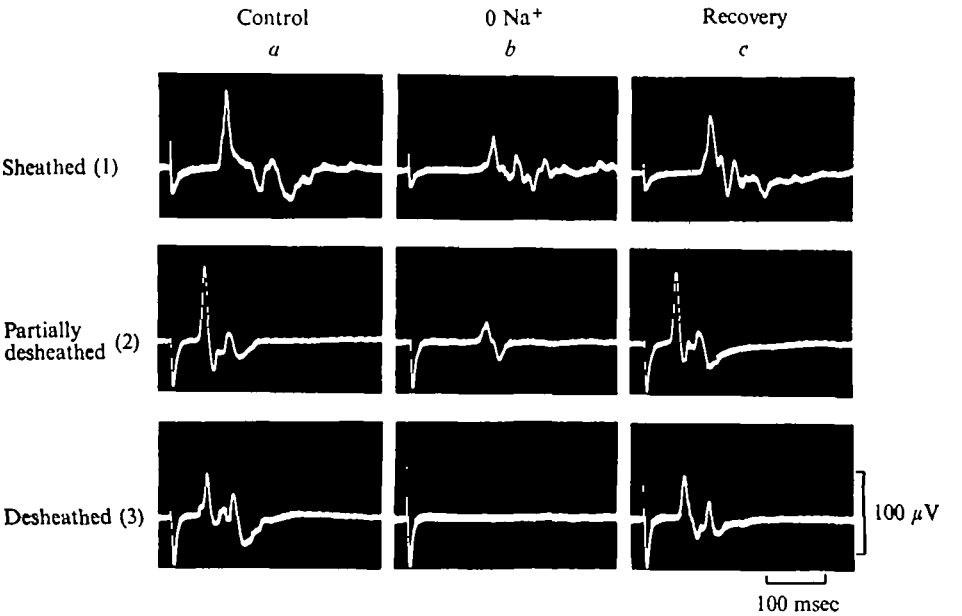

Fig. 2. Compound action potentials recorded from the cerebro-visceral connective of *Elliptio complanatus*. 1*a–c*: sheath intact; 2*a–c*: partially de-sheathed; 3*a–c*: completely de-sheathed. 1, 2 and 3*a*: controls; 1 and 2*b*: after 1 h in sodium-free solution; 3*b*: after 1 min in sodium-free solution; 1 and 2*c*: recovery after 10 min; 3*c*: recovery after 1 min. Calibrations: 100 μV, 100 msec.

Fig. 2 provides results obtained with *Elliptio*. A compound action potential was recorded before de-sheathing, after partial de-sheathing, and after complete de-sheathing. The action potential of the sheathed nerve altered only slightly after one hour in sodium-free solution. In the partially de-sheathed nerve, block was almost complete within 1 min, but certain nerve fibres characteristically continued to conduct for up to 1 h in sodium-free solution. The completely de-sheathed nerve, however, was blocked within 1 min in sodium-free solution. The same results were obtained with *Anodonta* nerve.

These results demonstrate that sodium deficiency causes a rapid, reversible block of the action potential in de-sheathed nerves of *Anodonta* or *Elliptio*. In the *Anodonta* nerve, high concentrations of manganese ions (10 mM) did not block the action potential, but tetrodotoxin (10^{-7} g/ml) did. In fact, tetrodotoxin blocked quite as rapidly and effectively whether or not the sheath was kept intact.

DISCUSSION

The evidence of the present study supports the conclusion of Carlson & Treherne (1969) that the action potential is generated by a sodium-dependent mechanism. However, the evidence presented in this paper implies that while an intact sheath does not limit tetrodotoxin penetration, it specifically restricts sodium loss. Contrary to the conclusions of Carlson & Treherne (1969), we conclude that for the regulation of sodium ions in the extracellular environment of the axons, an intact neural sheath is indeed as vital to *Anodonta* and *Elliptio* as it is to insects (Twarog & Roeder, 1956) and amphibia (Krnjević, 1954).

The nerve of the marine species, *Mytilus*, is invested with a tough, multilayered sheath; however, in sodium-deficient saline the action potential is blocked as rapidly when the sheath is intact as it is when the sheath is removed. As this would seem virtually to rule out the possibility that the *Mytilus* neural sheath supports a mechanism that restricts sodium loss, a detailed comparison of sheath function in the marine and freshwater bivalves would be of interest.

Carlson & Treherne (1969) have postulated that in *Anodonta* nerve: 'There is sequestered extra-axonal sodium fraction which can be utilized by the large axons to maintain action potentials in preparations bathed in sodium-free solutions.' But Treherne *et al.* (1969) rule out any role of the sheath in maintaining action potentials in such media: 'Electrophysiological and radioisotope evidence suggested that the ability to maintain action potentials in sodium-free solutions did not result from any appreciable restriction of ion movements to the axon surfaces.' They further state: 'Electron micrographs also showed no structures which would be likely to restrict intercellular ion movements between the bathing medium and the surfaces of the large axons.' Regarding ultrastructural evidence, Gupta *et al.* (1969) have demonstrated that no specialized cellular layer underlies the connective tissue sheath in *Anodonta*. Moreover, according to their studies, the thin layer of glial processes that separate the axon bundles from the neural lamella shows no specialized cell junctions other than the *macula adherens* type. Gupta *et al.* (1969) thus conclude that open extracellular channels exist between the glial processes.

However, the electrophysiological experiments of Carlson & Treherne (1969) were

not carried out on the de-sheathed nerve: they merely 'split' the sheath, having found it '...impossible to de-sheath the connectives...'. As the present study has found, however, partial de-sheathing is not equivalent to sheath removal. Moreover, regarding their radioisotope evidence, they reported that there is a small fraction of the total sodium content which exchanges very slowly, a fact which certainly suggests some restriction of sodium loss. Since they did not de-sheath, they did not test the effect of sheath removal on the rate of sodium exchange. Nor is the ultrastructural evidence (Gupta *et al.* 1969) decisive enough to rule out a functional role of the sheath. In the glutaraldehyde-fixed and osmium-fixed preparations shrinkage had occurred and was especially in evidence near the desmosomal attachments of neighbouring glial cells. And the glial terminals they describe are discernibly similar – in general organization at least – to the glial end-feet that surround the brain capillaries of vertebrates (Peters, 1962). These glial end-feet in vertebrates are typically connected by tight junctions at points where the extracellular space is either very narrow or, by virtue of membrane fusion, entirely absent. We suggest therefore, that the evidence for open channels in *Anodonta* is as yet inconclusive and that it remains quite possible that the surface layer of glial processes forms junctions that occlude the extracellular space.

Having found by direct experiment that an intact neural sheath is absolutely essential to maintain function in low-sodium bathing media, we question the findings and attendant assessments of Treherne *et al.* (1969) and Gupta *et al.* (1969). As stated earlier, we suggest that in *Anodonta*, as in vertebrates and insects, a firm grasp of the sheath function is necessary for an understanding of the ion-regulating mechanism; and there is as yet no evidence to support the existence of a mechanism that is unique to *Anodonta*. Possible mechanisms that have been suggested are listed and discussed below:

(1) *The sheath is an absolute barrier to ion diffusion.* This hypothesis would seem to have been ruled out by Treherne (1967) (see also Shantha & Bourne, 1968).

(2) *The cellular layer of the sheath actively regulates ion concentrations, presumably by selective ion pumps.* This regulatory function of the epithelium was first suggested for amphibia by Huxley & Stämpfli (1951). Krnjević (1954) and, more recently, Shantha & Bourne (1968) cited evidence that the perineural epithelium has an active regulatory function in vertebrates. Twarog & Roeder (1956) suggested that the perineurium may have an analogous function in insects. This active pumping mechanism remains an attractive hypothesis but has yet to be conclusively demonstrated in either vertebrates or insects. Obviously, such a hypothesis would require a continuous cellular layer between axons and the bathing medium. In *Anodonta*, there is no epithelial layer analogous to that of amphibia or insects. As noted, however, *Anodonta*'s highly organized layer of glial processes immediately surrounding the axons *is* similar to the layer of glial end-feet surrounding the vertebrate brain capillaries that are considered part of the 'blood-brain barrier'. Thus the possible function of the *Anodonta* sheath in pumping sodium ions merits further investigation.

(3) *The elastic properties of the sheath mechanically limit swelling, thereby restricting ion loss and limiting variations in extracellular space.* The osmoregulatory function of the sheath was first suggested by Lorente de Nó (1952) for amphibia and by Twarog & Roeder (1956) for insects. The *Anodonta* sheath, like that of the others, is highly

elastic. Restriction of swelling, combined with ion extrusion, should be seriously considered as a mechanism of sodium regulation. It would be possible to compare neural sheaths in species in which regulation occurs with sheaths in related species in which no regulation occurs (e.g. *Anodonta* and *Mytilus*).

(4) *The sheath itself has no special function in this regard but protects the structural integrity of the glia that surround individual axons.* The hypothesis that the glia have a special regulatory function has been put forward both for insects (Lane & Treherne (1969)) and for *Anodonta* (Treherne *et al.* (1969)). However, the speculation of Treherne *et al.* (1969) that sodium may be 'sequestered' intracellularly in glial granules, constituting a 'novel' element in glial cytoplasm of *Anodonta*, seems unlikely since similar granules are found in glial elements of many molluscs, including *Mytilus* (Twarog, 1967*b*), where apparently no sodium regulation takes place. The suggestion of injury to glia by sheath removal is an interesting one and deserves further study.

SUMMARY

1. Action potentials were observed in cerebrovisceral connectives of a marine bivalve, *Mytilus edulis* L., and two species of freshwater bivalve, *Anodonta cataracta* and *Elliptio complanata*.

2. In *Mytilus* nerve, responses to stimulation in both the intact and the de-sheathed nerve were blocked after two minutes in a sodium-free saline. Restoration of normal sodium levels reversed the block within 5 min.

3. In nerves of the freshwater species, *Anodonta* and *Elliptio*, the action potential of the sheathed nerve altered very slightly after one hour of stimulation in a sodium-free solution. The completely de-sheathed nerve, however, was blocked within one minute in sodium-free saline. The block was rapidly reversible.

4. It is concluded, contrary to conclusions of previous investigators, that the neural sheath in freshwater clams is just as vital to restriction of sodium loss as it is in amphibia and insects, and it would seem logical to seek out a common denominator of sheath function. Several possible mechanisms are put forth and discussed.

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REFERENCES

CARLSON, A. D. & TREHERNE, J. E. (1969). The ionic basis of the fast action potentials in the isolated cerebrovisceral connective of *Anodonta cygnaea*. *J. exp. Biol.* **51**, 297–318.

GUPTA, B. L., MELLON, D. & TREHERNE, J. E. (1969). The organization of the central nervous connectives in *Anodonta cygnea* (Linneus) (Mollusca: Eulamellibranchia). *Tissue and Cell* **1**, 1–30.

HUXLEY, A. F. & R. STÄMPFLI, R. (1951). Effect of potassium and sodium ions on resting and action potentials of single myelinated nerve fibres. *J. Physiol.* **112**, 496–502.

KRNJEVIĆ, K. (1954). The connective tissue sheath of the frog sciatic nerve. *Q. Jl exp. Physiol.* **39**, 55–72.

LANE, N. J. & TREHERNE, J. E. (1969). Peroxidase uptake by glial cells in desheathed ganglia of the cockroach. *Nature, Lond.* **223**, 861–2.

LORENTE DE NÓ, R. (1952). Role of epineurium. *Cold Spring Harb. Symp. quant. Biol.* **17**, 299–315.

PETERS, A. (1962). Plasma membrane contacts in the central nervous system. *J. Anat.* **96**, 237–48.

SCHARRER, B. C. J. (1939). The differentiation between neuroglia and connective tissue sheath in the cockroach (*Periplaneta americana*). *J. comp. Neurol.* **70**, 77–88.

SHANTHA, T. R. & BOURNE, G. H. (1968). The perineural epithelium – a new concept. In *The Structure and Function of Nervous Tissue*, ch. 10 (ed. G. H. Bourne). New York: Academic Press.

TREHERNE, J. E. (1967). Axonal function and ionic regulation in insect central nervous tissues. In *Insects and Physiology*, pp. 175–188 (eds. J. W. L. Beament and J. E. Treherne). Edinburgh and London: Oliver and Boyd.

TREHERNE, J. E., CARLSON, A. D. & GUPTA, B. L. (1969). Extra-neuronal sodium store in central nervous system of *Anodonta cygnaea*. *Nature, Lond.* **223**, 377–80.

TWAROG, B. M. & ROEDER, K. D. (1956). Properties of the connective tissue sheath of the cockroach abdominal nerve cord. *Biol. Bull. mar. biol. Lab., Woods Hole* **111**, 278–86.

TWAROG, B. M. (1967*a*). Excitation of *Mytilus* smooth muscle. *J. Physiol.* **192**, 857–68.

TWAROG, B. M. (1967*b*). The regulation of catch in molluscan muscle. *J. gen. physiol.* **50**, 157–69.