# PROPERTIES OF THE CONNECTIVE TISSUE SHEATH OF THE COCKROACH ABDOMINAL NERVE CORD [1, 2]

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Hoyle (1952, 1953) has drawn attention to the continuous sheath which surrounds nerve fibers and ganglia of *Locusta* and other insects. He has described the structure of this sheath and demonstrated that its effectiveness as a diffusion barrier enables the nerves of *Locusta* to function normally despite wide variations in the ionic composition of the surrounding fluid. His valuable work indicates basic similarity in the membrane properties of insect nerve and nerve of vertebrates and of invertebrates other than insects. In the present study of the ventral nerve cord of the roach, Hoyle's conclusions are confirmed. Normally sheathed and desheathed cords were compared with respect to interference with nervous function by variation in total salt concentration, sodium deficiency, excess potassium ions and acetylcholine. Certain structural details were studied histologically.

## Materials and Techniques

Adult male specimens of *Periplaneta americana* have minimal fatty deposits about the cord and were therefore used. For observations of the effects of ions on axonic conduction in the ventral cord, the head was crushed, and the cockroach was pinned, ventral side up, on a cork platform, with the legs taped down. Test solutions were applied and conduction examined in a segment of nerve cord comprising the fourth abdominal ganglion and the connectives between the fourth and fifth ganglia. Cuticle was removed over this region and a thin paraffin sheet was placed beneath the test segment (Fig. 1a). Drainage arranged from below the paraffin minimized mixture of hemolymph with the test solutions, which were perfused over the segment lying on the paraffin. In some experiments, it was possible to avoid cutting any large nerves or tracheal branches by locating the paraffin entirely under the connectives. Silver-silver chloride hook electrodes (Roeder, 1946) were placed below the cord and moved over the test area so that localized axonic block could be detected by changes in form of the compound action potential in the giant fibers (Fig. 1b).

Action potentials conducted into or through the test area could be elicited either directly via stimuli from a pair of silver electrodes (Fig. 1a, $S_1$) inserted under the nerve cord through a small cuticular opening near the first abdominal ganglion, or transynaptically via stimuli from a similar pair ($S_2$) inserted into the base of a cercus. An uninterrupted sequence of square pulses (0.5 per second; 0.2 msec.

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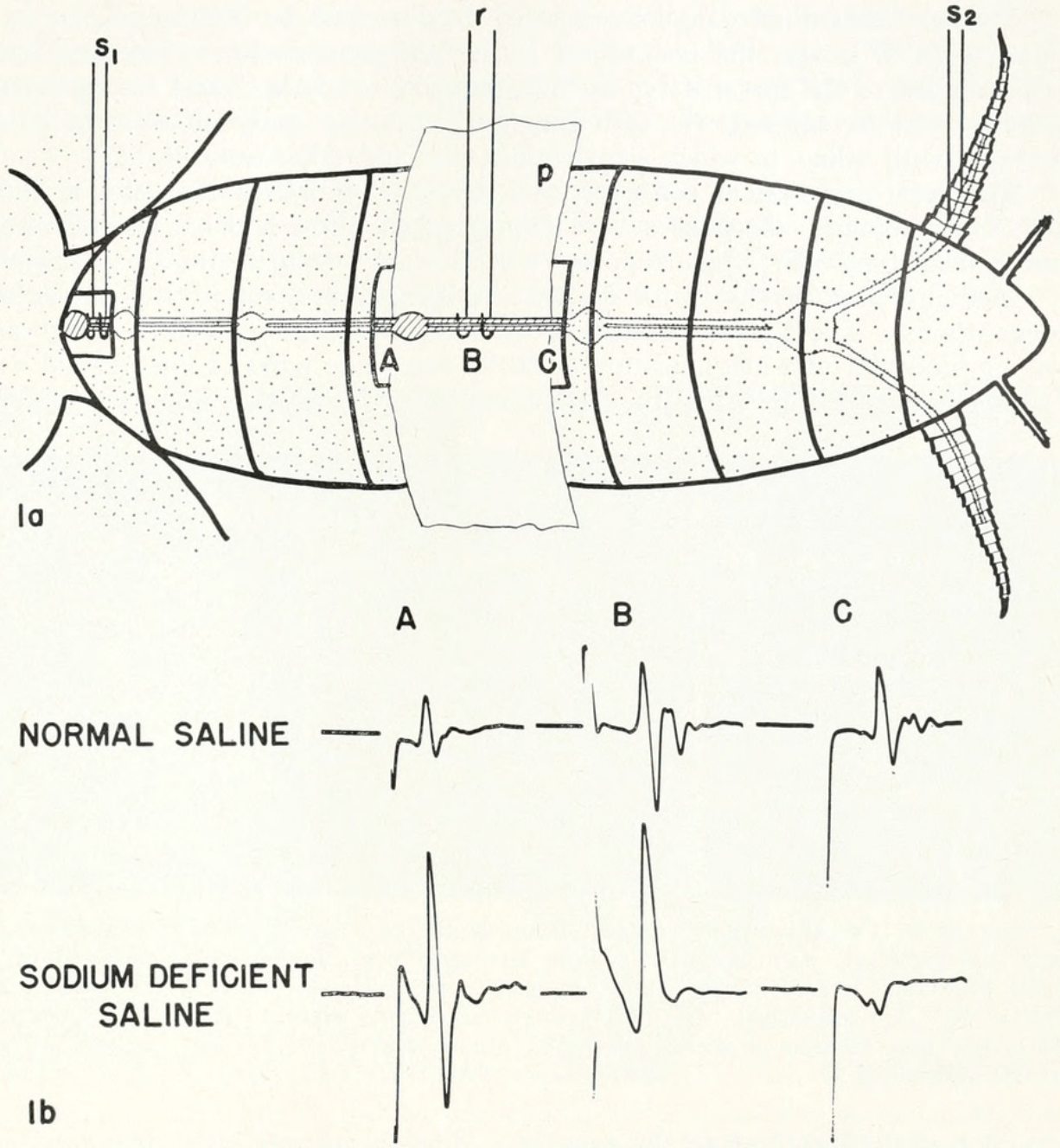


FIGURE 1. (a) $S_1$, stimulating electrode pair on cord near first abdominal ganglion; $S_2$, stimulating electrode pair on cercal nerve; p, paraffin sheet; r, recording electrode pair; A, B, C, sites in test area at which action potentials were recorded. (b) Records of action potentials at sites A, B, and C; stimulation at $S_1$ in a preparation desheathed at B. In sodium-deficient saline, the spikes are larger, due to decreased shunting by electrolyte. Localized block in the desheathed region between electrodes is indicated by the monophasic spike at B.

duration) was applied through $S_1$ except for brief periods when ascending conduction was checked through stimulation at $S_2$. In normal or potassium-free saline, this preparation responded uniformly well for many hours. "Normal" saline refers to Hoyle's (1953) basic mixture for *Locusta*, which proved most satisfactory in our experiments.[4] High potassium and potassium-free salines were made up as detailed by Hoyle.

[4]

| | | | |
|---|---|---|---|
| KCl | 10 mM./L. | $MgCl_2$ | 2 mM./L. |
| NaCl | 130 mM./L. | $NaH_2PO_4$ | 6 mM./L. |
| $CaCl_2$ | 2 mM./L. | $NaHCO_3$ | 4 mM./L. |

The last abdominal ganglion, exposed as described by Roeder, Kennedy and Samson (1947), was the test object in acetylcholine studies. Electrical stimuli were applied to the cercal nerve at low frequency (0.5/sec.) and the postsynaptic response was led off near the fifth ganglion. The last ganglion was continuously perfused with saline, to which acetylcholine was added for tests.

The recording system consisted of a Grass P-4 preamplifier and a Dumont 304-A oscilloscope. Responses were photographed with a Dumont oscillograph record camera, type 297. Square pulses were delivered from a Grass S-2 stimulator.

Methods were developed for desheathing ganglia and connectives in the above preparations. These operations were most conveniently performed under a dissecting binocular at a magnification of 80 ×, using two pairs of fine-ground watchmaker's forceps. Lowering the saline level briefly caused a barely perceptible

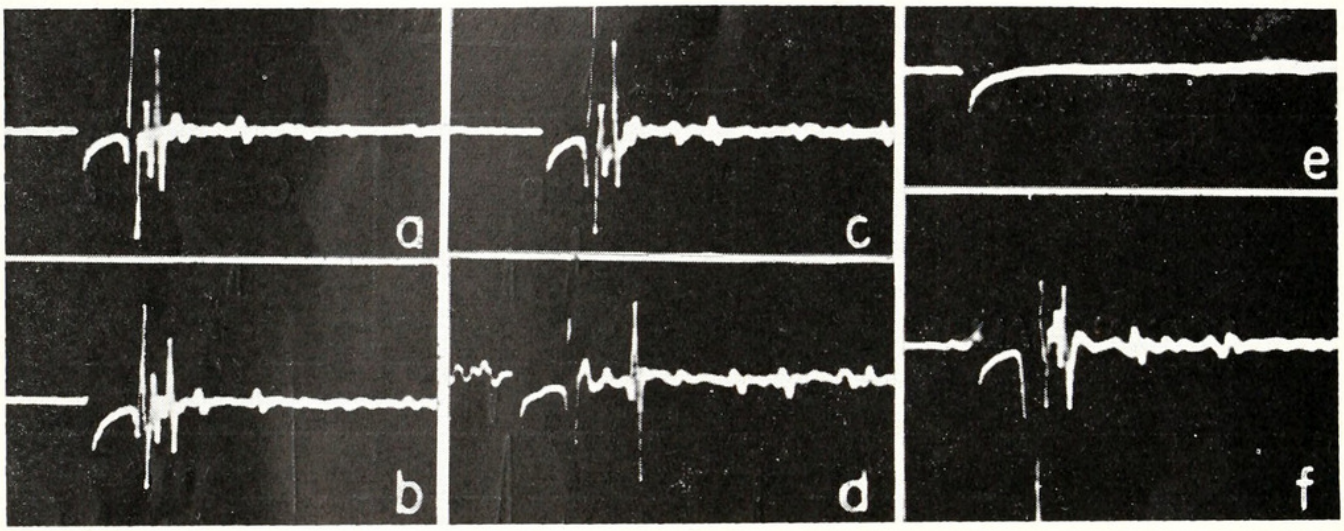


FIGURE 2. Cercal nerve stimulated. Postsynaptic responses recorded from abdominal cord near fifth ganglion. (a) In normal saline; last abdominal ganglion normally sheathed. (b) After 10 minutes in acetylcholine $10^{-2}$ *M;* normally sheathed. (c) After one minute in acetylcholine $10^{-2}$ *M;* desheathed. (d) After three minutes in acetylcholine $10^{-2}$ *M;* desheathed. (e) After four minutes in acetylcholine $10^{-2}$ *M;* desheathed. (f) After washing in normal saline; desheathed.

crinkling of the sheath about the ganglion. The sheath was lifted, torn, and gently pulled away from the entire dorsal surface of the ganglion. The saline level was rapidly adjusted to prevent drying of the desheathed ganglion. This procedure in no way altered the character of the postsynaptic response (see Fig. 2). Desheathing the connectives was more difficult since conduction failed if even a brief drying occurred, and visualization of the sheath under saline took some practice. Stretching the desheathed connective had to be avoided. The sheath, once torn, could be rolled back along the connective, which it enveloped in stocking-like fashion. The sheath is quite elastic and constriction of the cord by the rolled-up sheath had to be avoided by proper tearing. The stripping, successfully accomplished, did not alter the nerve action potential. In normal saline, desheathed preparations responded without change for hours.

The posterior portion of the abdominal cord, containing the fifth and last abdominal ganglia, was examined in 6-micron serial sections. A mercuric chloride-acetic acid mixture provided most satisfactory fixation. Ester wax (Steedman,

1947), with increased paraffin content, proved an excellent embedding medium. Conventional staining techniques included: Mallory's triple stain, Masson's trichrome, Gomori's chromium-hematoxylin-phloxin (Gomori, 1941) and Holme's silver as modified by Batham and Pantin (1951). Desheathed specimens were fixed after physiological tests.

A series of cords was stained by Laidlaw's method as described by Krnjević (1954) after the fifth ganglion and one half of the connective between fifth and sixth ganglia had been desheathed. The living cord was placed in 0.5% silver nitrate and observed under strong illumination. One of this series was fixed, sectioned and counterstained with Mallory's (Fig. 3b).

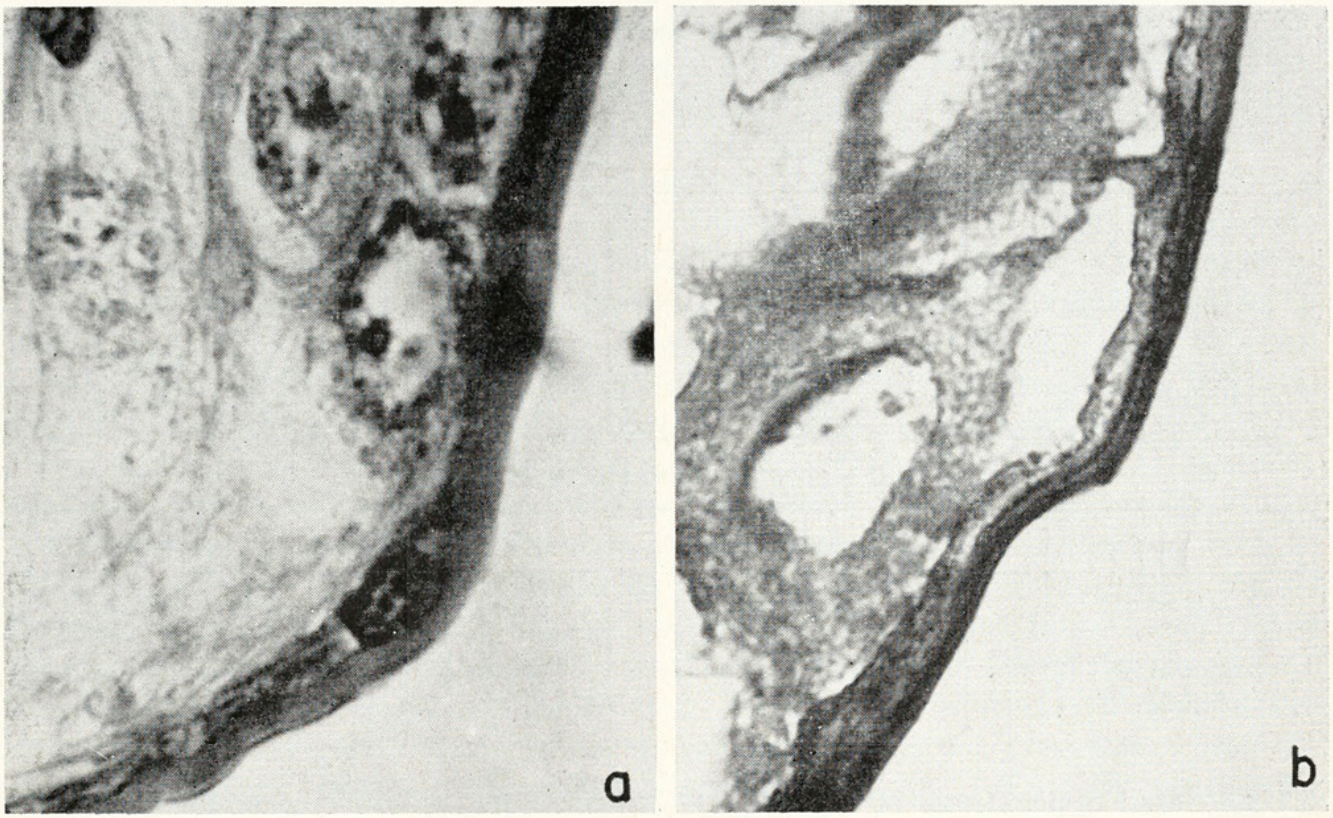


FIGURE 3. (a) Normally sheathed last abdominal ganglion stained with Masson's trichrome. Note outer homogeneous layer and inner nucleated granular layer of sheath. (b) Normally sheathed last abdominal ganglion after silver nitrate treatment. Note adherence of inner and and outer sheath layers in region of shrinkage. Note silver granules in sheath, absence of granules in interior of ganglion. Counterstained with Mallory's triple stain.

## OBSERVATIONS

### *Osmotic changes*

The intact, normally sheathed cord was not observed to swell or shrink and nervous conduction was unaffected when total solute concentration was reduced to the equivalent of 0.140 *M* NaCl by dilution or increased to 0.180 *M* by adding NaCl or sucrose to normal Hoyle's saline (0.156 *M*). Desheathing in the hypotonic (0.140 *M*) saline resulted in gross swelling and functional impairment. Synaptic conduction failed totally. Desheathing in the saline hypertonic to Hoyle's saline (0.180 *M*) resulted in no immediate structural or functional alternation.

*High potassium saline*

In Table I are listed typical times for total conduction block on perfusing a previously untreated cord with 0.180 *M* KCl or Hoyle's balanced saline containing 140 mM/L. $K^+$. Recovery was followed in $K^+$- free saline.

Blocking was more rapid in 0.180 *M* KCl. This could well be attributed to the absence of sodium and other ions rather than higher $K^+$ concentration. Desheathing obviously reduced recovery time as well as blocking time. The presence of the cut ends of small nerves (severed in dissecting the fourth ganglion) did not alter the blocking time appreciably.

TABLE I

*Effect of potassium ions on impulse conduction in intact and desheathed cords*

A. Intact segment

| Solution | Blocking time | Recovery time | Type preparation |
|---|---|---|---|
| 0.180 *M* $K^+$ | 12 min. | 10 min. | Connective only |
| 0.180 *M* $K^+$ | 15 min. | 10 min. | Ganglion included |
| 0.180 *M* $K^+$ | 18 min. | 20 min. | Ganglion included |
| 140 mM./L. $K^+$ | 30 min. | 25 min. | Ganglion included |
| 140 mM./L. $K^+$ | 22 min. | 33 min. | Ganglion included |

B. Test segment desheathed at B (see Fig. 1a)

| Solution | Blocking time | Recovery time | Type preparation |
|---|---|---|---|
| 0.180 *M* $K^+$ | 10 secs. | 4 min. | Ganglion included |
| 140 mM./L. $K^+$ | 60 secs. | 2 min. | Ganglion included |
| 140 mM./L. $K^+$ | 90 secs. | 2 min. | Ganglion included |

Repeated applications of high $K^+$ solutions to intact cord segments led to increasingly rapid block and delay in recovery. This effect was not seen in desheathed nerve.

*Sodium-deficient saline*

Substitution of sucrose for the sodium of the normal saline did not affect the normally sheathed segment in experiments continued several hours, although this caused conduction block in a stripped preparation within 30 seconds (Fig. 1b). Recovery was complete within two minutes in normal saline.

*Acetylcholine*

Treatment of the intact ganglion with extremely high concentrations of acetylcholine ($10^{-2}$ *M*) did not alter synaptic responses (Fig. 2 a, b), as had been reported by Roeder (1948). However, effects of $10^{-2}$ *M* acetylcholine on synaptic function in the desheathed ganglion were usually rapid and pronounced. In two of a series of seventeen experiments, only a moderate decrease in synaptic response

was noted. In all others, within one to five minutes bursts of asynchronous action potentials were followed by synaptic depression and block. Figure 2 shows the rapid and easily reversible block of a ganglion desheathed in $10^{-2}$ *M* acetylcholine. The lowest effective concentration of acetylcholine was between 3 and $5 \times 10^{-3}$ *M* in the uneserinized desheathed ganglion. After eserine, acetylcholine between $10^{-4}$ *M* and $10^{-3}$ *M* blocked the synapse. No effect on axonic conduction was noted.

*Sheath structure*

Since the foregoing experiments demonstrated the functional importance of the sheath, an attempt was made to examine its mechanical properties and structure.

It was extremely difficult to penetrate the sheath of the intact nerve cord with any object. Even a finely tapered capillary microelectrode merely dimpled the surface and broke. After desheathing, penetration of the cord and individual neurons presented no difficulty. During dissection, the sheath felt tough and elastic. As has been mentioned, in some dissections the sheath severely constricted the cord. The almost explosive bulging-out of nerve substance through a small hole made in the sheath when the preparation was immersed in hypotonic saline strikingly illustrated the mechanical resistance the sheath offers to swelling. Injected air bubbles were trapped beneath it. Histologically, the sheath was continuous and clearly double-layered. The outer, almost homogeneous layer, two to five micra in thickness, stained deeply with aniline blue. Occasional nuclei were evident in this layer, possibly representing fibroblasts. The inner layer consisted of granular squamous epithelial cells, one to three micra thick. The flattened nuclei of the inner layer were prominent in both cross and coronal sections (Fig. 3a). (The outer layer referred to above corresponds to the homogeneous, non-cellular, neural lamella described by Scharrer (1939) and Hoyle (1952), the inner layer to the thin, continuous cytoplasmic cylinder which Scharrer terms the perineurium and Hoyle terms the perilemma.) These layers were closely adherent to one another and pulled away together from the nerve substance when the preparation was desheathed or when shrinkage occurred in fixation (Fig. 3b). No other continuous structures lay external to these layers in our intact preparations. These two layers were always absent in areas functionally desheathed.

It should be mentioned that within the nerve cord itself, fibrous investments, almost capsular in appearance in silver-stained sections, but probably discontinuous, surrounded certain large cell bodies and groups of cell bodies. These fibrous investments may correspond to the glial elements described by Scharrer (1939). The giant axons were individually encased in a delicate (one micron in thickness) sheath-like structure throughout their length.

When a freshly-dissected, unfixed cord was observed during soaking in silver nitrate, the normally sheathed areas showed at first a fine network of black lines, then became stippled and blotched in appearance and finally almost uniformly black. The blackening was close to the surface and the deep black layer could be lifted off by desheathing. Desheathed cord soaked in silver nitrate did not blacken superficially, but individual neurons could be observed to stain black in these preparations. Sections of the intact last abdominal ganglion, treated while fresh with silver nitrate, showed black granules concentrated in the surface layer corresponding to

the sheath (Fig. 3b), while the desheathed fifth ganglion, after similar treatment, showed granules distributed throughout.

## Discussion and Conclusions

The sheath surrounding the nerve cord of *Periplaneta* clearly limits diffusion of ions, as Hoyle (1953) showed in *Locusta*. However, in efficiency of sheath function as measured by total time required for block in excess potassium, the intact *Periplaneta* cord is more comparable to the amphibian sciatic nerve, which will block in 13–20 minutes in isotonic KCl (Lorente de Nó, 1947; Feng and Liu, 1949) than to *Locusta* nerve, which resists block as long as four hours in saline containing 140 mM potassium, provided the tracheal supply is undamaged. Hoyle pointed out the importance of adequate oxygenation in maintaining sheath function. It may be that in our preparation, since abdominal movements were severely restricted, oxygenation through the tracheal supply was insufficient to maintain sheath function although efforts were made to avoid all damage to the tracheal supply.

The retardation of potassium diffusion is a two-way function of the sheath, judging by the large decrease in recovery time as well as blocking time in desheathed cord. The cumulative effect of repeated high potassium solutions, as well as the appearance of the silver nitrate-treated cord (Fig. 3b), suggests that ions penetrate the sheath readily but are accumulated there. Shanes (1954) showed with tracer techniques that the sheath of the frog sciatic nerve is entirely responsible for retardation of ion diffusion outward as well as inward.

Desheathed *Periplaneta* cord is soon blocked by sodium deficiency, although conduction in the intact cord is unaffected for long periods in sodium-free saline. This, then, is direct evidence that insect neurons are similar to the neurons of other groups not only in potassium sensitivity but in their susceptibility to inactivation by sodium deficiency, and that it is the sheath which masks this susceptibility just as does the sheath which invests vertebrate nerve (Huxley and Stämpfli, 1951).

Although much pharmacological evidence suggests the cholinergic nature of the synapse between cercal nerves and giant fibers in the last abdominal ganglion of *Periplaneta,* not even very high acetylcholine concentrations affect the intact ganglion. This total lack of effect is clearly due to restriction of acetylcholine penetration by the sheath. However, effective concentrations of acetylcholine are high even in desheathed preparations so that the synaptic specificity of the acetylcholine action is still in question. (See Twarog and Roeder, 1957 for further data.) The sheath-like structure which invests the giant fibers may represent another barrier to ion diffusion, or the synaptic terminations of the cercal nerves may be otherwise "protected."

Water diffuses through the sheath quite readily, as is evident from the immediate swelling-out of nerve substance which occurs when a small portion of the sheath is removed while the cord is soaking in hypotonic saline. It is obvious that the physical restraint exerted by the sheath in preventing swelling limits total water uptake and preserves structural integrity. Lorente de Nó (1952), Shanes (1953) and Krnjević (1954) have emphasized the possibility that the mechanical rigidity of the vertebrate sheath may serve an osmoregulatory function. The fluctuations in hemolymph water in insects are often very great (Mellanby, 1939), and an osmoregulatory function of the sheath may be as important as its role in salt regulation.

The actual site of ion regulation by the sheath is in one or both of the two layers described above, which were also described by Hoyle (1952) in *Locusta*. Huxley and Stämpfli (1951) suggested that ion regulation by the frog sheath is a function of the innermost epithelial layer first described by Ranvier in 1876 rather than the loose connective tissue of the epineurium. Krnjević (1954) showed conclusively that this is true. Within the epineurium he described the two-layered perineurium: an inner continuous layer of squamous epithelium, 4–6 micra thick, with an external layer of comparatively undifferentiated connective tissue. In some regions of the sciatic nerve additional cellular layers were seen, but these were not continuous over the entire nerve. He succeeded in showing that the silver granules are most concentrated in the epithelial layer. The analogy in structure between the insect sheath and the frog perineurium is rather striking. It is likely that it is the squamous epithelial layer which fulfills the important regulatory function in the insect. Krnjević (1954) has discussed the importance of epithelial layers in regulation of diffusion through capillary walls and through the connective tissue sheaths which surround nervous structures in vertebrates. The inner, epithelial layer of the sheath may well bear some physiological and structural resemblance to the so-called blood-brain barrier.

It must be mentioned here that the desheathing technique, in addition to having utility as a method of studying this interesting ion-regulating structure itself, presents advantages in investigating insect nervous function. It has been employed by Twarog and Roeder (1957) in pharmacological studies to avoid total failure of drug penetration or insufficiently rapid penetration to the site of action. Perhaps more important is the fact that it makes possible routine exploration of the insect nervous system with microelectrode techniques. Resting cell membrane potentials of 50 to 70 MV have been easily measured and sustained. Cell action potentials have been obtained but no systematic study has yet been made.

## Summary

1. A method is described for removing portions of the connective tissue sheath which invests the nervous system of the cockroach, making possible studies of sheath function and of basic physiological properties of the insect neuron.

2. This sheath restricts diffusion of potassium and sodium ions and of acetylcholine from the surrounding fluid to the interior of the cord.

3. The ability of the sheath to restrict swelling suggests a possible osmoregulatory function.

4. The functional sheath consists of an inner continuous squamous epithelial layer and an outer, almost homogeneous connective tissue layer.

5. This study confirms the conclusions of Hoyle (1952, 1953) with respect to ion regulation by the insect sheath, and indicates close functional and structural parallels in the sheaths investing insect and vertebrate nervous tissue as well as in the basic properties of the nervous tissues of insects and vertebrates.

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