16 | Recording from the Crayfish Abdominal Extensor Muscle Preparation with Microelectrodes

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Animals and Apparatus

For this experiment freshwater crayfish (e.g. *Procambarus clarki* (Girard), *Orconectes virilis* (Hagen), or other available species) should be obtained. The larger specimens are preferable. They can be maintained in fresh water (preferably chlorine-free) with occasional feeding (small pieces of liver or meat). A holding temperature of 10–15°C will generally be satisfactory.

To perform the experiments it is necessary to have equipment for manufacture and use of glass capillary microelectrodes as follows: a microelectrode puller; glass tubing of suitable diameter; a vacuum desiccator or side-arm vacuum flask; two micromanipulators; an electrode holder for microelectrode manipulation; a cathode follower input probe; and an oscilloscope.

Other items of equipment used in the experiments include: a binocular dissecting microscope and lamp; a compound microscope; a few slides and coverslips; an electronic stimulator, preferably with stimulus isolator included; stimulating electrodes; oscillograph camera with recording paper or film; developing tank and photographic solutions; a chamber (with wax bottom) to hold the preparation.

In order to put the equipment requirements into more concrete terms, some of the specific items used by students at the University of Toronto to perform this experiment are noted below. For most of the items many satisfactory alternatives are available; the list is given merely to serve as a frame of reference.

For pulling microelectrodes, the Micro-pipette Puller M I (Industrial Science Associates, New York) was used. (Electrodes can be pulled by hand or with various "home-made" pullers; see Donaldson (1958) or Nastuk

(1964).) The electrodes were pulled from Pyrex tubing of 2 mm outside diameter and 1 mm inside diameter, and had resistances when filled of 4–12 MΩ. For filling, a side-arm vacuum flask attached to a water suction pump with high-pressure rubber tubing was used (see Experimental details, p. 311).

The electrodes were used in conjunction with a Zeiss Jena sliding micromanipulator; others which could be used include those made by Prior and by Brinkman. The manipulation requirements of this experiment are less stringent than in many other microelectrode experiments. Electrode holders of the type shown in Fig. 1 were used. A Tektronix Type 502 A oscilloscope was used for electrical measurements, and records were made by means of a Cossor Model 1428 oscillograph camera and Ilford HP3 film (Tri-X film or photographic paper would also be suitable). Dissections and manipulations were viewed with the aid of a Zeiss Operation Microscope (working distance, 120 mm). For nerve stimulation, a Grass SD5 stimulator was employed.

There are a number of commercially available high-impedance input probes for use with microelectrodes (for example, the Negative Capacity Electrometer Amplifier, Model A-35, Medistor, Seattle, Washington, or the HFl Neutralized Capacity Amplifier, Bioelectric Instruments, New York). It is also possible to construct "home-made" probes from electrometer vacuum tubes or field effect transistors (e.g. Donaldson, 1958).

Chemical Solutions

1. For filling microelectrodes, a solution of 3 M potassium chloride (220·5 g/l) is required. This should be filtered into a clean bottle before use, to avoid introducing dirt into the microelectrodes.

2. The normal crayfish solution (after van Harreveld; 1936) has the following composition:

NaCl	12·0 g/litre
KCl (5·4 mM)	0·4 g/litre
$CaCl_2$	1·5 g/litre
$MgCl_2 \cdot 6H_2O$	0·5 g/litre
$NaHCO_3$	0·17 g/litre

In making up the solution, the first four substances should be added first, then enough distilled water to bring the volume up close to its final level, and lastly the $NaHCO_3$ and the necessary additional distilled water. After aeration and thorough mixing of the solution, the pH should be checked. If necessary, a few drops of dilute HCl or a small additional amount of $NaHCO_3$ may be added to bring the pH of the solution to 7·4. Another commonly adopted method is to buffer the solution at pH 7·4 by including

200 ml of 0·05 M Tris-maleate buffer as part of the volume of 1 litre of solution. The buffer is made as follows:

Tris acid maleate (24·2 g/l Tris and 23·2 g/l maleic acid)	50 ml
0·2 M NaOH (8·002 g/l NaOH)	54 ml
Distilled water	96 ml

3. For study of the effect of potassium ion on the muscle membrane potential, a solution containing high potassium is required:

NaCl (153 mM)	8·87 g/litre
KCl (60 mM)	4·42 g/litre

(Other ingredients as in the normal crayfish solution.)

4. For staining axons in the preparation, a solution of 1% methylene blue in normal crayfish solution should be prepared (1 g solid methylene blue in 100 ml crayfish solution).

5. For fixation of muscle fibres in the preparation, a mixture of formalin in crayfish solution is required (1 part commercial formalin, 40% HCHO, and 7 parts crayfish solution).

6. For the study of drug effects on muscle potentials, solutions containing γ-aminobutyric acid (GABA), picrotoxin and strychnine are required. Stock solutions from which suitable dilutions can be made are given below. It is suggested that 100 ml of each is probably adequate.

γ-Aminobutyric acid: 1·0 g per litre of crayfish solution, or 0·1 g/100 ml.
Strychnine nitrate: 5 g per litre of crayfish solution, or 0·5 g/100 ml.
Picrotoxin: 1·2 g per litre of crayfish solution or 0·12 g/100 ml.

All drug solutions should be stored in the dark in a refrigerator when not in use. Remember that they are *poisonous* and should be handled with due respect and caution.

IDEA AND PRINCIPLE

The crayfish abdominal extensor muscle preparation is easier to make than most other muscle preparations, and is thus a convenient one for application of microelectrode technique for the first time. Microelectrodes can be used to measure muscle membrane resting potentials and nerve-evoked muscle potentials. They can be used to investigate the effects of ions and drugs on muscle fibre membrane properties and neuromuscular transmission.

Crustacean muscle has become fairly popular material among electrophysiologists, partly because of the relatively large sizes of the muscle fibres

and motor axons. The abdominal extensor muscle preparation introduces many of the physiological and histological features of crustacean material, including polyaxonal and multiterminal innervation of muscle fibres (Hoyle, 1957), differentiation of muscle fibres into fast and slow-contracting types (Atwood and Dorai Raj, 1964; Kennedy and Takeda, 1965a, b), excitatory and inhibitory innervation (Wiersma, 1961), and variability of excitatory post-junctional events (Hoyle and Wiersma, 1958). The axons supplying the extensor muscles have a rather complicated pattern of distribution, which can be investigated by methylene blue staining and by microelectrode recording.

Several laboratory periods of 3 or 4 h can be alloted to this preparation.

Period 1. Preparation and testing of microelectrodes.
Period 2. Examination of the anatomy, innervation, and histology of the preparation.
Period 3. Study of the resting membrane potentials of muscle fibres, and effects of potassium ion.
Period 4. Study of nerve-evoked muscle potentials, including the distribution of those associated with individual axons.
Period 5. Effects of drugs (GABA, picrotoxin) on nerve-evoked potentials.
Period 6. Effects of drugs (strychnine) on nerve-evoked potentials.

Experimental Details

Microelectrodes

The manufacture and testing of glass capillary microelectrodes has been described in several references (e.g. Donaldson, 1958; Nastuk, 1964). A brief outline of procedure is included here for convenience.

The first step is to pull the electrodes on the pipette-pulling machine from glass tubing. It may be found convenient to cut the tubing into lengths of about 9 cm (thus yielding, for each machine pull, two electrodes of about 5 cm). As a precaution against blockage of electrode tips by dirt, the tubing may be washed with chromic acid, then with distilled water, and oven-dried.

In most pipette-pulling machines, the degree of heating of the coil, and the parameters of the force applied to pull the glass tubing, can be adjusted, and after a few trial pulls it should be possible to obtain electrodes of about the right shape (Fig. 1). These should be examined under the compound microscope to make sure that they taper smoothly to a sharp point with no break or ball at the end. Once the right setting for the machine has been determined, a large number of electrodes can be rapidly pulled.

To fill the electrodes, mount them tip downwards in filling solution (3 M KCl). As a rough-and-ready expedient, the electrodes can be mounted on a slice of rubber stopper with slits cut in the edges to accept the stems (Fig. 1). The filling solution and electrodes are then heated close to the boiling

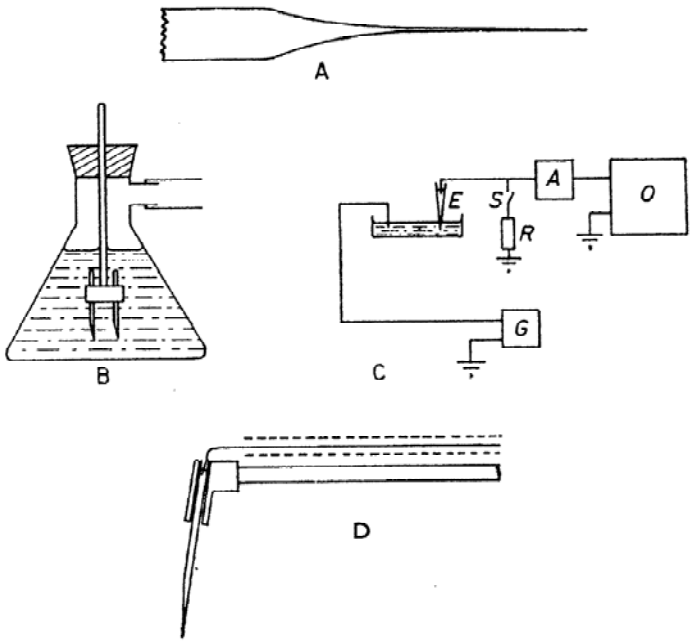

FIG. 1. Details of microelectrode technique. A. Sketch of microelectrode tip, showing the general shape. B. Arrangement for filling microelectrodes. The electrodes are mounted on a piece of rubber stopper attached to a glass rod. C. Arrangement for testing electrode resistance. Electrode (*E*) makes contact with saline in a dish, to which a signal is led from a generator (*G*). Pre- amplifier (*A*) is led to the oscilloscope (*O*). Resistor of known value (*R*) can be switched in (*S*) to shunt the input to earth. D. A holder for microelectrodes, made from plastic. Dotted line indicates cathodal screening of the input lead.

point (making sure that the container holding the electrodes is not sealed, otherwise an explosion could result). Then connect the desiccator or flask containing the electrodes to a water vacuum pump and allow bubble formation to occur. During this stage the air in the electrodes will be replaced by water vapour. After about 10 min of boiling under reduced pressure, reduce the vacuum and gradually readmit air to the container. (It is good policy to install a second flask as a trap between the water vacuum pump and the electrode container to prevent water being sucked into the latter). Hopefully, the vacuum treatment will have removed the air from the microelectrodes, leaving them filled with 3 M KCl after reduction of the vacuum. If air is obviously still present in the electrodes, the process should be repeated in an attempt to remove the last vestiges of air.

After filling, the electrodes should be removed and stored, for example in a Petri dish containing 3 M KCl and along the bottom a strip of plasticene grooved to hold the microelectrodes. Stored electrodes remain usable for about 2 weeks.

It is not possible to determine with certainty whether an electrode is usable by inspection under the light microscope. Instead, the suitability of an electrode is judged by the less direct method of measuring its electrical resistance. Usable electrodes (for this experiment) will generally be of 4–20 MΩ resistance. Very high resistance usually indicates a blocked or incompletely filled

electrode tip; low resistance, a broken tip. Such electrodes should be discarded.

To measure electrode resistance, a chlorided silver wire, borne by a manipulator, is first inserted into the open end of the electrode. The free end of the wire is connected to the input of the cathode follower or neutralized capacity pre-amplifier, which in turn is connected to the oscilloscope. The tip of the electrode is lowered into saline in a dish. A calibrated square-wave signal is introduced into the saline through a chlorided silver wire (e.g. from the square wave calibration source of the 502 oscilloscope).

At the input of the cathode follower, one or more resistors of known value (Fig. 1, R) can be arranged to shunt the input to earth (switch S). With S open, the full amplitude (V) of the square wave appears on the scope (with some "rounding off" at the corners due to loss of high frequency components of the signal). When S is closed, R forms a potential divider with the microelectrode resistance (E).

The proportion (V_1) of the original square wave, which now appears across R and on the oscilloscope, is:

$$V_1 = V.R/(R + E)$$

from which

$$E = R.(V - V_1)/V_1$$

Thus if R is 10 MΩ, and E is 10 MΩ the amplitude of V will be halved when S is closed. If E is very small (broken-tipped electrode), closing S will reduce V by a negligible amount; but if E is very large, V will be greatly reduced. Incompletely filled electrodes can usually be identified by instability of the scope trace or gross distortion of the square wave even before resistance is tested.

There are other ways to measure electrode resistance (see, for example, Donaldson, 1958), but the above method is widely used and easily arranged.

Ideally, the rise time of a square wave recorded through a usable microelectrode should be less than 100 μsec, to preserve the true characteristics of rapid bioelectric potentials. With a neutralized capacity pre-amplifier, the rise time can be made very much less than 100 μsec. In the case of conventional or "home-made" cathode followers, the input lead must be cathodally screened (Donaldson, 1958). The rise time of the signal will depend largely on the length of the input lead, which should be kept as short as conveniently possible to obtain a reasonable signal rise-time.

Dissection of the Abdominal Extensor Muscles

Remove head and abdomen from a crayfish. Cut through the shell along the lower lateral border of the abdomen on each side, along the series of small indentations (Fig. 2). When the shell has been separated into two parts, pry

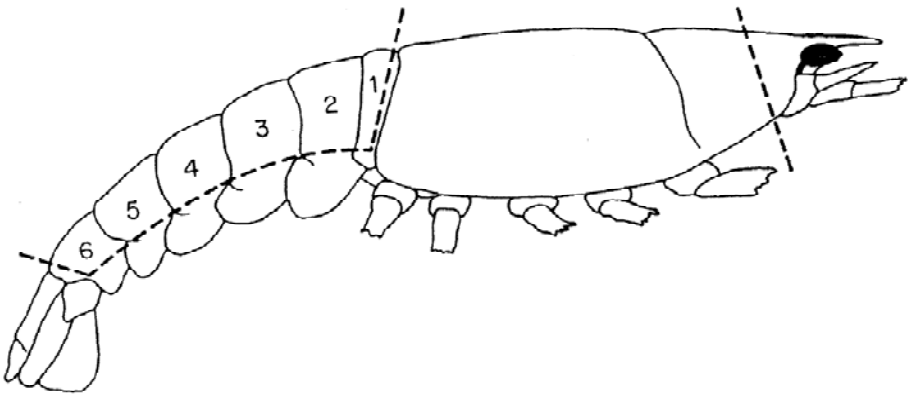

FIG. 2. Dissection of the abdominal extensor muscles. Dotted lines indicate the position of the cuts to be made. Numbers indicate segments of the abdomen.

them apart, starting at the anterior end. The lower half of the abdomen, together with the flexor musculature, will separate readily from the upper half of the abdomen and the extensor musculature. Sometimes it may be necessary to sever some of the flexor muscle connexions to the upper shell, but for the most part careful pulling will suffice to break the flexor connexions.

The extensor musculature comprises a relatively small mass of white muscle attached along the upper half shell. The preparation can be anchored (ventral, or muscle, side up) by pushing a straight pin through the shell at each end into a wax-bottom dish (Fig. 3). The preparation should be kept in crayfish solution, and will remain in good condition for several hours if the solution is periodically replaced, and provided the temperature of the solution is less

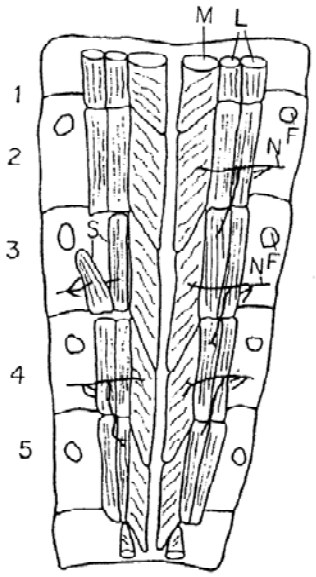

FIG. 3. A dissected preparation, viewed from the open (ventral) side. Segments are numbered from the front. *M*, *L*, medial and lateral deep extensor series; *N*, segmental nerves; *F*, attachment to flexor muscle; *S*, superficial extensor muscle (deep extensors removed in this segment).

than 20°C. For this purpose the replacement saline can be pre-cooled. At higher temperatures the preparations "age" more rapidly, but will still respond for at least an hour or two.

Anatomy of the Extensor Musculature

A careful preliminary study of the anatomy of the preparation should be made, as the subsequent physiological work largely depends on this knowledge.

The anatomy has been described in several papers (e.g. Pilgrim and Wiersma, 1963; Parnas and Atwood, 1966). Each abdominal segment has its own set of muscles, with a nerve supplying them on each side (Fig. 3). The larger *deep extensor muscles* are uppermost in the preparation. In these can be distinguished a *medial* muscle, with fibres twisted in a helix, and a *lateral* muscle (subdivided into L_1 and L_2) with straight-running fibres (Fig. 4). The attachments and delimitations of each muscle should be observed and studied. Are the muscles in all segments similar? How does contraction and shortening of these muscles make the tail extend?

Underneath the deep extensors are the smaller *superficial extensor muscles*; parts of them can often be discerned while the deep extensors are still in

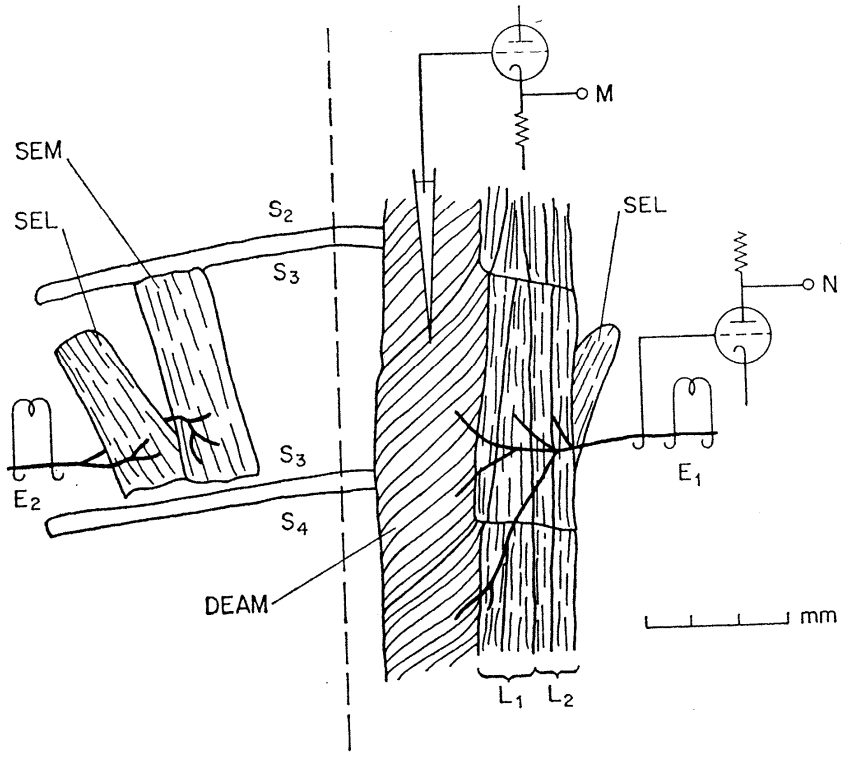

Fig. 4. Extensor muscle preparation showing positions of recording and stimulating electrodes. *DEAM*, medial deep extensor muscle; L_1, L_2, divisions of the lateral deep extensors; *SEM*, *SEL*, superficial extensor, medial and lateral heads; S_{2-4}, the abdominal segments; *M*, intracellular recording from medial deep muscle; *N*, recording of nerve potentials; E_1, E_2, stimulation of axons of the deep and superficial muscles respectively.

place, but they are fully revealed only by dissecting away the latter muscles (more particularly L_1 and L_2). Most of the present exercise will be concerned with the more accessible deep extensors, hence this further dissection will not usually be required.

Drawings should be made to illustrate the anatomical features of these muscles. It is worth bearing in mind that only a few species of crayfish (especially *Procambarus clarki*) have been studied in detail. In other species there may be some features which are not exactly the same as in *Procambarus*.

INNERVATION

A visual study of the innervation can be conducted by staining the axons in the nerve and on the muscle's surface with methylene blue. In relatively undamaged preparations good staining is readily achieved using the straightforward methylene blue solution. It is not necessary to use reduced methylene blue. Apply a few drops of methylene blue solution to the surface of the preparation while the latter is still covered by a shallow layer of saline. After 5–10 min, wash the stain away and examine the preparation under the microscope. If the axons are still faintly stained, repeat the staining procedure.

In well-stained preparations the axons will stain dark blue while the muscle remains light. The axons can be followed as they run on to the surface of the muscle and branch. Note that each segmental nerve divides into a branch supplying the deep extensors, a branch supplying the superficial extensors (and stretch receptors), and a sensory branch (Fig. 3). Study particularly the innervation of the deep extensors, noting that more than one segment of the musculature is innervated by each nerve, and that different axons supply different parts of the musculature; they do not all branch together, as in the classical description of the innervation of crustacean leg muscles (van Harreveld, 1939). Also, the pattern of branching is not the same in all segments.

Make drawings of the innervation, and attempt to determine the parts of the musculature (medial, L_1, or L_2; segment number) supplied by individual axons. The innervation scheme for certain segments of the abdomen of *Procambarus* is given under Results (p. 320), but this scheme does not necessarily apply to all segments or to all species. Be on the look-out for differences.

Histology of the Muscle Fibres

When observations on gross anatomy and innervation have been completed, the preparation may be fixed in formalin solution (24 h) for histological examination of fixed muscle fibres. (During fixation, the muscles should be held at rest length or even slightly stretched.) If preferred, muscle

fibres from a specimen prepared previous to the laboratory period may be used.

Dissect a few fibres, or parts of them, from the various parts of the deep muscles (medial, L_1, and L_2) and also from the superficial muscles; tease them with needles into fine, longitudinally separated shreds, mount them on a slide under a coverslip, and examine them under the compound microscope. Focus on the muscle fibre striations, and measure or estimate the sarcomere length (distance between recurring striations) in various fibres. Is there any marked difference in sarcomere length in fibres from various parts of the deep muscles, or in fibres from the superficial muscles as compared with those from the deep muscles? What significance can be assigned to these observations?

Measurement of Muscle Fibre Membrane Potentials

Set up a fresh preparation of the abdominal extensor muscles in normal crayfish solution. Pin it securely to the wax bottom of the preparation dish.

Mount a tested microelectrode in the electrode holder and make a connexion to the wire connected to the cathode follower input. Lower the electrode until the tip enters the solution above the preparation. Bring the beam of the oscilloscope to a known position on the tube face. Make sure that recording is d.c., at a vertical sensitivity of about 10–20 mV/cm. Then, under microscopic examination, lower the electrode towards one of the medial or lateral deep extensor muscles, in a region free of connective tissue or other obstacles. Carefully bring the electrode tip to the surface of the muscle. As the tip touches a muscle fibre, a slight movement of the oscilloscope beam will occur. A small further advance of the electrode will then usually effect penetration of the cell. The beam of the oscilloscope will indicate a negative potential (the membrane resting potential) of 60–75 mV. Ideally the resting potential should appear very abruptly without slow drift to the final level. Sometimes difficulty in penetration will be encountered; in this case, try another location, and/or small up and down excursions of the electrode tip, or a sharp tap to the table or support under the preparation dish. The electrode should not be forced too deeply into the muscle: the tip may break, or pass out of the cell, or cause damage. After a few penetrations have been made with success, sample membrane potentials of fibres in (a) the medial deep extensor, (b) the lateral deep extensors. Be sure to select fibres in undamaged regions of the muscle. Is there any statistical difference between the resting potentials in the medial and lateral muscles? What range of values is present?

Investigate the effect of raised potassium ion concentration on the resting potential, using several solutions made up by mixing various amounts of the high potassium solution with small amounts of the normal crayfish solution. (For example, 2 vol of the normal solution and 1 vol of the high

potassium solution give 3 vol of a solution containing 23·5 mM potassium ion.) Apply the solutions in order of increasing potassium content. After replacing one solution by the next in the series, let the preparation soak in it for 5–10 min, then measure the resting potentials of several cells in a selected region of the muscle. Make all measurements in the same region.

Make a graph of the results, plotting resting potential against log potassium ion concentration. According to the Nernst equation, the resting potential is related to the potassium ion concentration, roughly as follows (at 20°C):

$$\text{Resting potential} = 58 \log (K_{in}/K_{out}) \text{ mV}$$

where K_{in} is the potassium ion concentration inside the membrane, and K_{out} is the potassium ion concentration outside the membrane.

Check your plot to see how closely it follows this relationship. Is it a straight line? Does it have a slope of 58 mV per tenfold change in external potassium ion concentration? Draw through the average resting potential at 5 mM potassium ion, a line having the expected 58 mV/decade slope; compare with your plot. How would you deduce from this type of plot the potassium ion concentration inside the muscle fibres?

Stimulation of the Axons

The nerve supplying the deep extensor muscles in each segment contains a number of axons. These cannot readily be separated for individual stimulation, but it is possible to stimulate individual axons by careful adjustment of the stimulating pulse, and thus to determine which parts of the muscles are supplied by the stimulated axon.

For stimulation, use a thin (0·10–0·005 in.) platinum wire, insulated with varnish except at the very tip (through which current will flow to stimulate the axon). Position this by micromanipulator on the nerve, and connect or solder the end furthest from the preparation to a wire led from the negative side of the stimulator output. Return the positive side of the stimulator output to the bath (using a platinum or silver wire terminal). Setting the stimulating pulse duration to 0·05–0·1 msec, and the repetition frequency to 1/sec (or single shock mode), slowly increase the stimulating voltage, while watching the muscles through the dissecting microscope. If the preparation is reasonably fresh, and the equipment is working, parts of the musculature will give twitch contractions (often rather weak) at a fairly low voltage (0·5–3 V).

Penetrate the responding part of the musculature with a microelectrode (see Fig. 4 for a diagram of the general layout). Trigger the oscilloscope sweep with the stimulator; set the time base to 10 msec/cm. As the stimulation voltage is increased a point will be reached at which a muscle potential will appear:

this may be excitatory, in the form of a spike, graded response, or "pure" post-synaptic potential (see Results, p. 323), or it may be inhibitory, in the form of a small, relatively long-lasting, hyperpolarizing or depolarizing potential. Once a potential has been seen, at a low threshold, attempt to establish which parts of the musculature are responding, by several penetrations. Then try altering the stimulating voltage to see what changes in the potential can be induced. Changes may be related either to input from additional excitatory axons, or to input from inhibitory axons (see Results, p. 324, and Kennedy and Takeda, 1965a). Try altering frequency of stimulation (up to about 20/sec) to see if the potential appearing at the lowest threshold exhibits facilitation, or lack of it. When the above observations have been made, the position of the stimulating electrode on the nerve can be changed in an attempt to bring in a different axon at the lowest threshold. Other nerves in the preparation can also be tried. Obtain photographic records of the muscle potentials: be sure to note the oscilloscope calibrations associated with each record. In photographing spike-shaped responses, establish whether or not these overshoot the level of zero membrane potential, by withdrawing the microelectrode and photographing a sweep of the oscilloscope beam on the frame bearing the record of the muscle potential.

If time permits, try to penetrate fibres in the superficial extensor muscle (Fig. 4) and study the effects of stimulating the nerve supplying this muscle. Muscle potentials will be of the "pure" post-synaptic type, and rather small. The innervation pattern of individual axons is too complex to be worked out by the methods suggested in this exercise.

There are two difficulties which should be borne in mind when the above exercise is attempted. First, the microelectrode can be dislodged by muscle movement. In slightly aged or fatigued preparations the movement is much less vigorous and the difficulty not as serious as in fresh preparations. By suspending the microelectrode on a flexible wire, rather than placing it in a holder, more movement of the electrode is possible without withdrawal. Secondly, it is possible sometimes to stimulate two excitatory axons at about the same threshold, thus obtaining an incorrect distribution for a supposed "single axon". This difficulty can be coped with only by careful adjustment of the stimulation, careful attention to what is going on in the preparation and by repetition of the observations in other segments or other preparations.

Application of Drugs

Crustacean muscle differs from vertebrate striated muscle in its responsiveness to pharmacological agents. Some of the substances which affect the crustacean muscle fibres and neuromuscular transmission can be readily studied in the crayfish extensor muscle preparation.

Set up a preparation in the normal crayfish solution, and make a photographic record of muscle potentials evoked by one of the axons. Then replace the normal crayfish solution with one containing 10^{-5} g/ml GABA (1 vol of the original GABA solution to 99 vol of normal crayfish solution). Observe, and photograph, the effects on single muscle potentials (1/sec or single shock stimulation). Note also any effects on muscle contraction. After a few minutes, or when it appears that the full effect has occurred, replace the solution with normal saline, and note whether any recovery occurs. Repeat the experiment with other doses of GABA (e.g. 2×10^{-5} g/ml, 10^{-4} g/ml, 2×10^{-6} g/ml); are any differences in the rapidity or extent of the effect observed? If possible, study effects of GABA both on the excitatory postsynaptic potentials and on the spike-shaped or graded membrane responses.

After familiarity with effects of GABA has been obtained, try adding 6×10^{-4} g/ml picrotoxin (1 vol of the original picrotoxin solution to 1 vol of normal crayfish solution), and then a solution containing 10^{-5} g/ml GABA together with 6×10^{-4} g/ml picrotoxin (1 vol of the original GABA solution, 50 vol of the original picrotoxin solution, and 49 vol normal crayfish solution). How does the inclusion of picrotoxin affect the responsiveness to GABA?

Recently it has been found that strychnine, long known for its action on the vertebrate central nervous system has also an effect on neuromuscular transmission in the crayfish deep extensor muscle preparation (Parnas and Atwood, unpublished). Study the effects of strychnine by adding a solution containing 10^{-3} g/ml (1 vol of the original strychnine solution to 4 vol normal crayfish solution) to a preparation in which excitatory muscle potentials have been recorded. Observe and photograph the effects, which may develop slowly (allow about 10 min after addition of the drug). Determine whether or not the effect can be reversed by restoring the normal crayfish solution. If time permits, try other dosages of the drug (e.g. double and half).

Other drugs may also be tested on the preparation (see below).

RESULTS

HISTOLOGY

In *Procambarus clarki*, the histological features of the deep and superficial extensors are very different. Sarcomeres of 2 to 5 μ are encountered in the deep extensors, the shortest sarcomeres being found in the medial deep extensors (Abbott and Parnas, 1965). In the superficial extensors, much longer sarcomeres of 8–12 μ are always found. When cross sections are cut through the muscles, marked differences again appear: the fibres in the deep extensors are tightly bound together in a compact mass, whereas those in the superficial extensors are separated; within the fibres the contractile elements

are differently arranged (Parnas and Atwood, 1966). A similar situation has been established in the deep and superficial flexor muscles of the abdomen (Kennedy and Takeda, 1965a,b). In both extensors and flexors, the superficial muscles are slow-contracting tonic ones, used in postural activity; the deep muscles are fast-contracting phasic ones, used in the tail flick (for escape or swimming). Physiological differences between the two muscle groups parallel the morphological differences.

INNERVATION

The innervation of the deep extensor muscles in middle abdominal segments (3 and 4) of *Procambarus clarki* has been worked out by the methods of methylene blue staining and selective stimulation of single axons, as outlined above. In each segmental nerve, five excitatory axons and one inhibitory

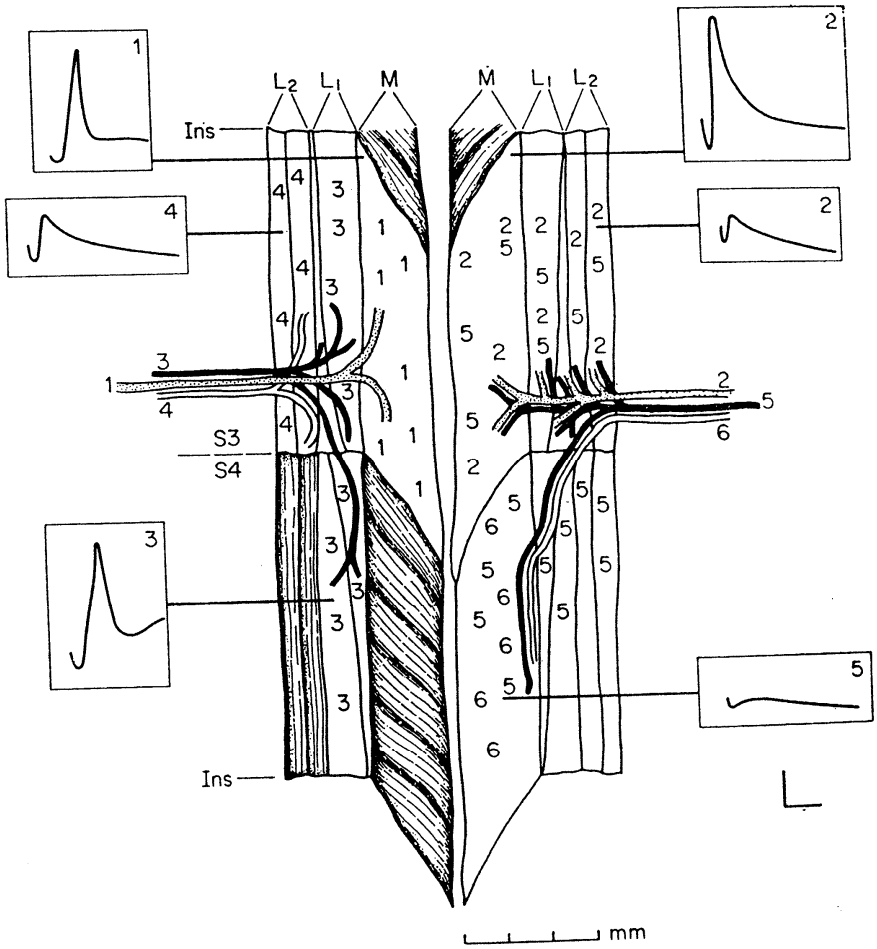

FIG. 5. Innervation of the deep extensors (segments 3 and 4, *Procambarus clarki*), showing the main branches of axons entering segment 3. Each axon is numbered, together with the parts of the musculature which it supplies. Tracings of representative electrical responses are shown for each axon. Calibration for the electrical responses: time, 20 msec; voltage, 1, 3, 4, 2 (top), 20 mV; 2 (bottom), 10 mV; 5, 4 mV. Note that both post-synaptic potentials and spike-shaped responses occur. (After Parnas and Atwood, 1966.)

axon appear. The distributions of the individual axons, and some of the muscle potentials evoked by them, are illustrated in Fig. 5 (after Parnas and Atwood, 1966).

Individual axons supply specific regions of the musculature, and some of them supply parts of two segments. The muscle potentials, even those evoked in different fibres by the same axon, are rather variable.

RESTING POTENTIALS

Table I provides an example of resting potential measurements from the medial and lateral deep extensors in normal saline. Use of the *t* test shows that there is no significant difference between the two muscles in this respect.

TABLE I

Resting potentials in lateral and medial deep extensor muscles in normal crayfish solution

	Lateral muscle resting potentials (mV)		Medial muscle resting potentials (mV)
Fibre 1	76·5	Fibre 1	70·5
2	84·5	2	70·5
3	78·0	3	70·5
4	76·0	4	67·5
5	62·0	5	70·5
6	73·5	6	73·5
7	73·5	7	73·5
8	76·0	8	73·5
9	73·5	9	74·5
10	76·0	10	70·5
Mean	75·0 ± 1·8 (S.E.)	Mean	71·5 ± 0·7 (S.E.)

t test: $t = 1{\cdot}94$.

(No difference between means at $P = 0{\cdot}05$).

Note. The values were taken from a student experiment. It is evident that the standard error is less for the second series than for the first; this may reflect the fact that more experience in making penetrations had been gained by the time the second series of measurements was made.

In Table II and in Fig. 6 results are presented from an experiment in which potassium ion concentration was increased to study the effect on membrane potential measurements. The plot is not a perfectly straight line; in particular, the slope decreases at lower potassium ion concentrations, perhaps due to decreased permeability to potassium ions (Hoyle, 1957; Hodgkin, 1964).

TABLE II

Variation in resting potential with external potassium ion concentration in medial deep extensor fibres

Potassium ion (mM/l)	Resting potentials (mV)	Mean (mV)
5·0	74, 76, 76, 72, 74, 76	74·7
8·9	68, 68, 64, 66, 66, 68	66·7
12·9	60, 60, 62, 64, 58, 64	61·3
20·7	52, 48, 52, 52, 50, 52	51·0
25·6	44, 46, 44, 46, 44, 42	44·3
32·5	36, 40, 40, 38, 40, 40	39·0
38·0	34, 38, 36, 36, 32, 34	35·0
60·0	28, 26, 28, 28, 30, 30	28·3

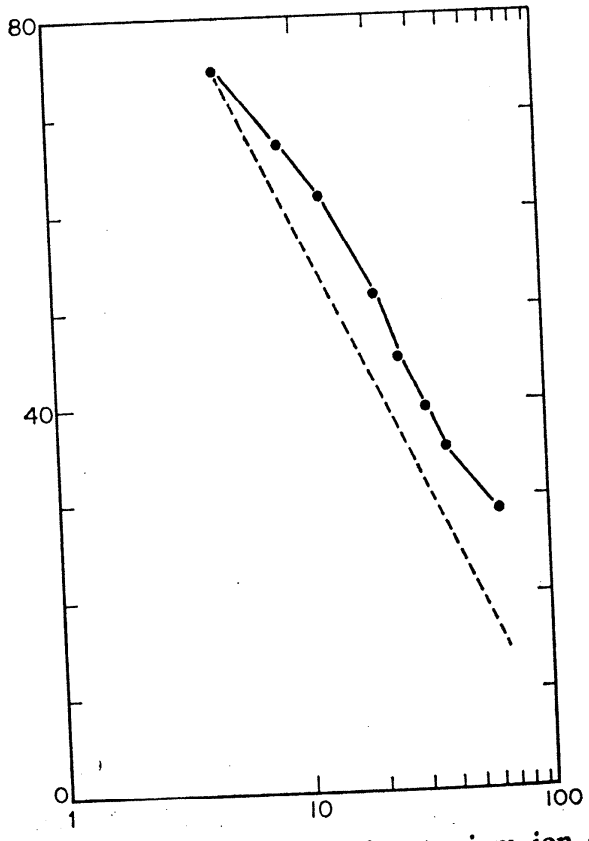

FIG. 6. Plot of relationship between external potassium ion concentration (abscissa, log scale) and membrane potential, for medial deep extensor muscle fibres. The dotted line indicates a slope of 58 mV per ten-fold change in potassium ion.

The slope of the plot in its linear region is not much different from the theoretically expected 58 mV per decade change in K_{out}.

NERVE STIMULATION

Single excitatory muscle potentials recorded from the deep extensors of *Procambarus clarki* are of three general types: "pure" post-synaptic potentials, post-synaptic potentials with superimposed graded membrane responses, and large spike-shaped responses which may approach or overshoot the zero membrane potential level (Figs 5 and 7). It should be noted that the graded

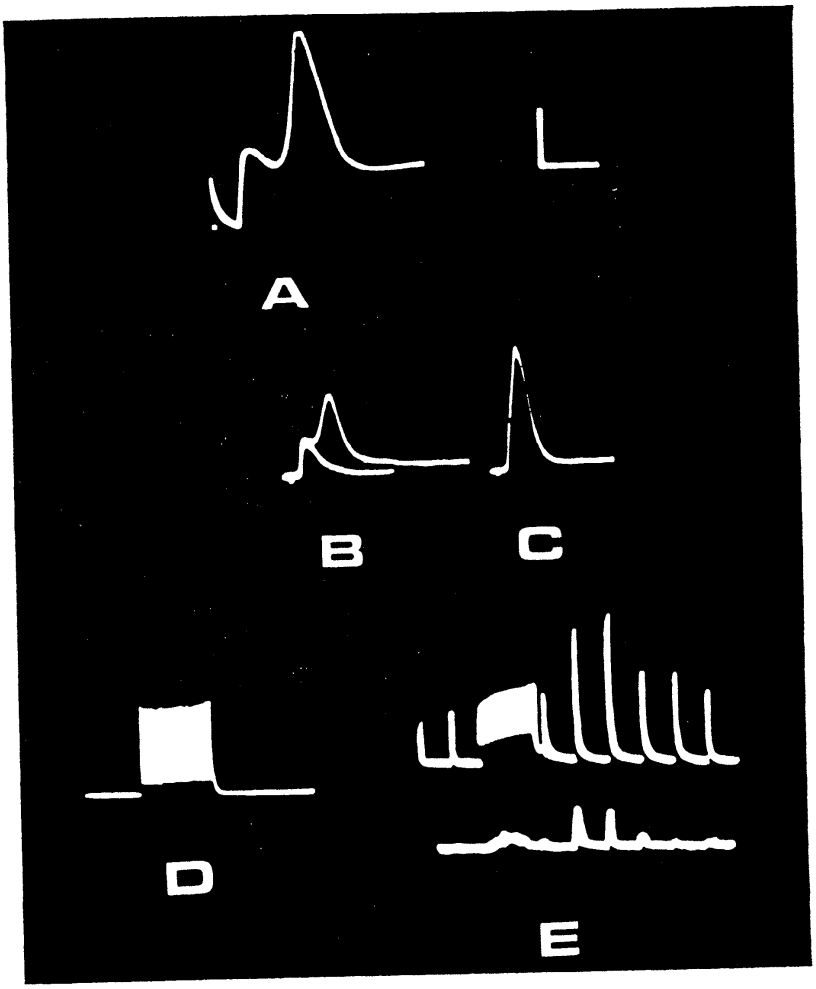

FIG. 7. Excitatory potentials from deep extensor muscles. A. Post-synaptic potential with a delayed secondary response appearing during the decay phase. The response occurred in the medial muscle with a stimulus to axon 1. B. Record from L_1 responding to axon 3. Two stimuli were given; the first elicited a post-synaptic potential, the second a delayed secondary response. C. A spike-shaped response occurred in the same location after a short burst of stimuli at 20/sec (facilitation). D. Lack of facilitation of the post-synaptic potential generated in L_2 by stimulation of axon 2 at 30/sec (note slower time base for this record). *E*. Post-tetanic potentiation in L_2 with stimulation of axon 4. The lower trace shows the tension responses of the muscle. After a short train of closely spaced stimuli, the electrical and mechanical responses increase in size. Calibration: time, (A) 10 msec, (B, C) 20 msec, (D, E) 1 sec; voltage, (A) 10 mV, (B, C, E) 20 mV, (D) 5 mV.

membrane responses and spike-shaped responses are all excited by post-synaptic potentials set up by the chemical activity of the transmitter substance. These post-synaptic potentials are distributed along the length of the muscle fibre (Fatt and Katz, 1953b). If the post-synaptic potentials are large enough they can initiate an additional "electrically excited" membrane response.

Some of the axons (e.g. axon 2, Fig. 5) typically give spike-shaped responses in one part of the innervation field, and post-synaptic potentials in other parts. With fatigue or ageing of the preparation, the number of spike-shaped responses decreases, as does the vigour of the muscular contraction. In fact, fatigue can be induced by a short period of continuous repetitive stimulation (at, say, 10 or 20/sec); these muscles are incapable of supporting prolonged, uninterrupted contractions. Normally, in the tail flick, the contractions are intermittent.

The post-synaptic potentials of the deep extensor muscles show moderate facilitation, or sometimes none, with repetitive stimulation (Fig. 7). However,

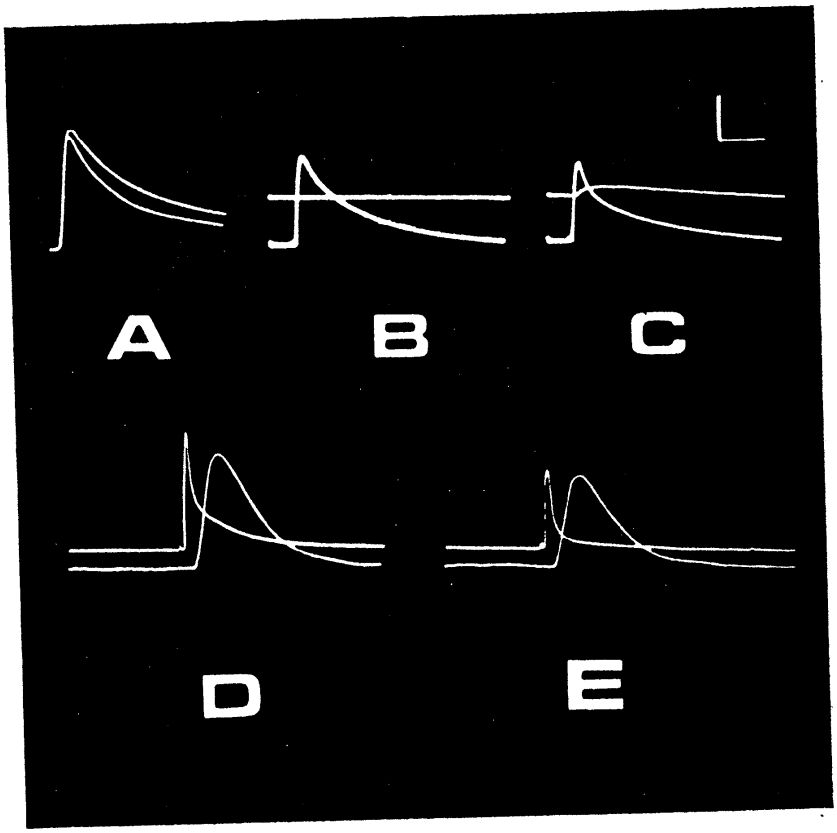

FIG. 8. Manifestations of inhibitory activity in the deep extensor muscles. A. Post-synaptic potential (L_2, axon 4), with (bottom trace) and without (top trace) a simultaneous inhibitory stimulus. Note increased decay rate of the potential with inhibition. B and C. Simultaneous records made from the medial muscle (top) and L_2 (bottom). A stimulus to axon 4 produces a post-synaptic potential in L_2 (B). Simultaneous stimulation of axon 5 gives rise to an inhibitory potential in the medial muscle and to increased rate of decay of the excitatory potential in L_2 (C). D and E. Electrical record (top) and tension record (bottom) of activity in the medial muscle. In D, axon 1 was stimulated; in E, axon 5 was also stimulated, and the responses produced by axon 1 show attenuation. Calibration: time, (A) 10 msec, (B, C) 20 msec, (D, E) 40 msec; voltage, (A, B, C) 10 mV, (D, E) 20 mV.

post-tetanic potentiation (increase in amplitude of the response to a single shock following a burst of closely spaced stimuli) can be quite pronounced (Fig. 7).

Activation of inhibitory axons may give rise to inhibitory post-synaptic potentials, if the inhibitor is active at the lowest threshold, which is usually not the case. More often, the inhibitor acts to reduce the amplitude of excitatory potentials, when it is stimulated at the same time as a lower-threshold excitatory axon (Fig. 8). Spike-shaped responses are often reduced to graded responses with simultaneous inhibitory input; graded responses tend to disappear entirely. Excitatory post-synaptic potentials characteristically show an increased rate of decay when the inhibitor is simultaneously activated, even though the amplitude of the excitatory response may be little reduced.

In the superficial extensor muscles there are five motor axons and one inhibitor supplying each muscle, but fibres within the muscle receive a variable number of the total available axons. The excitatory post-synaptic potentials are smaller than in the deep muscles, and for the most part show more pronounced facilitation with repetitive stimulation (Fig. 9). Graded responses

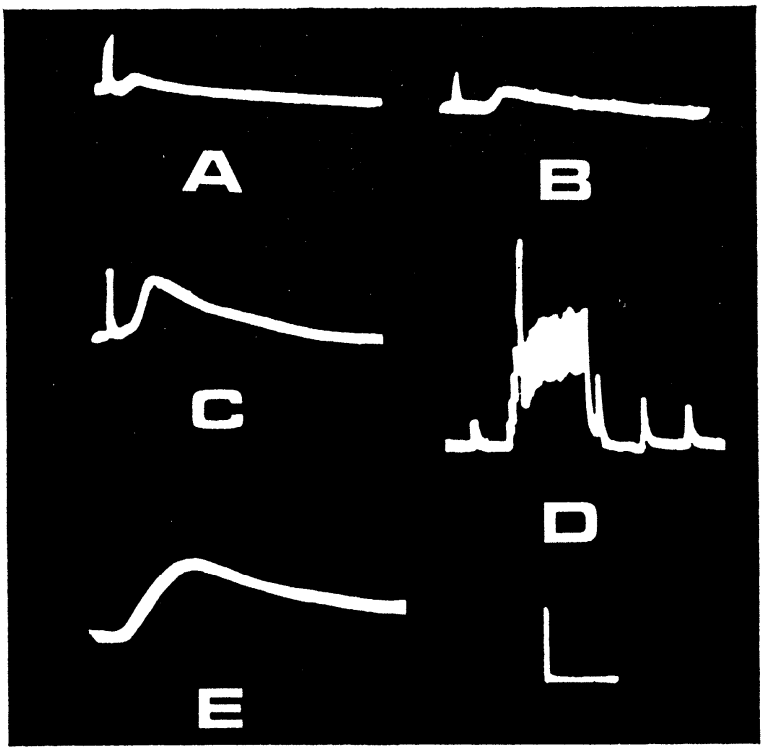

FIG. 9. Responses of the superficial extensor muscle to stimulation of the nerve. A–C. Single post-synaptic potentials produced in a muscle fibre by three different axons. D. Responses to stimulation of the third axon at 20/sec, showing facilitation of the post-synaptic potentials and a single graded response. E. Tension development with stimulation of the nerve at 20/sec. Note slow increase and relaxation. Calibration: time, A–C, 20 msec; D, 1 sec: E, 4 sec; voltage, A–C, 10 mV; D, 20 mV. (After Parnas and Atwood, 1966.)

are occasionally seen with repetitive stimulation in some fibres, but not with single stimuli. No twitch-like contractions occur in these muscles; instead, the contraction develops and relaxes very slowly, and its speed and magnitude increase with increasing frequency of the indirect stimulation.

EFFECTS OF DRUGS

GABA acts to reduce the amplitude of the excitatory muscle potentials and to increase their rate of decay. The associated muscle contraction is also reduced (Fig. 10).

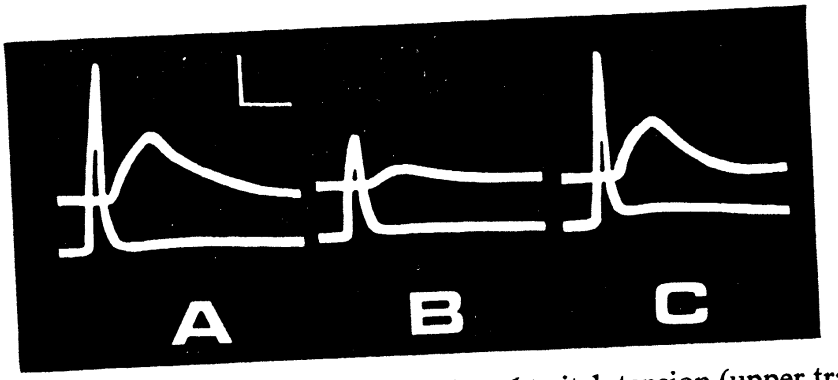

FIG. 10. Effect of GABA on spike (lower trace) and twitch tension (upper trace) produced by axon 1, in the medial deep extensor of a rock lobster. A. Control in normal saline. B. Soon after addition of 10^{-5} g/ml GABA. C. Recovery after restoration of normal saline. Calibration: time, 20 msec; voltage, 20 mV.

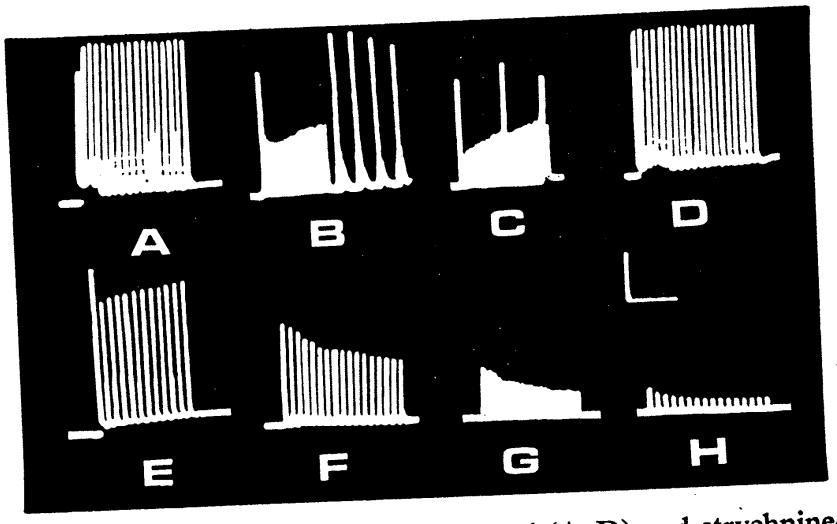

FIG. 11. Effect of stimulation of axon 1 on normal (A–D) and strychnine-treated (E–H) deep extensor preparations. A. Spike-shaped responses at 3/sec stimulation. B and C. After fatigue, a post-synaptic potential showing some facilitation at 20/sec stimulation, and post-tetanic potentiation (B), was obtained; occasional graded responses occurred. D. Recovery of the spike-shaped response after a period of rest. E. Spike-shaped responses in another preparation. F. Response after 2 min exposure to 1 mg/ml strychnine nitrate, showing antifacilitation of the electrical responses. G. Persistence of antifacilitation during stimulation at 20/sec. H. After a rest period of 1·5 min the response continued to decline; complete block occurred at 5 min. Calibration: 20 mV, 2 sec.

At 10^{-5} g/ml GABA these effects are usually quite well marked, and are even more pronounced at higher doses. The preparation recovers in normal saline, at least from 10^{-5} g/ml GABA (Fig. 10). Picrotoxin antagonizes the action of GABA and prevents reduction of the excitatory muscle potentials. It is thought that this effect is attributable to inactivation of the inhibitory receptor areas on the muscle, which are normally activated by GABA (Grundfest *et al.*, 1959).

Strychnine also reduces the excitatory muscle potentials, but more slowly and less reversibly (Fig. 11). The mechanism of action is very likely on the pre-synaptic nerve terminals rather than on the receptor areas of the muscle. Glutamic acid stimulates contraction in a strychnine-blocked preparation, as it does in the normal muscle (cf. Van Harreveld and Mendelson, 1959). The peculiar antifacilitation of the muscle potentials during repetitive stimulation also suggests a pre-synaptic action.

TROUBLE-SHOOTING

The most common sources of trouble are associated with the microelectrode technique itself. Failure to penetrate a cell, or low resting potential values, may be due to a broken electrode (check electrode resistance). Instability of the trace may be caused by lack of contact of the saline in the electrode with the wire leading to the cathode follower, or to blockage of the electrode tip (check electrode resistance, and discard the electrode if the resistance is unduly high), or to lack of contact of the bath with the grounding wire.

Another source of trouble is 60 or 50 c/s interference from the mains. This may be hard to eliminate. Attention should be given to correct grounding procedures, arrangement of equipment, etc. Sometimes higher frequency interference comes from fluorescent overhead lights, which may have to be turned off.

The preparation, if poorly made, or too old, or allowed to heat up too much, may fail to respond to stimulation of the nerves. Efforts should be made to get clean, undamaged preparations and to maintain them below 20°C.

Pulling out of the microelectrode during a muscle contraction is common. Since the muscle potential occurs before the contraction, most of it can be recorded even when the electrode pulls out. Some parts of the muscles contract or are displaced less than others, and there is an advantage in attempting to place the electrode there. With fatigue of the preparation there is less contraction but of course fewer of the spike-shaped responses are then recorded.

Conclusions

The main points which the above experiments serve to illustrate, are as follows.

(1) In the abdominal extensor muscles, there is separation of the contractile elements into the *deep* muscles and the *superficial* muscles. These differ in structure, innervation, and electrical and contractile properties.

(2) In the deep muscles, the individual axons entering from a segmental nerve supply discrete areas of the musculature, in some cases in two segments.

(3) The excitatory axons, when stimulated, typically evoke large post-synaptic potentials, graded responses, or spike-shaped responses in the areas of the musculature which they innervate. Impulses in the inhibitory axons reduce the amplitude of the excitatory responses, and increase the rate of decay of excitatory post-synaptic potentials.

(4) In the superficial extensors, the post-synaptic potentials are smaller, and graded membrane responses uncommon. Facilitation of excitatory post-synaptic potentials is usually more pronounced than in the deep extensors.

(5) GABA activates the inhibitory areas of the muscle membrane, which are blocked by picrotoxin.

(6) Strychnine blocks neuromuscular transmission, probably by an effect on the pre-synaptic terminals of the excitor axons.

(7) The membrane potential of fibres in the deep extensor muscle is sensitive to potassium ion concentration, in approximate agreement with the Nernst equation.

(8) The abdominal muscles are different from the more usually studied leg muscles in many ways.

Further Ideas

Other experiments which could be attempted on this preparation include the following.

(1) Simultaneous recording of tension and electrical events in the deep extensors. This requires a strain gauge or mechano-electronic transducer of suitable sensitivity, fitted with a probe to make contact with the transverse segmental ligaments associated with the deep extensors.

(2) Effects of other drugs and ions on the muscle potentials; for example, acetylcholine (10^{-5} g/ml), strontium or barium chloride (20–50 mM, replacing osmotically equivalent sodium chloride), choline chloride (replacing sodium chloride).

(3) Further analysis of the effects of strychnine in the deep extensors, for example, by recording the nerve potential in the strychnine-treated preparation (Fig. 4).

(4) Effects of strychnine on the potentials of the superficial extensors. The effect is much less pronounced than in the case of the deep extensors.

(5) More thorough study of the effects of ions on the resting potential; for example, by replacement of sodium chloride by potassium in conjunction with a non-penetrating anion (acetate or propionate) rather than chloride.

Cleaning Up

All equipment, both mains and battery operated, should be switched off at the end of the experiment. Carefully wipe up any spilled saline and KCl solution, especially from equipment. Unused microelectrodes should be stored in a safe place. Remains of preparations and animals should be wrapped up in paper towels and deposited in the appropriate disposal bag or container. Wash out preparation dishes with fresh water, and leave them to dry for the next laboratory period.

Bibliography

Abbott, B. C. and Parnas, I. (1965). Electrical and mechanical responses in deep abdominal extensor muscles of crayfish and lobster. *J. gen. Physiol.* **48**, 919–931. [An account of propagated spikes in deep extensor muscle fibres; see Parnas and Atwood (1966) for further information.]

Atwood, H. L. and Dorai Raj, B. S. (1964). Tension development and membrane responses in phasic and tonic muscle fibres of a crab. *J. cell. comp. Physiol.* **64**, 55–72. [A study of the different kinds of muscle fibre encountered in a crab muscle. Note that in this muscle, unlike the case of the abdominal muscles, diverse types of fibre occur in the same muscle.]

Donaldson, P. E. K. (1958). "Electronic Apparatus for Biological Research." Butterworths, London. [A very useful description of microelectrode technique is included in this book, together with much information on apparatus of various types.]

Fatt, P. and Katz, B. (1953a). The electrical properties of crustacean muscle fibres. *J. Physiol., Lond.* **120**, 171–204. [Effects of certain ions on directly-evoked crustacean muscle potentials: effect of potassium ion on the resting potential; much information on the electrical properties of the muscle fibres.]

Fatt, P. and Katz, B. (1953b). Distributed "end-plate potentials" of crustacean muscle fibres. *J. exp. Biol.* **30**, 433–439. [Demonstration of the fact that post-synaptic potentials occur at multiple sites along crustacean muscle fibres, instead of at one point as in many vertebrate muscle fibres.]

Grundfest, H., Reuben, J. P. and Rickles, W. H. (1959). The electrophysiology and pharmacology of lobster neuromuscular synapses. *J. gen. Physiol.* **42**, 1301–1323. [An account of the effects of GABA, picrotoxin, and other substances on crustacean muscle fibres.]

Hodgkin, A. L. (1964). "The Conduction of the Nervous Impulse." Thomas, Springfield. [A readable review of information relating to resting and action potentials of nerve; some of the findings apply also to muscle.]

Hoyle, G. (1957). "Comparative Physiology of the Nervous Control of Muscular Contraction." Cambridge University Press. [Although some of the material is now out-dated, this book contains a good account of the resting and action potentials, and of the earlier work on arthropod muscles.]

Hoyle, G. and Wiersma, C. A. G. (1958). Excitation at neuromuscular junctions in Crustacea. *J. Physiol., Lond.*, **143**, 402–425. [Illustration of the variability of nerve-evoked potentials in crustacean muscle fibres.]

Kennedy, D. and Takeda, K. (1965a). Reflex control of abdominal extensor muscles in the crayfish. I. The twitch system. *J. exp. Biol.* **43**, 211–227. [A description of the fast abdominal flexor muscles, which are similar in many respects to the deep extensor muscles.]

Kennedy, D. and Takeda, K. (1965b). Reflex control of abdominal flexor muscles in the crayfish. II. The tonic system. *J. exp. Biol.* **43**, 229–246. [A description of the innervation and certain other properties of the superficial flexor muscles, which are similar to the superficial extensors.]

Nastuk, W. L. (Ed.) (1964). "Physical Techniques in Biological Research," Vol. 5. Electrophysiological methods, Part A. Academic Press, New York and London. [Contains a chapter on the microelectrode techniques of value as a reference.]

Parnas, I. and Atwood, H. L. (1966). Phasic and tonic neuromuscular systems in the abdominal extensor muscles of the crayfish and rock lobster. *Comp. Biochem. Physiol.* **18**, 701–723. [An account of the innervation and physiology of the abdominal extensor muscles in *Procambarus* and *Panulirus*; of particular relevance to the present experiments.]

Pilgrim, R. L. C. and Wiersma, C. A. G. (1963). Observations on the skeleton and somatic musculature of the abdomen and thorax of *Procambarus clarkii* (Girard), with notes on the thorax of *Panulirus interruptus* (Randall), and *Astacus*. *J. Morph.* **113**, 453–587. [Contains further details of the anatomy of the abdominal extensor muscles.]

Van Harreveld, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. exp. Biol. Med.* **34**, 428–432. [The original recipe for crayfish solution.]

Van Harreveld, A. (1939). The nerve supply of doubly-and-triply-innervated crayfish muscles related to their function. *J. comp. Neurol.* **70**, 267–284. [A description of the pattern of innervation in crustacean leg muscles.]

Van Harreveld, A. and Mendelson, M. (1959). Glutamate-induced contractions in crustacean muscle. *J. cell. comp. Physiol.* **54**, 85–94. [Description of the stimulating effect of glutamate on crustacean muscle fibres.]

Wiersma, C. A. G. (1961). The neuromuscular system. *In* "The Physiology of Crustacea" (T. H. Waterman, ed.), Vol. 2. Academic Press, New York and London. [A review of earlier work on crustacean neuromuscular physiology.]