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INVESTIGATIONS ON EXCITATION AND CONTRACTION IN CRUSTACEAN MUSCLES

THESIS

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Degree of Doctor of Philosophy

in the

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by

H. L. ATWOOD, B.A. (Toronto),
M.A. (California at Berkeley).

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The motor nerve impulse arrives at the terminal point of the motor nerve, the nerve-muscle junction or end-plate. "Twitch" muscle fibres generally receive only one or two end-plates.

The nerve impulse causes the release of a chemical transmitter substance (in the case of vertebrate skeletal muscles, acetylcholine) from the nerve ending. The transmitter substance is released in discrete quanta. The released transmitter substance reacts with the post-junctional area of the muscle fibre membrane, presumably combining with special "receptor" sites. This reaction results in an increase in membrane permeability to more than one ion. A lowering of the membrane potential in the end-plate region results; this depolarization is termed the "end-plate potential."

If the depolarization is large enough, the surrounding non-junctional membrane is excited, and a regenerative muscle spike is triggered. The muscle spike is propagated rapidly along the length of the muscle fibre, causing the entire muscle fibre membrane to undergo rapid depolarization.

A series of rapid events, about which little is known, but which can be referred to as the "excitation-contraction coupling" process, is set in motion by the muscle spike. The excitation-contraction coupling process translates the electrical activity of the muscle fibre membrane (or, more generally, the release of transmitter substance) into mechanical activity of the muscle. As a result of the excitation-

contraction coupling process, the muscle changes from its resting state to its "active state" (Hill, 1938) i.e. it acquires the ability to contract and to do work.

Contraction of the muscle then occurs. Again, the actual mechanism of contraction is as yet unknown, although a large amount of biochemical knowledge has been accumulated. Older theories of contraction were based on the chemical reaction between the muscle proteins actin and myosin (Szent-Gyorgyi, 1951); more recent thinking has as its basis the "sliding-filament" hypothesis (Hanson and Huxley, 1955). The contractile response of the muscle is influenced by the mechanical properties of the muscle and by interaction of the muscle with its load.

Finally, breakdown of "active state" occurs; the muscle relaxes and returns to its resting state. Relaxation is generally considered to be a passive process, but it has been suggested (e.g. Ramsey, 1944) that relaxation is active.

Although this generalized model provides a framework for the consideration of the problems of muscular contraction and its control, and for comparison of frog "twitch" muscle with other muscles, knowledge of many of the stages is very incomplete. In particular, little is known about the excitation-contraction coupling processes. It is thought that excitation-contraction coupling probably occurs more rapidly than can be accounted for by diffusion of a substance from the surface of a muscle fibre to its interior (Hill, 1949).

It has been argued (Bay et al, 1953) that the longitudinal current flow associated with differential depolarization of different parts of the muscle fibre is the factor which initiates contraction. A variation of this hypothesis (Csapo and Suzuki, 1957; Sakai and Csapo, 1958) attributes excitation-contraction coupling to the transport of an activating substance by longitudinal current flow. However, this type of argument has been shown to be inconsistent with many experimental findings (Sten-Knudsen, 1954; Watanabe, 1958; Buchtal and Sten-Knudsen, 1959; Sten-Knudsen, 1960; Close, 1962), and is not in general acceptance.

Excitation-contraction coupling is more often explained in terms of transverse, rather than longitudinal, transfer of excitation. Very cogent experimental evidence for this viewpoint stems from the phenomenon of contracture.* When a frog muscle or muscle fibre is uniformly depolarized, for instance by application of excess potassium, or certain drugs, tension is developed (Kuffler, 1946; Sandow, 1955). This tension can be graded according to the extent of the depolarization,

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*As defined by Sandow (1955) for vertebrate "twitch" muscle, contracture is a "prolonged, reversible, non-propagated" contraction, as distinct from rigor (an irreversible contraction) and tetanus (propagated). This definition does not apply very well to crustacean muscles, since here the element of propagation is often not present in normal contractions. Therefore, contracture would be better defined for these muscles as "a prolonged, reversible, non-propagated contraction not produced by normal nervous activity."

and only occurs when the muscle fibre has been depolarized past a "threshold" membrane potential (Hodgkin and Horowicz, 1960b). In frog "twitch" muscle fibres the threshold for tension development is slightly greater than that for production of spikes in the normal muscle. The threshold for tension development can be lowered by substitution of nitrate, bromide, iodide or thiocyanate for chloride (Hodgkin and Horowicz, 1960c); these anions are also known to potentiate the twitch contractions of the muscle.

Evidence of this type has led to the widely held view that a primary step in the excitation-contraction coupling process is the depolarization of the muscle fibre membrane by the muscle spike rather than longitudinal current flow (Kuffler, 1946; Katz, 1950; Sten-Knudsen, 1954; Sandow, 1955; Hodgkin and Horowicz, 1960b). However, it has become clear that other factors besides depolarization *per se* play a part in excitation-contraction coupling. If calcium ions are removed from the solution surrounding the muscle, depolarization (by potassium) does not cause contraction (Frank, 1958, 1961). This result has led to the conclusion that external calcium ions act as a link in the excitation-contraction coupling process. Other experiments support this view (e.g. Bianchi and Shanes, 1959; Bianchi, 1962). However, contraction in frog muscle can occur in the absence of external calcium ions under certain conditions (as in response to caffeine; Frank, 1962). In such cases it has been postulated that an intra-

cellular supply of "bound calcium" is utilized during contraction.

Further support for the role of calcium as a link in excitation-contraction coupling is provided by work of Jenkinson and Nicholls (1961), who found that depolarized denervated skeletal muscle (of rat) contracts on application of acetylcholine, and that an uptake of calcium accompanies this contraction.

A recent development in the study of excitation-contraction coupling has been the focussing of attention on the ultrastructure of muscle. It has been shown (Huxley and Taylor, 1958) that locally applied depolarization is effective in activating the contractile mechanism only when it is applied at particular spots on the muscle fibre membrane (in frog muscle, these spots were located at the Z lines; in crab and lizard muscles, the spots were located at the boundaries between A and I bands). The location of the active spots in frog and lizard muscles corresponds to the location of certain components of the sarcoplasmic reticulum (the "triads" of Porter and Palade, 1957). Therefore, it appears possible that part of the sarcoplasmic reticulum having contact with the external solution may be concerned in the conduction of excitation inwards from the surface of the fibre (Huxley and Taylor, 1958).

Although the information which has been gathered about excitation-contraction coupling is fragmentary and cannot yet

be integrated into a satisfactory model, one point deserves emphasis: it appears probable that in frog "twitch" muscle fibres depolarization of the muscle fibre membrane normally acts as an essential link in the coupling process. The frog muscle spike thus has two functions: conduction of excitation from the end-plate region(s) to distant parts of the muscle fibre, and initiation of excitation-contraction coupling via depolarization.

Much less is known about the neuromuscular systems of the decapod crustaceans than about that of the frog. However, it is well established that the mechanisms of excitation and contraction differ in many respects in the two groups (Hoyle, 1957; Wiersma, 1957, 1961).

Using the outline previously presented for frog muscle as a frame of reference, a comparative scheme can be prepared for crustacean muscles:

(a) The motor nerve impulse travels down the motor axon to the nerve-muscle junctions.

In the leg muscles of the decapod crustaceans the motor axons split into many branches and innervate the muscle fibres at many points along their lengths (multiterminal innervation). The nerve-muscle junctions (described by Lavallard, 1960) are located along the fine terminations of the motor axons and can be found all over a given muscle fibre.

(b) The nerve impulse causes the release of transmitter substance. The chemical nature of crustacean transmitter

substances is unknown, although it has been suggested by van der Kloot (1960) that a substituted derivative of nicotinamide may be involved. As in frog muscle, the transmitter substance is released in discrete quanta (Dudel and Orkand, 1960). Facilitation at crustacean nerve-muscle junctions can be formally described as an increase in the probability of release of transmitter-substance quanta (Dudel and Kuffler, 1961).

(c) Reaction of the released transmitter agent with the post-junctional membrane of the muscle fibre occurs.

(d) This reaction results in the appearance of an electrical change in membrane potential, which typically takes the form of an "end-plate potential" (Fatt and Katz, 1953b) "junctional potential" (Hoyle, 1957) or "post-synaptic potential" (Grundfest, et al., 1959).*

This electrical change occurs simultaneously in all parts of the muscle fibre (Fatt and Katz, 1953b), reflecting the distribution of the nerve endings.

(e) In some crustacean muscles, but not in all (Furshpan, 1955; Hoyle and Wiersma, 1958a) sufficient depolarization gives rise to a muscle spike, which can be graded rather than all-or-none.

(f) When present, the muscle spike can appear simultaneously in all parts of the muscle fibre, so the fact that it

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*Throughout the present study the latter term will be used.

may be propagated (Fatt and Katz, 1953a) is apparently of little functional significance (Hoyle and Wiersma, 1958a). In crustacean muscle the distribution of nerve terminals ensures uniform depolarization of the muscle fibre. In fibres where spikes are not present, summation of successive post-synaptic potentials (p.s.ps.) can result in depolarization "plateaus."

(g) Excitation-contraction coupling processes of an unknown nature take place.

(h) The muscle can be assumed to pass into an "active state." Nothing is known about active state in crustacean muscle.

(i) Contraction of the muscle takes place. Very little is known about the specific biochemistry of contraction in crustaceans.

(j) The contractile response of the muscle is presumably influenced by the muscle's mechanical properties and by interaction with the load. No work has been done on problems of this type in crustaceans.

(k) Relaxation occurs.

Certain further differences between vertebrate and crustacean neuromuscular systems deserve mention. Decapod leg muscles receive from one to four motor axons (Wiersma, 1941; Wiersma and Ripley, 1952; Wiersma, 1961, etc.) Two muscles, the "stretcher" and the "bender" are supplied by a single motor axon and can thus be considered as a single motor

unit. The pattern of distribution of excitatory axons to decapod leg muscles, as worked out by Wiersma and his collaborators, is shown in Figure 1.

In muscles supplied by two motor axons (closer, bender, extensor), the mechanical and electrical events produced by separate stimulation of the two axons often show marked differences. The speed of the contraction evoked by one of these axons is usually much faster than that evoked by the other, provided that the load on the muscle and the frequency of stimulation are the same. The axons are therefore termed "fast" and "slow" respectively. In many muscles, most of the muscle fibres are innervated by both axons (polyneuronal innervation), but in some muscle it has been found that some of the muscle fibres are innervated exclusively by either the "fast" or the "slow" axon (Hoyle and Wiersma, 1958a).

"Fast" and "slow" neuromuscular systems have been found also in certain frog muscles,* but here polyneuronal innervation is probably not present (see, however, Shamarina, 1962). The "slow" (or "small-nerve") motor axons innervate a group of muscle fibres having membrane properties quite distinct from those of the more common "twitch" muscle fibres (Burke and Ginsborg, 1956a). The contractile properties and morphological appearance of the "slow" muscle fibres are also quite distinct (Kuffler and Vaughan Williams, 1953a,b;

*Also, more recently, in vertebrate extraocular muscles (Hess, 1961).

Kruger, 1952; Gray, 1958). Frog and crustacean "slow" and "fast" systems have little in common at first glance. In the frog, differences between the two systems have been explained by differences in the "slow" and "fast" muscle fibres, which react to the same transmitter substance, acetylcholine. In crustaceans, the same muscle fibres have been thought to give both slow and fast contractions, and it has been easier to propose separate "slow" and "fast" transmitter substances acting on the same muscle fibres than to account for the "slow" and "fast" phenomena in terms of a single excitatory transmitter substance.

Even less is known about excitation-contraction coupling in crustacean muscle than in frog muscle. The most logical initial assumption to make is that depolarization of the surface membrane initiates the excitation-contraction coupling process, as it probably does in frog muscle. It has been suggested (Fatt and Katz, 1953c) that the tension is controlled by the mean level of membrane depolarization produced by the summated post-synaptic potentials. As depolarization increases past a supposed "threshold" tension becomes greater, according to this hypothesis. This hypothesis is similar to those proposed for the frog "slow" system (Kuffler and Vaughan Williams, 1953a,b) and for certain smooth muscles (Bulbring, 1955).

However, the suggestion of Fatt and Katz has been undermined by observations made by Hoyle and Wiersma (1958c) who came to the conclusion that it was untenable for crustacean muscles. The main basis of their criticism is the "paradox"

phenomenon in muscles of *Randallia*, *Blepharipoda*, and *Cambarus*. Low frequency stimulation applied to the "fast" axon of muscles in the "paradox state" evokes comparatively large electrical responses but no contraction, whereas the same frequency of stimulation applied to the "slow" axon produces much smaller electrical responses, but a pronounced mechanical response. This phenomenon was originally described by Wiersma and van Harreveld (1938), who used external recording electrodes. Since maintained slow depolarizations would not be recorded by this method, it was possible that the total depolarization produced by "slow" axon stimulation was greater than that produced by "fast" axon stimulation. Hoyle and Wiersma (1958c) re-examined the phenomenon using intracellular electrodes, and found that in *Randallia* electrical events during the "paradox" appeared to be similar in all the muscle fibres which they examined. Throughout the muscle, "fast" electrical responses appeared to be larger than "slow" electrical responses, but contraction occurred only in response to "slow" axon stimulation.

It is of interest that the paradox state did not become apparent in either *Randallia* or *Cambarus* until the preparations had aged and become partially fatigued. The phenomenon may therefore be largely an artificially induced one.

Hoyle and Wiersma also used the paradox phenomenon as the major proof of their contention that separate "fast" and "slow" transmitter substances must be postulated to explain "fast" and "slow" transmission in all crustacean muscles.

However, the results of Hoyle and Wiersma (1958c) can be severely criticised on the grounds that the number of muscles studied by them was small, and that correlation of electrical events recorded from single cells with mechanical events recorded from the whole muscle is risky.

Only two nerve-muscle preparations of Randallia, the species in which the paradox state was most convincingly apparent, were studied. This amount of investigation is almost certainly not sufficient to determine whether or not all of the muscle fibres in the muscle gave the same responses. In Carcinus (see this study, Results) certain specialized muscle fibres were not discovered until over twenty muscles had been examined.

Furthermore, it is impossible to be absolutely certain of correlation between electrical and mechanical events unless both are recorded from a single muscle fibre. The paradox phenomenon cannot be regarded as definitely proven until it has been demonstrated from a single intact, normally functioning muscle fibre. The preparation of such a unit is a formidable task in crustacean muscle. However, steps towards obtaining such a preparation have been taken by Hoyle (personal communication) and his method has been used by the author in the present study.

A more general reason for doubting the importance of membrane depolarization per se in excitation-contraction coupling in crustacean muscles is the extremely small size of the

electrical events (often less than a millivolt) associated with many indirectly produced contractions. This problem has become even more apparent since the demonstration by Orkand (1962) and shortly afterwards, by the author (Atwood, 1962)* that certain crayfish muscles must be depolarized past a threshold of 60 mV (from resting potentials of about 80 mV) before tension is developed.

It must be concluded, therefore, that at the present time almost nothing is definitely known about excitation-contraction coupling in crustacean muscles. It is not even known whether indirectly produced depolarization of the muscle fibre membrane is an essential step in the normal excitation-contraction coupling process.

The object of the present study was to obtain more information about excitation and contraction in crustacean muscles and about the excitation-contraction coupling processes. In particular, attention was paid to the related questions: (1) Is depolarization of the muscle fibre surface membrane an essential link in the excitation-contraction coupling process? (2) Does "fast" axon stimulation bring about tension development by a different mechanism (transmitter substance or coupling process) than "slow" axon stimulation?

Several different but interdependent methods of investigation were used to obtain information about the excitation and

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*This study was complete and submitted for publication before Orkand's paper appeared.

contraction mechanisms in crustacean muscles. Briefly, these were:

(a) Study of the electrical and mechanical events in intact muscles. This type of study is inherently limited in that electrical events recorded from single muscle fibres by microelectrodes cannot be correlated with the mechanical responses of those muscle fibres, but only with the contraction of the whole muscle. Nevertheless, much valuable information can be obtained by this method provided the muscle under investigation is studied thoroughly.

(b) Study of the electrical properties of the muscle fibre membrane and correlation of these properties with "fast" and "slow" electrical responses in the same muscle fibre.

(c) Study of the membrane potential threshold at which contraction is initiated (1) by potassium depolarization (in whole muscles and in single muscle fibres), and (2) by directly applied electrical depolarization (in single muscle fibres). The ultimate aim of this type of study was to compare directly produced depolarization and indirectly produced depolarization with respect to their ability to bring about muscular contraction.

(d) Study of indirectly produced electrical and mechanical responses in single isolated muscle fibres with intact motor axon innervation.

(e) Application of experimental solutions modified by addition or substitution of various ions, with the purpose of

observing the effects of artificial conditions on the excitation-contraction coupling processes.

Throughout the investigation, muscles from several different animals were used. This comparative approach was dictated partly by the circumstance that the study was begun at the University of Glasgow and completed at the University of Oregon. The animals used at Glasgow were the shore crab _Carcinus maenas_, the small lobster or "crayfish" _Nephrops norvegicus_, and the fresh-water crayfish _Astacus pallipes_. In Oregon the experimental animals were the shore crab _Pachygrapsus crassipes_ and the large edible crab _Cancer magister_. Most of the work was done on the three animals used at Glasgow.

The choice of a semi-comparative approach was also partly deliberate, since it was considered advantageous to compare muscles from different animals in order to judge better which mechanisms are specific and which are of general occurrence. This use of the comparative approach to the problems of crustacean muscles has previously been strongly advocated by Hoyle and Wiersma (1958a).

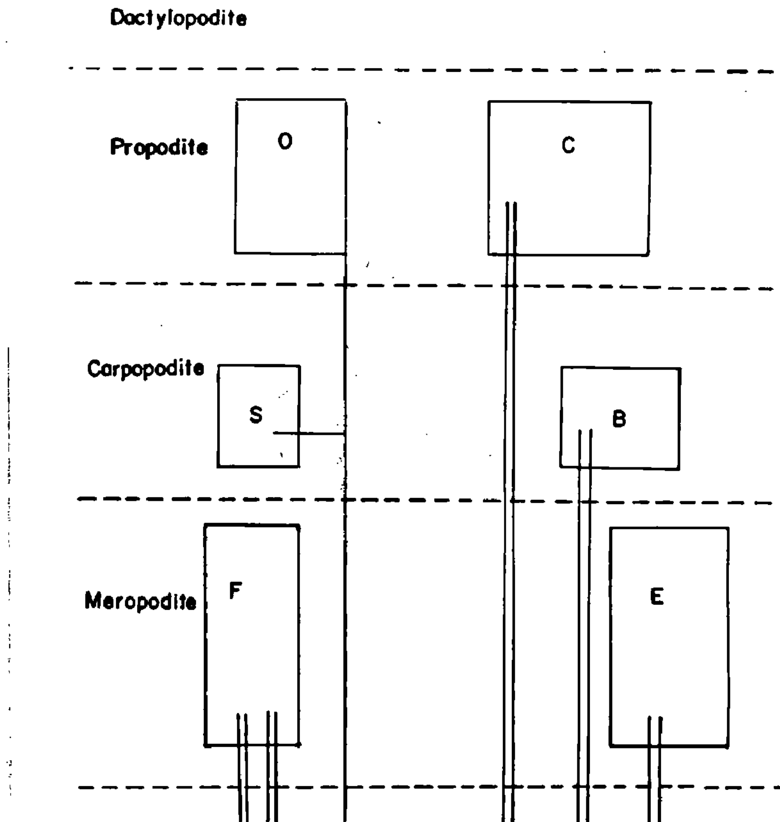


Figure 1.

Distribution of excitatory axons to decapod leg muscles (after Hoyle and Wiersma, 1958a). O, opener, (abductor of the dactylopodite); C, closer (adductor of the dactylopodite); S, stretcher, (extensor of the propodite); B, bender, (flexor of the propodite); F, flexor (of the carpopodite); E, extensor (of the carpopodite). The muscles are represented by rectangles, and each axon by a single line.

GENERAL METHODS

a) Recording and Stimulating Techniques

Most of the electrical recording in this study was done using intracellular glass microelectrodes of the type invented by Graham and Gerard (1946). They were machine-pulled and filled with 3 M potassium chloride by heating under reduced pressure. Only electrodes having resistances of 6 to 20 megohms and tip potentials (Adrian , 1956) of less than 8 mV were used.

When in use, the recording microelectrode was placed in a holder filled with saline agar. A chlorided silver wire was led from the agar to the recording system. Potential changes were recorded between the microelectrode tip and chlorided silver wire, embedded in saline agar, which made contact with the bath.

The cathode follower stage used with the microelectrodes was built from a design obtained from Dr. Greer of the Department of Physiology, Glasgow. Two EF37A valves (Tungsram) were used in this circuit. The screen potential of these valves could be varied to reduce grid current to a low value. Grid current was checked frequently by observing the potential across a 200 megohm resistor switched between grid and earth. The two sides of the output could be adjusted to zero potential with respect to ground.

Early experiments were done using an AC=DC preamplifier

built after the design of Donaldson (1958) in connection with a Cossor 1049 oscilloscope. In later experiments a Tektronix 502 oscilloscope was used. The high gain of this instrument made it possible to apply the output of the cathode follower to the amplifier of the oscilloscope without an intermediate preamplifier.

The time constant of rise of a square pulse recorded through a microelectrode with a resistance of 8 megohms was about 65 microseconds. This value was not large enough to seriously affect the recordings.

A calibrator of the type described by Wood (1957) was attached to one grid of the cathode follower for measurement of membrane potentials and other electrical phenomena.

Stimulation of nerves was done through platinum wire electrodes using, in most cases, transistorized stimulators built after a design by Greer (1960). These stimulators were modified by the author so that they could be gated by a second similar stimulator. Trains of pulses of various frequencies and durations could be obtained. Since the transistorized stimulators can operate in isolation from earth, no further stimulus isolation was necessary.

In stimulating motor axons, care was taken to keep the pulse duration short (less than 0.4 msec) to avoid repetitive firing of the axons.

In many experiments a second microelectrode was inserted into a muscle fibre to pass hyperpolarizing or depolarizing current. The circuit used to measure the current delivered

through the microelectrode was similar to that described by Fatt and Katz (1953a). Current was measured as the voltage drop across a 15K ohm resistor connected between the preparation and earth.

Electrodes used for passing current into single muscle fibres were often filled with 2M potassium or sodium citrate (Boistel and Fatt, 1958) or soaked in these solutions prior to use (Orkand, 1962) to minimize difficulties encountered while passing outward currents.

A disadvantage of the transistorized stimulators is that they cannot give an output of more than about 19 volts amplitude. When single muscle cells are stimulated by current passed through microelectrodes, greater voltages are sometimes necessary. A Grass SD5 stimulator was used in most cases for this type of stimulation; some experiments were done (at Oregon) using a Tektronix pulse generator (Type 161).

Mechanical recording was accomplished by means of an RCA 5734 transducer fitted with a suitable recording shaft, or (in the case of large muscles) by means of a Grass FT03 strain gauge. Some kymographic recording was also done, using isotonic and auxotonic lever systems. In most cases, mechanical recording was done so that the muscle contracted under isometric conditions. This minimized displacement of intracellular microelectrodes.

b) Experimental Animals

At the University of Glasgow the three species used were: *Carcinus maenas* Pennant, the shore crab; *Nephrops*

norvegicus L., small lobster; and Astacus pallipes Lereboullet, the fresh-water crayfish.

The first two species were kept in aerated sea water at an average temperature of 15° C. Provided the sea water was changed regularly, survival was good, and both species could be kept alive for at least two months, although in most cases specimens were used in experiments about one to two weeks after they had been received from the Marine Biological Laboratory at Millport.

Fresh-water crayfish were kept in running Glasgow tap-water at an average temperature of 12° C. They were observed to survive many months.

At the University of Oregon two species were used: Cancer magister Dana, and Pachygrapsus crassipes Randall. Specimens of Cancer were kept in tanks of aerated sea water maintained at a temperature of about 13° C. by refrigeration. Specimens of Pachygrapsus were kept in aquaria containing one inch of sea water and objects on which the animals could climb out of the water. The aquaria were kept in a cold room maintained at 12° C.

c) Dissections.

Walking legs removed from experimental animals were dissected to expose the main leg nerve in the meropodite. Surrounding muscles were removed. "Fast" and "slow" motor axons were isolated from the main nerve as described by van Harreveld and Wiersma (1937). The muscle to be studied was then exposed either by removing part of the overlying shell to which no

muscle fibres were attached, or by removing the antagonistic muscle.

Perfusion of the muscle under investigation was often carried out. Closer muscles of chelate walking legs were perfused through the index of the propodite. Other muscles were perfused through the dactyl or by means of needles inserted through the shell near the muscle being studied.

The normal saline used for the fresh-water crayfish was van Harreveld's (1936) solution. For marine crustaceans, the normal solution had the following composition (mM per litre): NaCl 490, KCl 10, $CaCl_2$ 17, $MgCl_2$ 10, $NaHCO_3$ 3; pH adjusted to 7.2 to 7.6 by addition of drops of dilute HCl. When experimental solutions having excess or foreign anions or cations were made up, equivalent sodium (in the case of cations) or chloride (in the case of anions) was removed to maintain a constant osmotic pressure.

d) Mechanical Recording from Single Muscle Fibres.

In many experiments attempts were made to record electrical and mechanical activity from the same muscle fibre. Recording of this nature has been done in frog muscle by Hodgkin and Horowicz (1960b), and in crayfish muscle by Orkand (1962).

In the present study a relatively simple method was employed at the University of Glasgow to obtain mechanical recordings from single *Carcinus* muscle fibres depolarized by a current-passing microelectrode. The tip of a finely tapered shaft attached to an RCA 5634 transducer was pressed against the central tendon of the muscle adjacent to the muscle fibre

to be recorded from (Fig. 2a). In some cases fibres on the opposite side of the tendon were removed, and cuts were made in the tendon on either side of the desired muscle fibre, but not too close to it. This resulted in a piece of tendon hinged on one side to the rest of the tendon, with several muscle fibres attached, including the one to be recorded from. A small amount of tension was applied to stretch the muscle fibres slightly beyond their resting length. Measurements of tension could then be made when depolarizing current was applied through a current-passing microelectrode inserted in the muscle fibre. A second microelectrode was used to record membrane potential changes along the length of the muscle fibre.

Visual observation of the contraction of the muscle fibre showed that in some cases small contractions were not recorded by the transducer, but that in other cases the visually observed "threshold" corresponded with that measured by the transducer. In general, therefore, the average "threshold" for contraction measured by the transducer is probably higher than the true "threshold."

This method, like that of Orkand (1962), cannot be used to measure tension of single muscle fibres in response to indirect stimulation. However, a method which can be used for this purpose has recently been worked out by Professor Hoyle at the University of Oregon. This method was used by the author to measure tension in single muscle fibres of the stretcher muscle of _Cancer magister_.

The muscle fibre to be studied is isolated from the

underlying tendon by careful dissection around its insertion on the tendon. When correctly performed, the operation yields an undamaged muscle fibre, attached to the shell at one end, but free at the other. Care must be taken to leave a piece of the tendon attached to the free end of the muscle fibre. This piece of tendon is gripped in a pair of fine spring-steel forceps attached to the plate shaft of an RCA 5634 transducer. (Usually this step is performed while the muscle fibre is still attached very slightly to the main tendon; after the forceps have been placed, the last link with the main tendon is cut). The isolated muscle fibre can be pulled out to any desired length. Recording and stimulating electrodes can be inserted (Fig. 2b). If the operation has been carefully done, the innervation of the muscle fibre remains largely intact, and tension responses to indirect stimulation can be measured in the isolated muscle fibre (The main part of the muscle must, of course, be held firmly in place).

There is no doubt that partially damaged muscle fibres are often obtained by this method, but some of the resulting preparations appeared to be relatively intact.

At the University of Oregon, building vibrations created serious interference with mechanical recordings of the tension of single muscle fibres. An attempt to minimize such interference was made by mounting the preparation and transducer on shock absorbers. Unfortunately this method did not completely eliminate "pick-up" in mechanical recordings.

e) Calculation of Muscle Fibre Membrane Properties.

In studying the electrical properties of the muscle

fibre membrane, use was made of the standard technique of applying hyperpolarizing square pulses to the membrane by means of a current-passing microelectrode, and studying the amplitude and time course of the potential change along the length of the muscle fibre with a second microelectrode (Hodgkin and Rushton, 1946; Fatt and Katz, 1951, 1953a).

In most cases the current-passing electrode was placed as close as possible to one end of the muscle fibre. In these circumstances current spreads in only one direction from the stimulating electrode.

In muscle fibres which were long compared with their length constants, the potential declined exponentially with distance from the stimulating electrode (Fig. 60). In such cases the equations worked out for a cable sealed at one end and extending to "infinity" could be applied (Weidmann, 1952):

$$V = \exp ({}^{-x}/\lambda) \qquad (1)$$

$$\frac{Vo}{Io} = r_i \lambda \qquad (2)$$

$$\lambda = \sqrt{r_m / r_i} \qquad (3)$$

$$R_i = \pi a^2 r_i \qquad (4)$$

$$R_m = 2 \pi a \, r_m \qquad (5)$$

where x is the distance between the current-passing and recording electrodes;

V is the potential recorded at x;

Vo is the potential recorded at the current-passing electrode (x=o)

Io is the polarizing current;

λ is the length constant of the muscle fibre;

r_i is resistance of the myoplasm per unit length;

r_m is the membrane resistance times unit length;

R_i is the specific resistance of the myoplasm;

R_m is resistance per unit area of the membrane;

a is the radius of the fibre.

In muscle fibres which were short compared with their length constants, the potential did not decline exponentially with distance (Fig. 60). Cases of this sort required use of the equations worked out by Weidmann (1952; see also Orkand, 1962) for a "cable" of short length with infinite resistance at both ends:

$$V = Vo \frac{\cosh(L-x/\lambda)}{\cosh(L/\lambda)} \qquad (6)$$

$$\frac{Vo}{Io} = r_i \lambda \coth(L/\lambda) \qquad (7)$$

where L is the length of the fibre.

Equation (6) can be simplified by substitutions. If V/Vo= n, $\lambda/L = 1/m$, and $x/L = 1 - u$, equation (6) can be reduced to:

$$n = \frac{\cosh m u}{\cosh m} \qquad (8)$$

In this form the equation still does not yield a simple general solution, but it can be solved easily for particular conditions. If the recording electrode is placed at the end of the muscle fibre opposite to the stimulating electrode, u becomes o. Let n (u = o) be n_a.

$$n_a = 1 / \cosh m \qquad (9)$$

This equation can be solved for m:

$$m = \log e((1/n_a + \sqrt{(1/n_a^2 - 1)})) \qquad (10)$$

If the recording electrode is placed in the middle of the muscle fibre, $u = 1/2$. Let n ($u = 1/2$) be n_b.

$$n_b = \frac{\cosh m/2}{\cosh m} = \frac{\cosh m/2}{2 \cosh^2 m/2 - 1} \qquad (11)$$

Equation (11) can be solved for cosh m/2.

$$\cosh m/2 = \frac{1 + \sqrt{(1+8\, n_b^2\ )}}{4\, n_b} \qquad (12)$$

Let cosh m/2 be B. Then, solving for m:

$$\exp\ (\ m/2\ ) + \exp\ (\ -\ m/2\ ) = 2\ B \qquad (13)$$

$$\text{and } m = 2 \log_e ((\ B + \sqrt{(B^2 - 1)}\ )) \qquad (14)$$

In practice use was made of equations (10) and (14) in the following way. Measurements of the potential at several points along the length of a muscle fibre were plotted against distance (x). . A curve was drawn through these points, and the values of the potential at the end of the fibre opposite to the current-passing electrode and at the mid-point of the muscle fibre were found. The length constant could then be found by means of equations (10) and/or (14).

At the University of Oregon, use was made of an IBM 1620 computer to calculate numerical values for the length constant. A program for this purpose, based on "Newton's method", was written by Dr. G. Struble of the University of Oregon. This program saved much time-consuming manual calculation.

In muscle fibres with exponential decay of potential, values for the membrane time constant could be calculated by finding the time of rise of the polarizing potential at the stimulating electrode to 84% of its final value, or by plotting the time of half-decay of the potential against distance

from the current-passing electrode (Hodgkin and Rushton, 1946; Fatt and Katz, 1953a). In muscle fibres with short ratios of length to length constant, it was necessary to calculate the rise and fall of total membrane charge (Hodgkin and Rushton, 1946; Fatt and Katz, 1951: Weidmann, 1952). An example of this type of plot is shown in Fig. 60 .

After the time constant had been found, the membrane capacitance could be calculated.:

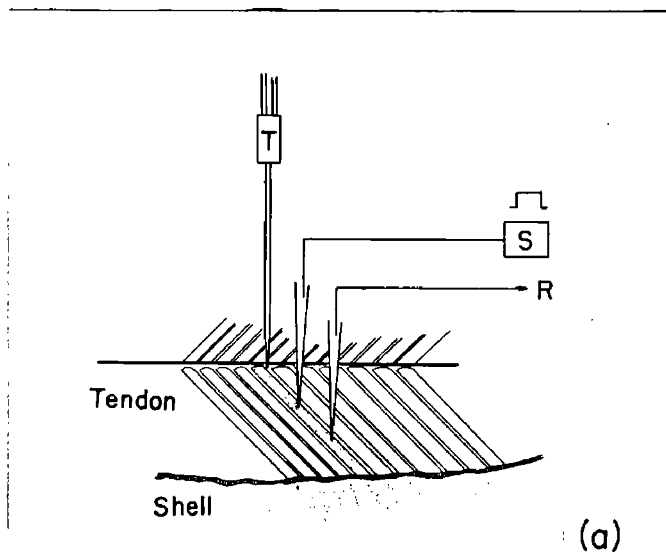
$$Cm = \tau m \, / \, Rm \qquad (15)$$

where Cm is the membrane capacitance per unit area; τm is the membrane time constant.

Probably the biggest source of error in calculations of membrane constants was uncertainty in measurement of fibre diameter. Crustacean muscle fibres are often not circular in cross-section. However, an approximate idea of the fibre diameter could be obtained by assumming for <u>Carcinus</u> muscle fibres a value of 55 ohm cm. for R_i (c.f. Fatt and Katz, 1953a., Shaw, 1955), and calculating fibre diameter from the following equation (Fatt and Katz, 1951).

$$\text{diameter} = \sqrt{4 \; R_i \; / \pi \; r_i} \qquad (16)$$

By this means a rough check on the measured fibre diameter could be made.



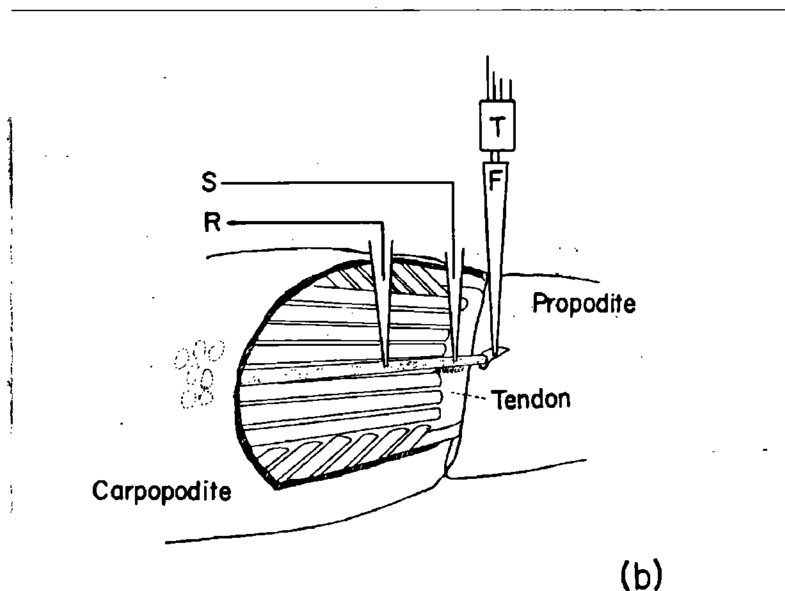


Figure 2.

(a) Diagramatic illustration of the method of recording tension from single muscle fibres in the *Carcinus* closer muscle. R, electrical recording of membrane potential; S, stimulator; T, transducer for recording tension.

(b) Diagramatic illustration of the method of recording tension from single muscle fibres in the stretcher muscle of *Cancer*. R, electrical recording of membrane potential; S, stimulator; T, transducer for recording tension; F, steel forceps for gripping tendon attached to the isolated muscle fibre.

RESULTS

a) Artefacts

Records of electrical and mechanical activity of crustacean muscles are often distorted by various types of artefact. Those most frequently encountered in the present study will be dealt with briefly here.

Influence of nearby muscle fibres: It has been pointed out by Easton (1959) that electrical events in muscle fibres close to the one recorded from can influence the electrical records. In many crustacean muscles this problem is relatively minor, because individual excursions of membrane potential are often small. However, if a microelectrode is introduced into a quiescent muscle fibre close to a fibre responding to motor axon stimulation, small hyperpolarizing potentials can often be seen (Fig. 3a). In muscle fibres close to those in which spikes are produced, this effect can be quite pronounced. In Fig. 3(d), the muscle fibre recorded from gave a spike in response to the second stimulus delivered via the "fact" axon, but not to the first stimulus. However, spikes in nearby muscle fibres produced a hyperpolarizing potential during the first electrical response, at the same relative time at which the spike occurred in response to the second stimulus in the muscle fibre being recorded from. A

measurement of spike height in this fibre must be augmented by about 5mV to compensate for the hyperpolarizing potential.

Other records in which hyperpolarizing potentials caused by voltage fields of nearby fibres were apparent are shown in Fig. 3 (b, c).

Effects due to the influence of nearby fibres* are more pronounced in circumstances in which the volume of solution surrounding the muscle fibre is reduced, such as when recordings are made from fibres deep in the muscle.

When the recording electrode is at or near the surface of a muscle fibre, a hyperpolarizing potential is also recorded on occasion (see del Castillo and Katz, 1956). An example is shown in Fig. 4 (a). The electrode pulled out of the fibre being examined to its surface, and a hyperpolarizing potential was then recorded in response to nerve stimulation.

Pulling out of the microelectrode: As has been pointed out by Fatt and Katz (1953a), artefacts can be caused by the recording electrode pulling out of the muscle fibre (presumably due to mechanical activity of the muscle fibre). This effect is shown in Fig. 4 (a,b,g). In Fig. 4 (g) the final membrane potential, after the electrode has re-entered

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*No evidence for electrotonic connections between adjacent muscle fibres was found in the muscles used in the present study. It is unlikely that artefacts were attributable to this cause. Reuben (1960) has described electrotonic connections between lobster muscle fibres, but states that they have not been found in *Carcinus* or *Cancer* muscles.

the muscle fibre, is greater than it was initially; this may indicate that at the start of the stimulation the electrode was not firmly implanted in the muscle fibre, or it could indicate a change in tip potential of the electrode due to a broken tip or to bending. In cases where this type of indication was present, it was often necessary to replace the microelectrode.

Other artefacts which may also have been caused by pulling out or bending (cf. Ogato, 1960) of the microelectrode during contraction are shown in Fig. 4. In Fig. 4 (c,d) records were made from muscles giving brisk twitches. In Fig. 4 (d) the electrical response of the muscle fibre was minute, but a subsequent much larger potential change with a time course the same as that of the muscle twitch, was observed. In Fig. 4 (c) the postsynaptic potential is followed by a marked hyper-polarization, with a time course similar to that of the muscle twitch.

Artefacts which were attributable to similar effects are shown in Fig. 4 (e,f,h,i).

There was no sure remedy for this type of artefact, which was by far the most troublesome of those encountered. The main precautions which could be taken were to ensure that recording conditions were as isometric as possible, and to guard against partial denervation of the muscle. In muscles

which were partially denervated, differential movements of separate parts of the muscle greatly augmented the number of artefacts obtained.

Transducer drift: In some cases mechanical records were affected by electrical drift in the R C A 5734 transducer employed. Usually such drift was due to inadequate H T batteries, but it is also possible that thermal drift due to uneven heat dissipation of the transducer may have been responsible on occasion (see Talbot et al., 1951).

When the Grass FT03 strain gauge was used, drift of this nature was not a problem. If too light a spring was used in the strain gauge, however, the response sometimes showed an uneven return to base line. This could be attributed to insufficiently isometric recording conditions, and could be remedied by making the recording conditions more nearly isometric.

Uneven passage of current by the stimulating microelectrode: When a microelectrode was used to pass current into a muscle fibre, it was frequently found that different amounts of current were passed at different times during an applied pulse. This held true particularly for depolarizing current, but was also seen on occasion for hyperpolarizing current. In Fig. 5 (a), the potential change across the muscle fibre membrane is distorted by uneven passage of current, which can be seen in the lower trace. In Fig. 5 (b),

the depolarizing potential change is uneven. Although current was not monitored in this record, the effect was also reflected in an uneven tension recorded from the muscle fibre.

It was possible to reduce effects of this nature by soaking electrodes in citrate solutions prior to use (see Methods), but even when this was done artefacts of this type were still frequently observed. Much depended on the particular electrode used in an experiment.

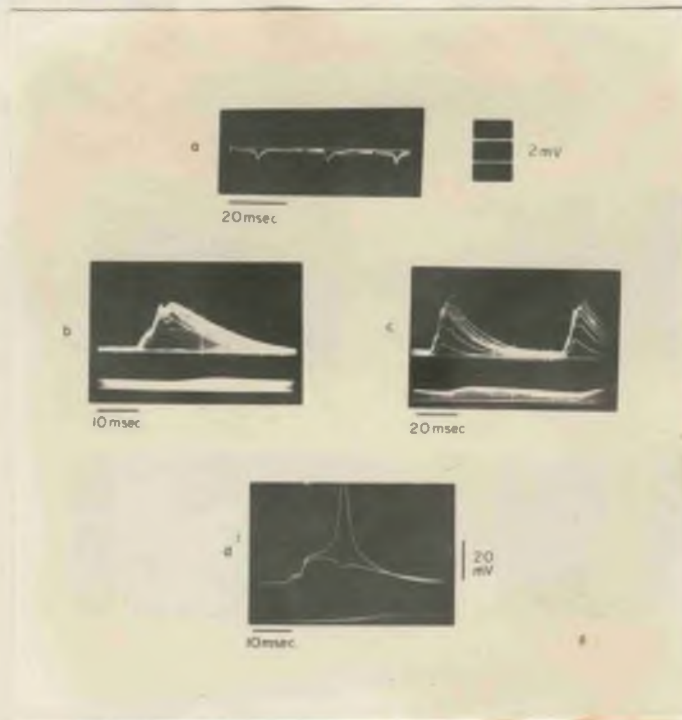


Fig. 3. Artefacts in electrical recordings produced by activity of adjacent muscle fibres. (a) Recording from a muscle fibre in the opener muscle of Carcinus during stimulation of the "fast" axon of the closer muscle. (b,c) Responses of muscle fibres in the closer muscle to double pulses (3 msec. separation) delivered at 15 per sec. via the "fast" axon. (d) Response of a muscle fibre to double pulses delivered at 1 per sec. via the "fast" axon. Lower traces indicate mechanical activity of the whole muscle. Voltage scale the same for (b,c,d).

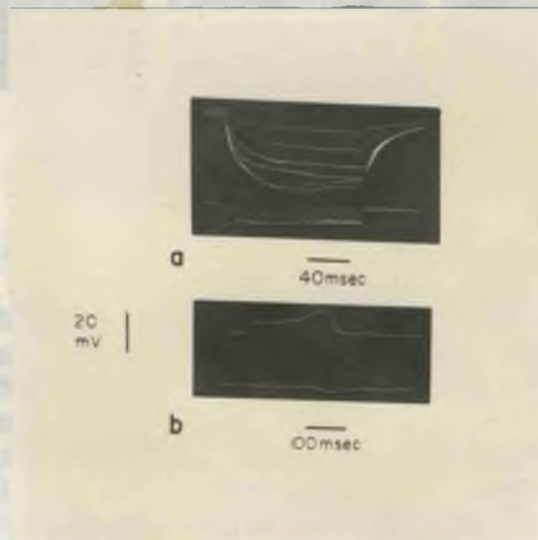


Fig. 5. Electrical artefacts. (a) Artefact caused by irregular hyperpolarizing current passed by the polarizing electrode; Carcinus closer muscle fibre; lower trace, current. (b) Uneven electrical and mechanical responses in a Cancer stretcher muscle fibre, probably due to a faulty current-passing electrode; lower trace, tension of the muscle fibre.

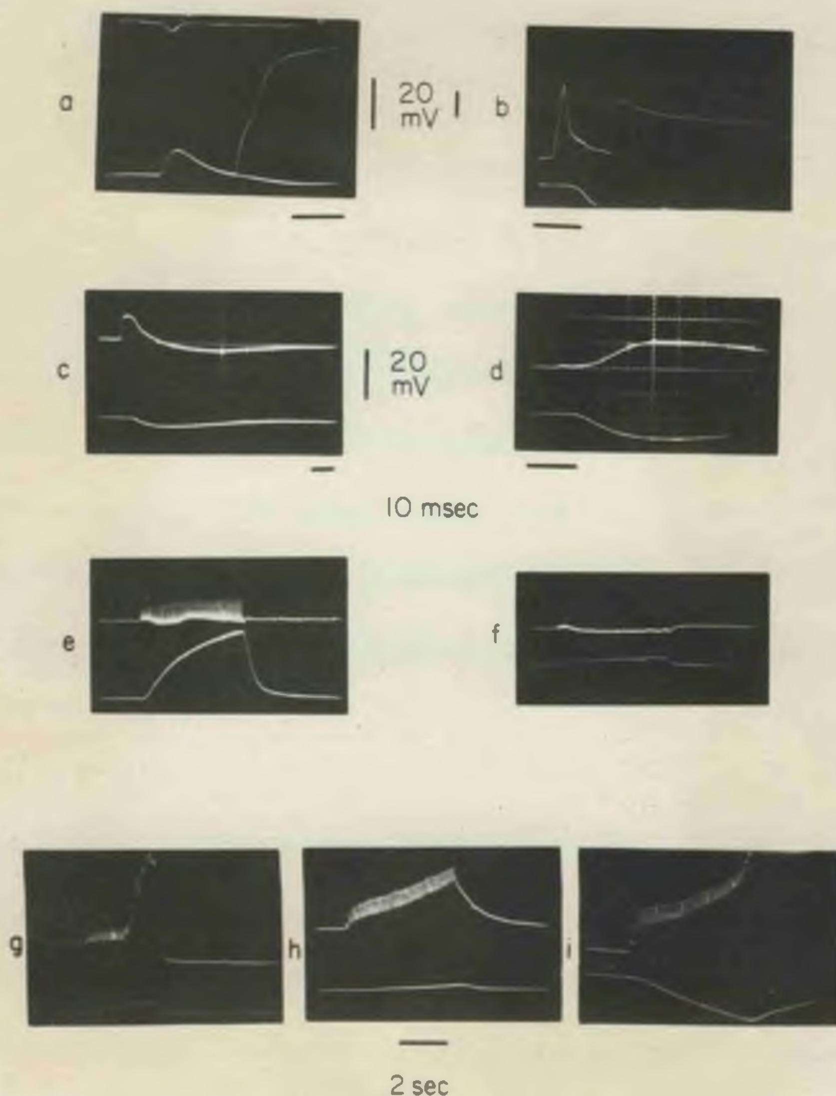


Fig. 4. Electrical artefacts. (a) Record from a fibre in a Carcinus closer muscle during "fast" axon stimulation at 10 per sec., showing electrode pulling out of the fibre. (b) Record from a fibre in a crayfish leg closer muscle during "fast" axon stimulation at 1 per sec., showing electrode pulling out. (c,d) Records from closer muscles of crayfish legs during "fast" axon twitches, showing the influence of mechanical events on electrical records. (e-i) Records from Carcinus closer muscles showing various effects due to mechanical activity of the muscle: (e) "slow" axon stimulation, double pulses of 3 m sec. separation delivered at 15 per sec; (f) "fast" axon stimulation at 10 per sec; (g) "fast" axon stimulation at 10 per sec: (h,i) "slow" axon stimulation at 10 per sec. (Lower traces, tension of the muscle).

b) Responses of Whole-Muscle Preparations to Indirect Stimulation

The initial step in the present study of excitation and contraction in crustacean muscles was to observe the electrical responses of individual muscle fibres and the corresponding mechanical responses of the whole muscle. Muscles from several crustacean species were examined for comparative purposes. The purpose of this method of investigation was to obtain as good a correlation as possible between the electrical and mechanical events of the intact muscle during stimulation of excitor axons.

In this section the material presented will be mainly descriptive in nature. Conclusions regarding the relation of excitation to contraction will be reserved until experiments bearing on the excitation-contraction coupling process have been presented.

1) Carcinus: Closer muscle

The closer muscles of walking legs 2, 3 and 4 of *Carcinus* were studied thoroughly; observations were made on over 100 preparations. This thoroughness of study proved to be justified, since important features of the responses to indirect stimulation were not immediately apparent.

The *Carcinus* closer muscle has been studied previously by Fatt and Katz (1953b), but their observations were concerned

mainly with the problem of the distributed innervation of crustacean muscle fibres, rather than with specific effects of "fast" and "slow" motor axons. However, they observed that the two motor axons supplying excitatory innervation to the muscle produced "end-plate potentials" (postsynaptic potentials) with very different decay rates in some of the muscle fibres innervated by both axons. They did not determine which axon was "fast" and which was "slow."

In the present study interest was focused on the mechanism by which the "fast" and "slow" axons produce different mechanical responses in the same muscle. Can the different mechanical responses be explained in terms of differences in the electrical responses, or must one invoke (as do Hoyle and Wiersma:1958c) differences in the excitation-contraction coupling processes, perhaps brought about by different "fast" and "slow" transmitter substances? This question can be finally answered only by the study of innervated single muscle fibre preparations, but the study of whole-muscle preparations can yield important preliminary information.

Electrical responses: Exploration of different muscle fibres in the closer muscles of Carcinus with a recording microelectrode during separate stimulation of "fast" and "slow" motor axons revealed a great variety of electrical responses. These responses could, however, be grouped into three main categories.

In one type of muscle fibre, stimulation of the "slow" axon, even at high frequencies of stimulation, produced very small electrical responses, or, in most cases, none at all. A single stimulus applied to the "fast" axon gave large postsynaptic potentials (p.s.ps.) in these fibres (Fig. 6a, b; Fig. 7a, c). These p.s.ps. were usually 8 to 20mV. in magnitude, but as the preparation became older the responses in any given muscle fibre became smaller. In some cases a small electrically excitable membrane response in the form of a hump following the peak of the p.s.p. could be observed in response to a single stimulus (Fig. 7c, d, e, f) but usually the response was limited to a p.s.p.

At stimulation frequencies of 3 to 10 per second the p.s.ps. showed facilitation, and graded electrically excitable responses usually became evident, increasing in size with successive stimuli (Fig. 6c; Fig. 7d, f).

Even larger responses (up to 70mV), could be obtained when two closely spaced stimuli were given (Fig. 6d, e). The spikes thus obtained were followed by a pronounced "hump" in some cases (Fig. 6d). Paired shocks did not always give rise to pronounced active membrane responses of this nature in these muscle fibres. Initially the response of Fig. 7 (a,b) was a p.s.p. of 11mV. Two closely spaced stimuli elicited an additional response which was probably a very large p.s.p. (Fig. 7b). This response did not have the initial rapid repolarization observed in the electrically excitable responses

of other muscle fibres of this type, but it is possible that some degree of electrical excitability was involved (see p.).

The muscle fibres described above were discovered only by introducing the recording electrodes deep into the muscle through the outer layers of muscle fibres. Because of their inaccessibility they were difficult to record from, but they appeared to be present in all muscles where they were looked for carefully. They were apparently localized in a group of very large muscle fibres (300 to 800 μ in diameter, 10 to 20mm. long) which could most easily be approached by removing the antagonistic (opener) muscle.

For convenience these muscle fibres will be referred to subsequently as Type A muscle fibres.

Responses of an entirely different character were recorded from a second type of muscle fibre (Figs. 8 to 12). Single stimuli applied to the "slow" axon elicited p.s.ps. of comparatively large size (2 to 10mV) and with very slow decay rates. The "slow" p.s.p. shown in Fig. 9(a), for example, had a time constant of decay of about 200 msec. In other fibres of this type values of 200 to 30 msec. were observed. Often "fast" p.s.ps. could not be found in these fibres (Figs. 8c, 10b), but when present they were usually very small (Fig. 9b) and showed the slow rate of decay typical of the "slow" p.s.ps. in these fibres. In Fig. 10 (d) an exceptionally large "fast" p.s.p. was observed, which was

only slightly smaller than the "slow" p.s.p. in the same muscle fibre (Fig. 10c). Both p.s.ps. had time constants of decay of about 110 msec.

When repetitive stimulation was delivered to the "fast" axon, the "fast" p.s.p. typically showed almost no facilitation, and the total electrical response remained small (Fig. 9e). In the majority of the muscle fibres of this type, there was no electrical response at all to "fast" axon stimulation, even at high frequencies of stimulation (Fig. 8c).

Frequencies of stimulation as low as 4 per second applied to the "slow" axon produced depolarization "plateaus" in many of these muscle fibres (Fig. 8b, 9c). Some facilitation of the response was always evident during the first few impulses. As the frequency of stimulation was increased above 10 per second, the individual p.s.ps. became smaller, and not larger as in other muscle fibres. Examples of this behaviour are shown in Figs. 8, 9, 11. Depolarization "plateaus" tended to reach a maximum size at stimulation frequencies of about 30 per second and did not become much larger at higher stimulation frequencies. After an initial rising phase of depolarization, the "plateaus" often showed a tendency to decline in magnitude during a prolonged period of stimulation at a high frequency. An extreme example is shown in Fig. 11 (j, k). After stimulation of this sort it was observed that the first p.s.p. in response to a succeeding train of stimuli was often smaller than it had

been before prolonged stimulation. It is reasonable to infer, therefore, that exhaustion of the transmitter substance plays a part in the decline in magnitude of the depolarization "plateau" during a train of stimuli. The fact that individual p.s.ps. become smaller in size as soon as a substantial depolarization "plateau" is built up, cannot be explained in this manner (see p. 85).

When two closely spaced shocks were applied to the "slow" axon, summation and facilitation of the electrical responses occurred in these fibres (Fig. 12). No electrically excitable membrane responses were observed.

The muscle fibres responding in the manner outlined above were always found at the proximal end of the muscle and never anywhere else. They were observed to be rather small (80 to 150 μ in diameter). They will be referred to as Type B muscle fibres.

The major part of the _Carcinus_ closer muscle was made up of muscle fibres which showed electrical responses intermediate between Types A and B. These muscle fibres will be referred to subsequently as Type C.

There was considerable variation in "fast" and "slow" p.s.ps. from fibre to fibre in this category. In many cases the "fast" p.s.p. was larger than the "slow" at low frequencies of stimulation, but the reverse situation also occurred. In Fig. 13, examples are given of "fast" and "slow" p.s.ps.

recorded from different fibres in the same muscle during stimulation of the motor axons with single shocks. There is no correlation in size between "fast" and "slow" p.s.ps. in these muscle fibres; sometimes a large "fast" p.s.p. occurs with a large "slow" p.s.p. (Fig. 13e, f), whereas in other cases a large "fast" p.s.p. occurs with a small "slow" p.s.p. (Fig. 13, i, j). Sometimes the "slow" p.s.p. is larger than the "fast" (Fig. 13g, h). In general, "slow" p.s.ps. were less than 4mV, and usually 0 to 2mV, in Type C fibres. "Fast" p.s.ps. averaged larger, but seldom exceeded 5mV.

It was estimated that about 95% of the Type C muscle fibres gave responses to both "fast" and "slow" axon stimulation. Occasionally, muscle fibres were encountered which did not show electrical responses during high frequency stimulation of one of the two motor axons (Fig. 16, r,s).

When the frequency of stimulation applied to the "slow" axon was greater than about 2 per sec., the electrical response showed facilitation, usually quite marked (Figs. 14, 15, 16, 18, 19, 25). In most muscle fibres, depolarization "plateaus" were built up at stimulation frequencies of 15 to 30 per sec. and higher. Usually the size of the individual "slow" p.s.ps. remained about the same at stimulation frequencies greater than 50 per sec., but depolarization "plateaus" continued to increase in size with increasing frequency of stimulation up to 100 per sec., or even higher.

In some cases "slow" p.s.ps. grew by facilitation to very large sizes without giving rise to a depolarization "plateau." In Fig. 15, an example of this behavior is shown (a,d,f,h). At similar frequencies of stimulation, other muscle fibres (e.g. Fig. 18 a,b) showed depolarization "plateaus," even though individual p.s.ps. were several times smaller. It was observed that the larger p.s.ps. in such instances had more rapid rates of decay. For example, the p.s.p. of Fig. 15 (f) was estimated to have a time constant of decay of 13 msec. whereas the time constant of decay for the p.s.p. of Fig. 18 (b) was about 25 msec. In general, therefore, three factors contribute to the total extent of indirectly produced depolarization in a muscle fibre: (1) the size of the p.s.p.; (2) the decay rate of the p.s.p.; (3) the frequency of recurrence of the p.s.p.

When the "fast" axon was stimulated at frequencies greater than 2 or 3 per sec., the electrical responses of some Type C muscle fibres showed marked facilitation, similar to that observed for "slow" p.s.ps. More frequently, "fast" p.s.ps. showed little or no facilitation.

In Fig. 14, an example is given of a "fast" electrical response showing almost no facilitation, even at high frequencies of stimulation (a,c,f). The total electrical response remains very small. In the same fibre the "slow" p.s.p. shows marked facilitation, and the total depolarization

due to "slow" axon stimulation is considerable at high frequencies of stimulation.

Further examples of this type of behavior are given in Fig. 16 (a to p). In these records, the "fast" response is almost the same size at high frequencies of stimulation as it is at low frequencies of stimulation, whereas the "slow" responses from the same muscle fibres show marked facilitation.

An entirely different type of behavior is shown in Fig. 16 (u,v,w). In this case the "fast" electrical response shows marked facilitation with increasing frequency of stimulation. Other examples of this type of behavior are shown in Fig. 17, 19 (f). Cases in which facilitation was present, but not marked, are shown in Figs. 19(a,c), 25(b,d).

It is apparent that "fast" electrical responses in Type C muscle fibres can show a wide range of behavior. The differences of behavior within the observed range of "fast" electrical responses are almost as great as the differences between "fast" electrical responses as a whole, and "slow" electrical responses as a whole.

In general, the "fast" electrical responses of Type C muscle fibres were smaller than "slow" electrical responses at frequencies of stimulation greater than about 20 per sec., although at lower frequencies of stimulation the reverse situation often occurred (Fig. 16). In some cases depolarization "plateaus" were built up by stimulation of the "fast" axon at

frequencies above 30 per sec. (Fig. 17); in many other instances total depolarization remained minute (Fig. 14).

It was observed that in most of the muscle fibres recorded from, "fast" and "slow" p.s.ps. had similar shapes when of the same size (Figs. 16, 19). However, in some muscle fibres differences occurred. The "fast" p.s.p. sometimes showed a more rapid decay than the "slow" p.s.p. recorded from the same muscle fibre; but the reverse situation was also observed. These differences will be discussed later (p. 83).

When "fast" and "slow" axons were stimulated together, the resulting electrical response was increased in size (Fig. 19). The size of this response appeared to be approximately equal to the algebraic sum of "fast" and "slow" responses.

When double pulses of stimulation were applied to the "slow" axon, the electrical response to the second shock showed facilitation, and summed with the first (Figs. 20, 23). Large depolarizations could be obtained by delivering double shocks at high frequencies of stimulation. On no occasion were electrically excitable membrane responses seen during this type of stimulation.

In most cases the same result was obtained when double pulses of stimulation were applied to the "fast" axon (Figs. 21, 23). However, in a few fibres it was found that electrically excitable membrane responses could be produced by double pulses of stimulation applied to the "fast" axon

(Fig. 22). In these particular muscle fibres responses to "slow" axon stimulation were rather small.

The responses of Type C muscle fibres to indirect stimulation indicate that, in general, these muscle fibres do not show electrically excitable membrane responses even when considerable depolarization of the muscle fibre membrane has been produced. A few exceptional fibres did give electrically excitable membrane responses when the "fast" axon was stimulated with two closely applied shocks. These fibres appeared to be similar to Type A fibres in many respects.

Mechanical responses: The mechanical responses of the whole muscle were usually recorded simultaneously with the electrical responses of individual muscle fibres.

The slow contraction was detectable at frequencies of stimulation of 5 to 10 per sec. Examples of slow contractions at these frequencies of stimulation are shown in Figs. 9, 11, 25. In most cases the contraction was a slowly developing and smooth tetanus, but in a few instances small "twitches" could be seen in the record (e.g. Fig. 25, g). The slow contraction increased greatly in rate of development and in magnitude at higher frequencies of stimulation (Figs. 9, 11, 18). The rate of decay of tension was rather slow; complete return to the base line of zero tension always took several (3 to 10) seconds.

A single stimulus applied to the "fast" axon usually

evoked a small twitch from the muscle (Figs. 9, 10, 24, 25). This twitch often showed some facilitation when the frequency of stimulation was greater than about 3 per sec. (e.g. Fig. 25, a,b,f). In other cases the twitch remained the same size (Fig. 25,l) or showed irregular increases and decreases in magnitude (Figs. 9,e; 25,i).

As the frequency of stimulation was increased above 10 to 15 per sec. the individual twitches became superimposed on a tetanic contraction, which increased in size with increasing frequency of stimulation (Figs. 17, 19). However, even at high frequencies of stimulation individual twitches could be seen in the mechanical response (Fig. 17, g). The fast mechanical response showed a more rapid rate of development and a much more rapid rate of decay than did the slow mechanical response. Tension usually returned to the base line within half a second.

Occasionally single stimuli applied to the "slow" axon resulted in small "twitches" (Fig. 24,c) which were smaller and had slower rates of rise and fall than "fast" axon twitches in the same muscle (Fig. 24,b). These slow twitches were only found in recently moulted animals which had been kept previously at a temperature of about 20°C. "Fast" axon twitches recorded from these muscles had slower rates of decay than did fast twitches recorded from muscles of other animals (Figs. 10d, 24a). The physiological state of the experimental animal apparently influences the excitability and responsiveness

of the nerve-muscle preparation.

In most muscles, double pulses of stimulation applied to the "slow" axon at frequencies of 5 to 15 per sec. gave rise to small twitch responses of the muscle (Fig. 23). The same type of stimulation applied to the "fast" axon produced larger twitches (Figs. 21, 22, 23).

When both "fast" and "slow" axons were stimulated at the same time, the resulting tension was equal to the algebraic sum of the tensions developed by separate stimulation of the motor axons (Fig. 19).

The amount of tension developed in response to stimulation of the "slow" axon was typically considerably greater than that developed in response to "fast" axon stimulation (Fig. 26), except at low frequencies of stimulation (below 5 per second). At these low frequencies the "slow" response was usually not detectable, whereas the "fast" response was a small twitch.

In the experiment of Fig. 26, stimulation was applied to the two motor axons in three-second bursts. Virtually all of the "fast" tension was developed within a second, and most of the "slow" tension appeared by three seconds, except at low frequencies of stimulation. An interval of two minutes was allowed between each period of stimulation; the muscle was perfused continuously. As the frequency of stimulation was increased, "slow" tension increased in magnitude more rapidly than "fast" tension. The latter levelled off at 30 per sec. stimulation, whereas the "slow" tension showed a

continuous increase throughout. After the highest frequency had been attained, measurements were again made at 40 per sec. stimulation. It was observed that the "slow" response was reduced to 3/4 of its original size and the "fast" response to about 1/3 of its original size. Fatigue had taken place during high frequency stimulation of both axons, and was more marked in the case of the "fast" system. Similar observations on the relative sensitivities to fatigue of the two systems in other muscles have been made by Hoyle and Wiersma (1958a). Perhaps the "levelling off" of "fast" tension at higher frequencies of stimulation can be partly attributed to the onset of fatigue.

Mechanics of the closer muscle: Muscle tension was recorded at the tip of the dactyl in the *Carcinus* closer muscle (and in the other closer and opener muscles studied). Force recorded in this manner gives a relative measure of muscle tension, but the real tension developed by the muscle can be determined only by taking into account the geometry of the muscle and moving skeletal parts.

In Fig. 26A, a diagram to illustrate the mechanics of the closer muscle is given. The recording system (r) measured n, the vertical component of the force at the tip of the dactyl. The total force (F) exerted by the tendon as a result of the activity of the muscle can be found:

$F = mL/a \cos \beta$ (17)
(The meanings of the symbols are given in the caption of Fig. 26A.)

Values for m and cos β can be found by direct measurement. Values for L and a can also be found by direct measurement, but the possible error is considerable since a is small and the pivot point of the dactyl on the propodite cannot be definitely ascertained. The ratio L/a can be determined accurately by applying a known force to the tendon and measuring the resulting force at the tip of the dactyl. Six such determinations on different legs give an average value for L/a of 18 (range, 14.5 to 22). With a value for β of 25°, the force exerted by the tendon as a result of muscular contraction can be estimated to be approximately 20 times the measured value of n.

In order to find the tension developed by the muscle, the force exerted by the tendon must be divided by the average cosine of the angles made by the muscle fibres with the tendon (α). Observations made on the muscles showed that an approximate average value for α of 30° could be used. The tension developed by the muscle is therefore about 23 times the measured value of n, but was observed to vary from 18n to 30n in individual muscles.

In order to compare tension from different muscles, a measure of the size of the muscle was necessary. The most convenient procedure was to measure the size of the propodite

(length times width). This measurement was found to be related to the cross-sectional area of the muscle in the legs used in these experiments (Fig. 26A). When the tension per cross-sectional area of the muscle had to be found, use was made of the graph of Fig. 26A and the measured propodite size of the leg used in the experiment.

Calculated values for the tension developed by the closer muscle during maximum stimulation of the motor axons ranged from 2300 to 4200 gm/cm^2 (five determinations). These values are similar to those found for other types of striated muscle (e.g., Hodgkin and Horowicz, 1960b).

Measurements of the tension of single "fast" axon twitches ranged from 0.015 to 0.08 gm. at the tip of the dactyl (eight measurements). Assuming that all the tension during a fast twitch is attributable to Type A fibres, and using values for α and β of 10° and 25° respectively, it can be estimated that the Type A fibres develop 0.32 to 2.0 gm. tension during a twitch. Since the diameters of these fibres are large (up to 800 μ), it is evident that only a few Type A fibres could develop all the tension observed during a fast twitch, even if each fibre were only slightly activated. Also, the differences in size sometimes observed between successive fast twitches (Figs. 9, e; 25, i) could easily be due to intermittent activation of a single muscle fibre, or to occasional occurrence of additional electrical activity in a Type A muscle fibre (see Fig. 6, c).

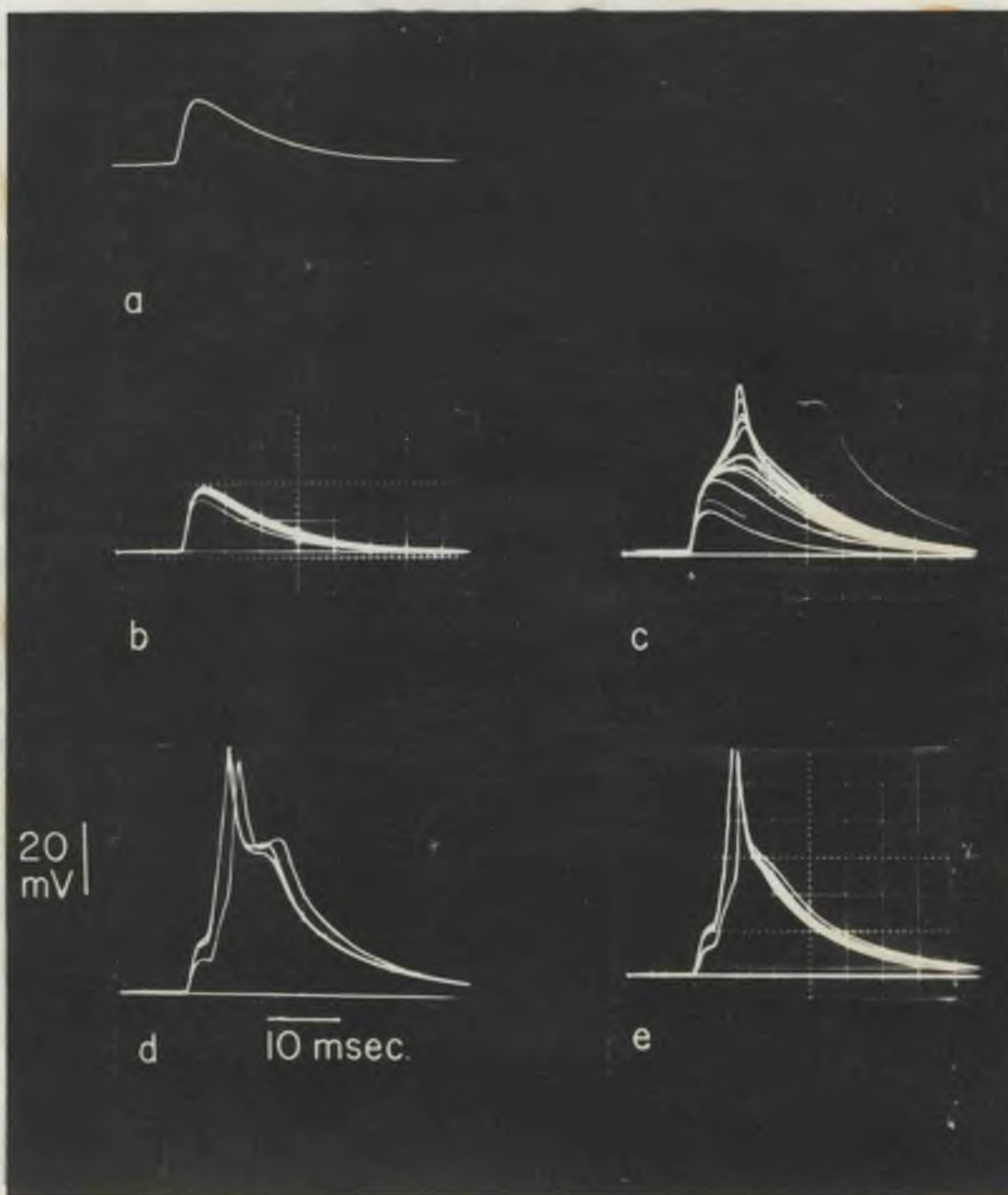


Fig. 6. Carcinus: Electrical responses of Type A muscle fibres. (a) p.s.p. in response to a single stimulus applied to the "fast" axon. (b) "Fast" p.s.ps. in another muscle fibre in response to stimuli at 3 per sec. (c) Response of the same muscle fibre to stimuli delivered at 8 per sec. (d) Response of the same muscle fibre to pairs of shocks (2.5 msec. separation). (e) Response to similar stimulation in another muscle fibre.

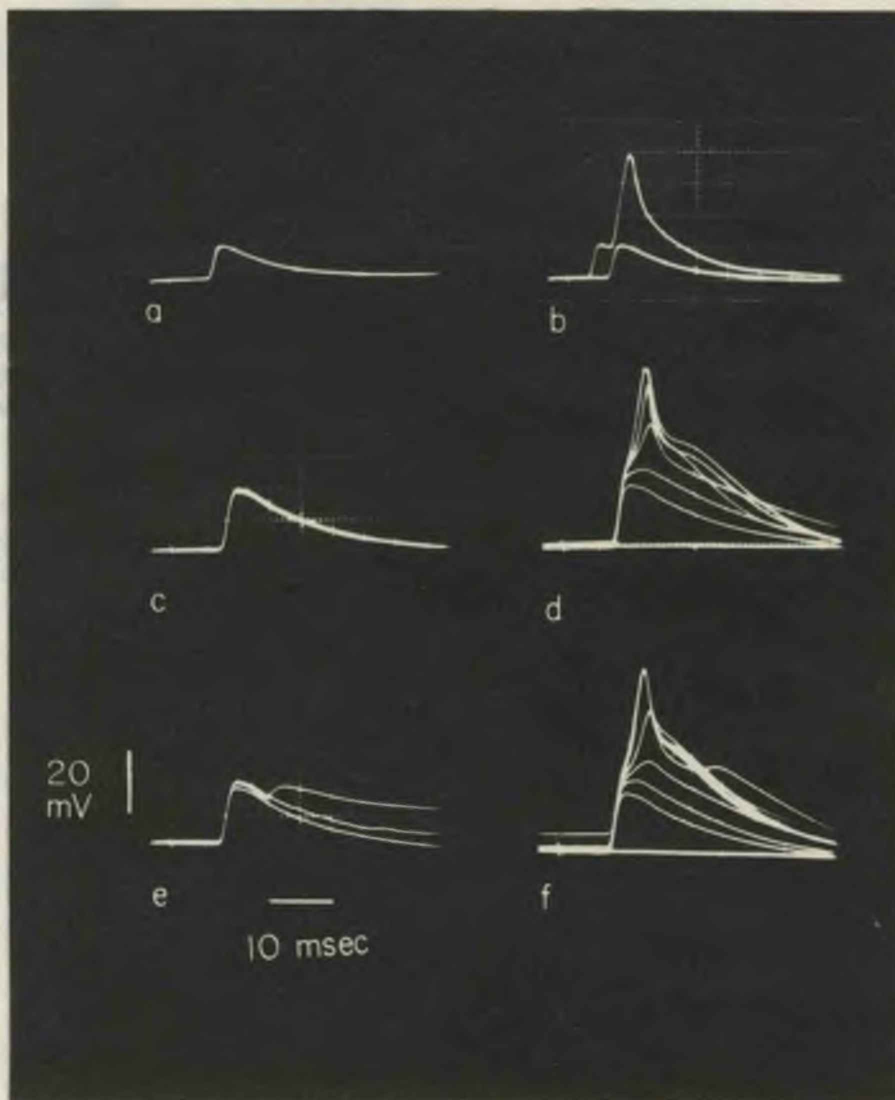


Fig. 7. Carcinus: Electrical responses of Type A muscle fibres. (a,b) Responses of a Type A muscle fibre to a single stimulus applied to the "fast" axon (a) and to a single stimulus followed by a paired shock (b). (c,d) Responses of another muscle fibre to stimulation of the "fast" axon at 1 per sec. (c) and 5 per sec. (d). (e,f) Responses of another muscle fibre to "fast" axon stimulation at 2 per sec. (e) and 8 per sec. (f).

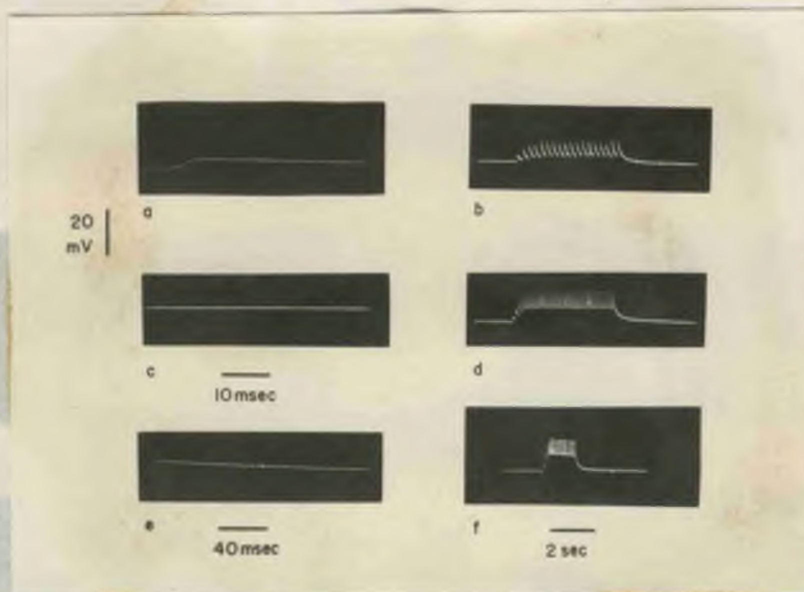


Fig. 8. Carcinus: Electrical responses of a Type B muscle fibre. (a,e) "Slow" p.s.p. in response to a single stimulus; the time constant of decay is about 140 msec. (c) Response to "fast" axon stimulation at 20 per second; no response was observed. (b,d,f) Responses to "slow" axon stimulation at 4 per sec. (b), 8 per sec. (d), and 16 per sec. (f).

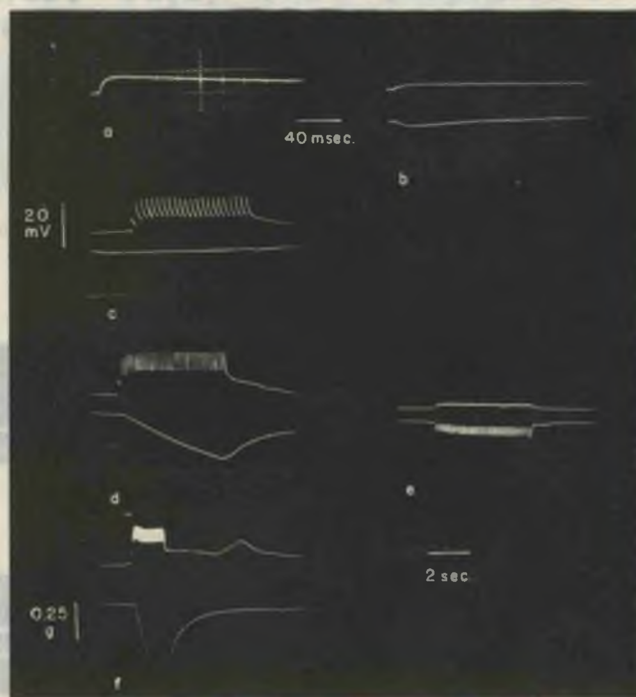


Fig. 9. Carcinus: Responses of a Type B muscle Fibre. (a) "Slow" p.s.p. in response to a single stimulus. (b) "Fast" p.s.p. recorded from the same muscle fibre in response to a single stimulus. (c,d,f) Responses to "slow" axon stimulation at 4 per sec. (c), 10 per sec. (d), and 20 per sec. (f). (e) Response to "fast" axon stimulation at 10 per sec. Lower traces, mechanical activity of the whole muscle.

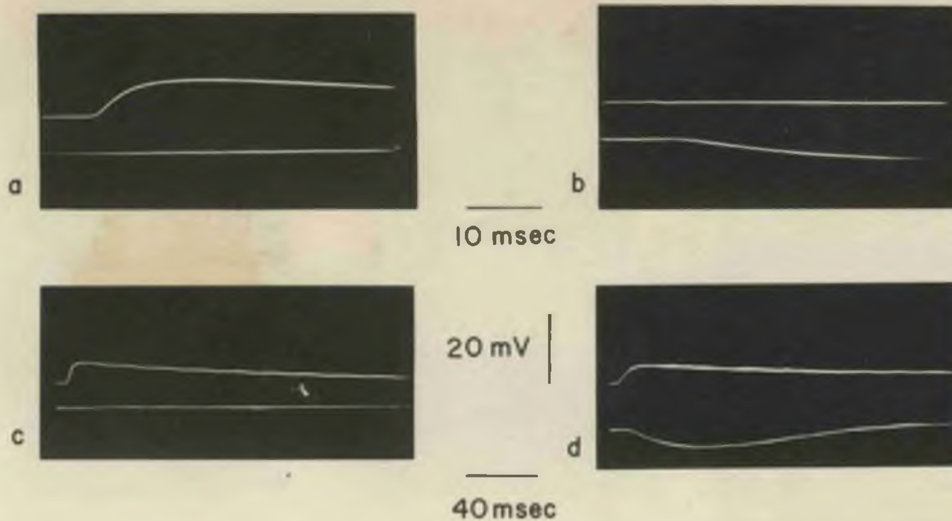


Fig. 10. Carcinus: "Fast" and "slow" responses of Type B muscle fibres in response to single stimuli applied to the respective axons. (a,c) "Slow" responses from two different muscle fibres. (b,d) "Fast" responses of same muscle fibres. Lower traces, mechanical activity of the whole muscle.

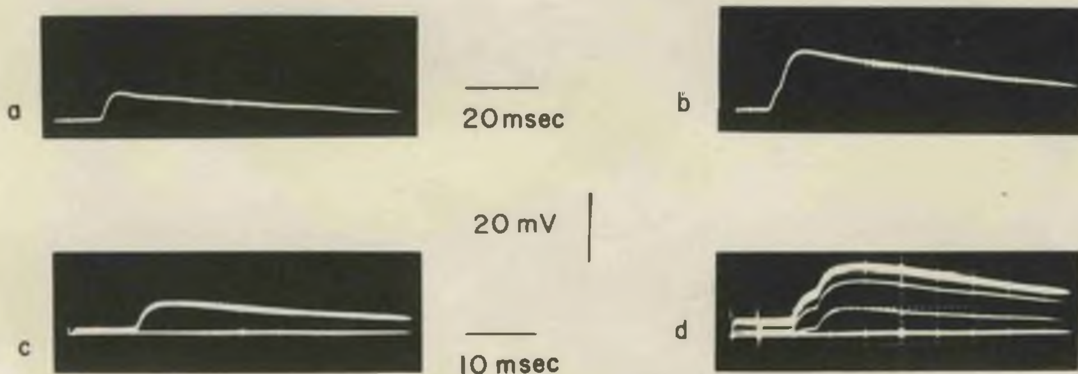
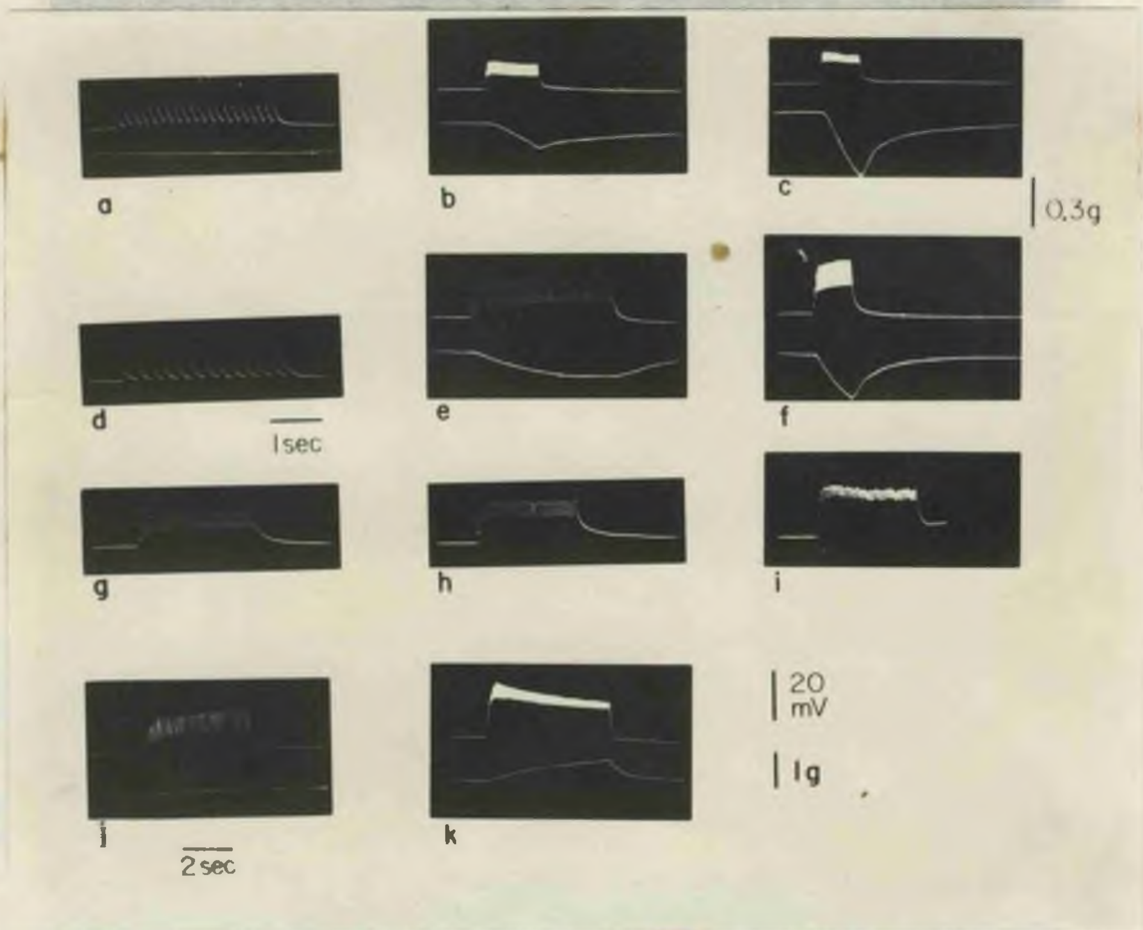


Fig. 12. Carcinus: Responses of two Type B muscle fibres to paired shocks (3 msec. separation) applied to the "slow" axon. (a,c) Responses to single shocks; (c,d) Corresponding responses to double shocks. In (d) stimulation was repeated at 5 per sec.



<u>Fig. 11</u>. <u>Carcinus</u>: Responses of Type B muscle fibres to repetetive stimulation of the "slow" axon at various frequencies. In each horizontal row recordings were from the same muscle fibre. When present, lower traces indicate tension. Frequencies of stimulation were: (a) 6 per sec.; (b) 15 per sec.; (c) 25 per sec.; (d) 3 per sec.; (e) 10 per sec.; (f) 20 per sec.; (g) 6 per sec.; (h) 10 per sec.; (i) 25 per sec.; (j) 10 per sec.; (k) 40 per sec.

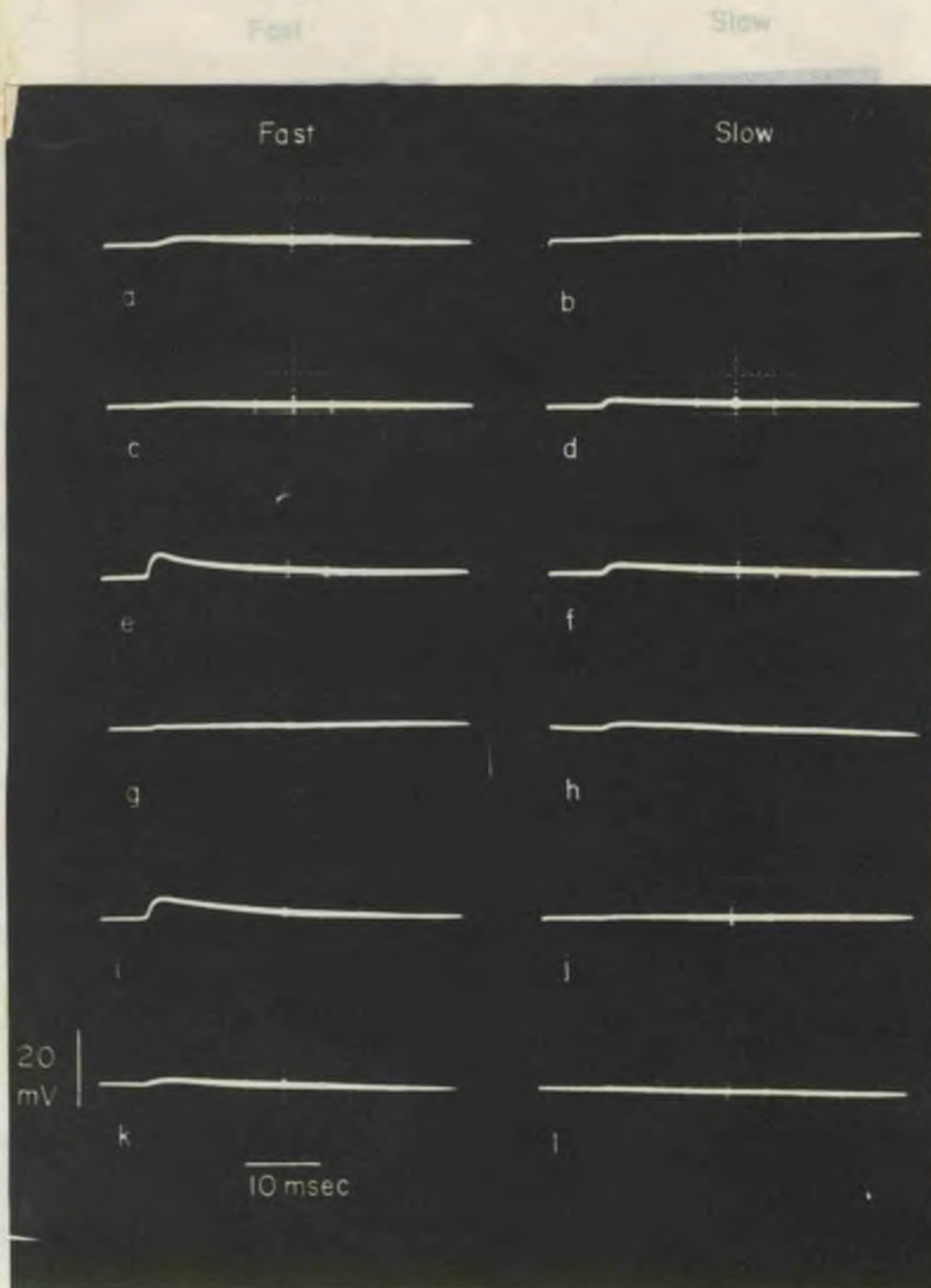


Fig. 13. Carcinus: P.s.ps. of Type C muscle fibres in response to single stimuli applied to "fast" and "slow" axons. Paired records (a and b, c and d , etc.) are from the same muscle fibre. All records are from the same muscle.

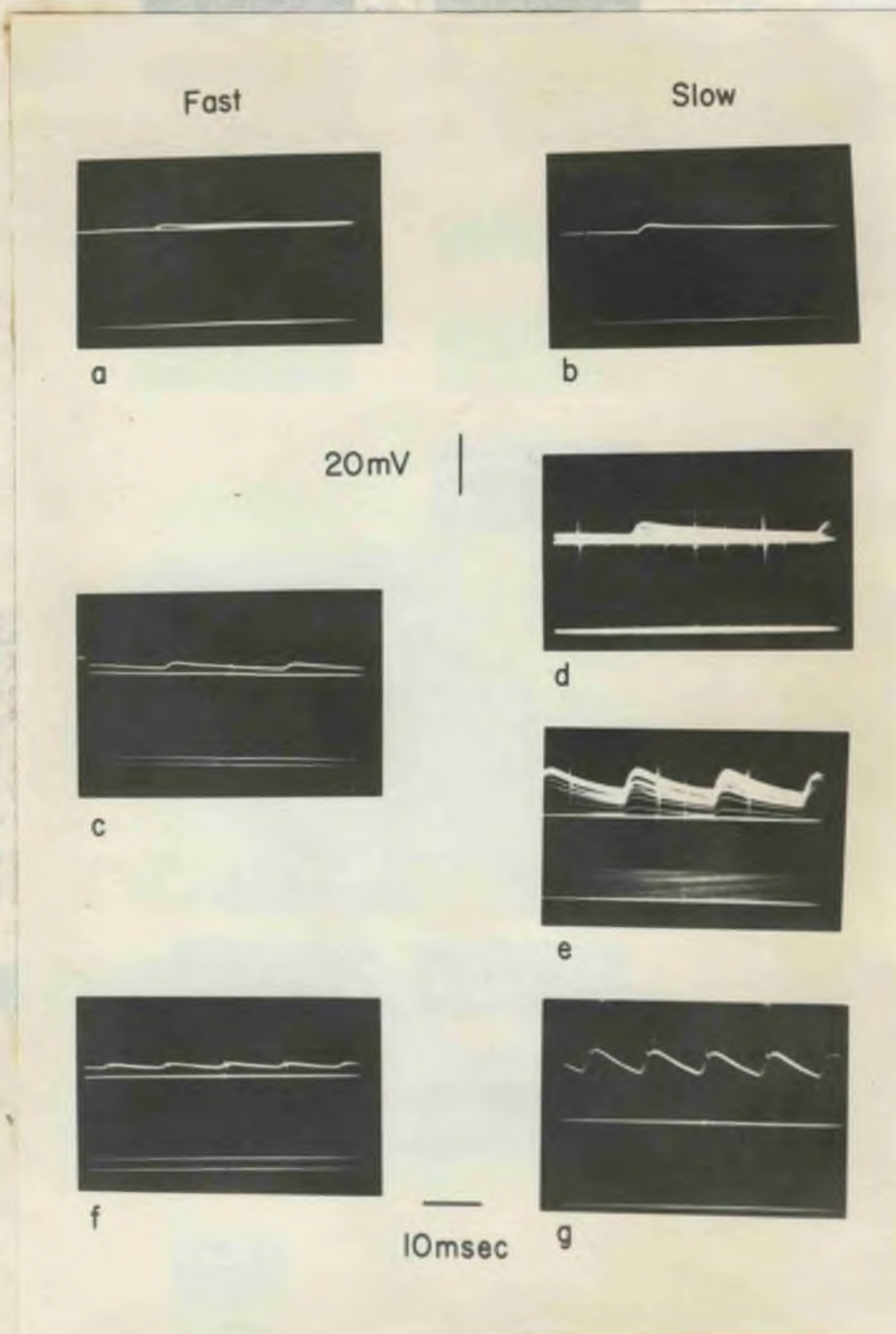


Fig. 14. Carcinus: Comparison of "fast" and "slow" responses in a Type C muscle fibre. (a,c,f) "Fast" response to stimulation at 10 per sec. (a), 50 per sec. (c), and 90 per sec. (f). (b,d,e,g) "Slow" response to stimulation at 10 per sec. (b), 25 per sec. (d), 70 per sec. (e), and 90 per sec. (g). Tension of the whole muscle is seen in the lower parts of (c,e,f). The electrical base line appears below the electrical response to indicate the total extent of the depolarization.

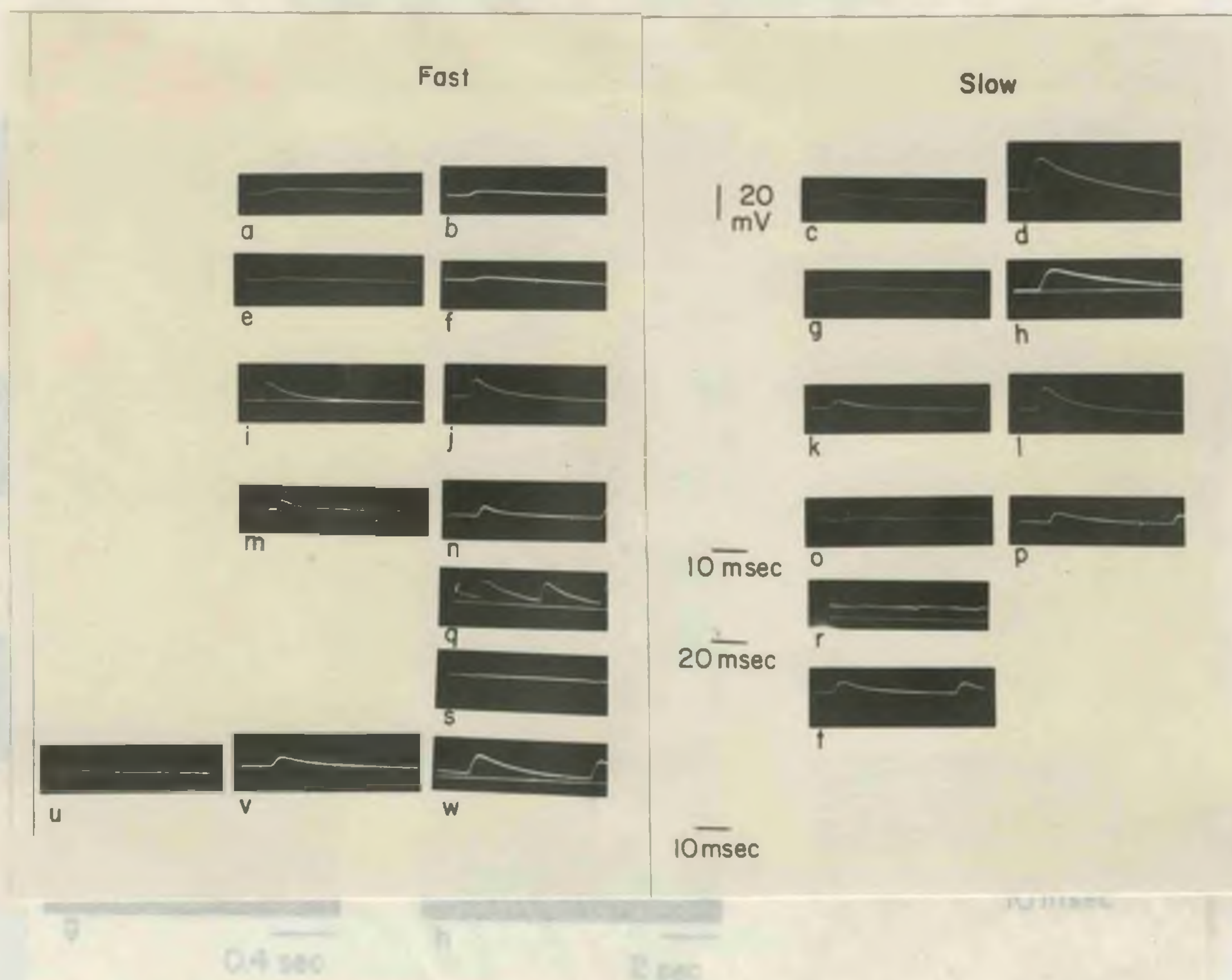


Fig. 16. Carcinus: Patterns of facilitation and shapes of p.s.ps. in Type C muscle fibres. Frequency of stimulation was 5 per sec. in (a,e,i,m,u) and (c,g,k,o); 15 per sec. in (b,f,j,v) and (d,h,l); 30 per sec. in (n,s,w) and (p,t); 40 per sec. in (q,r). Examples were taken from several different muscles. All of the records in a horizontal row are from the same muscle fibre. In (r) and in (s) small hyperpolarizing potentials indicate an influence of electrical responses of nearby muscle fibres and as absence of response in the muscle fibres recorded from.

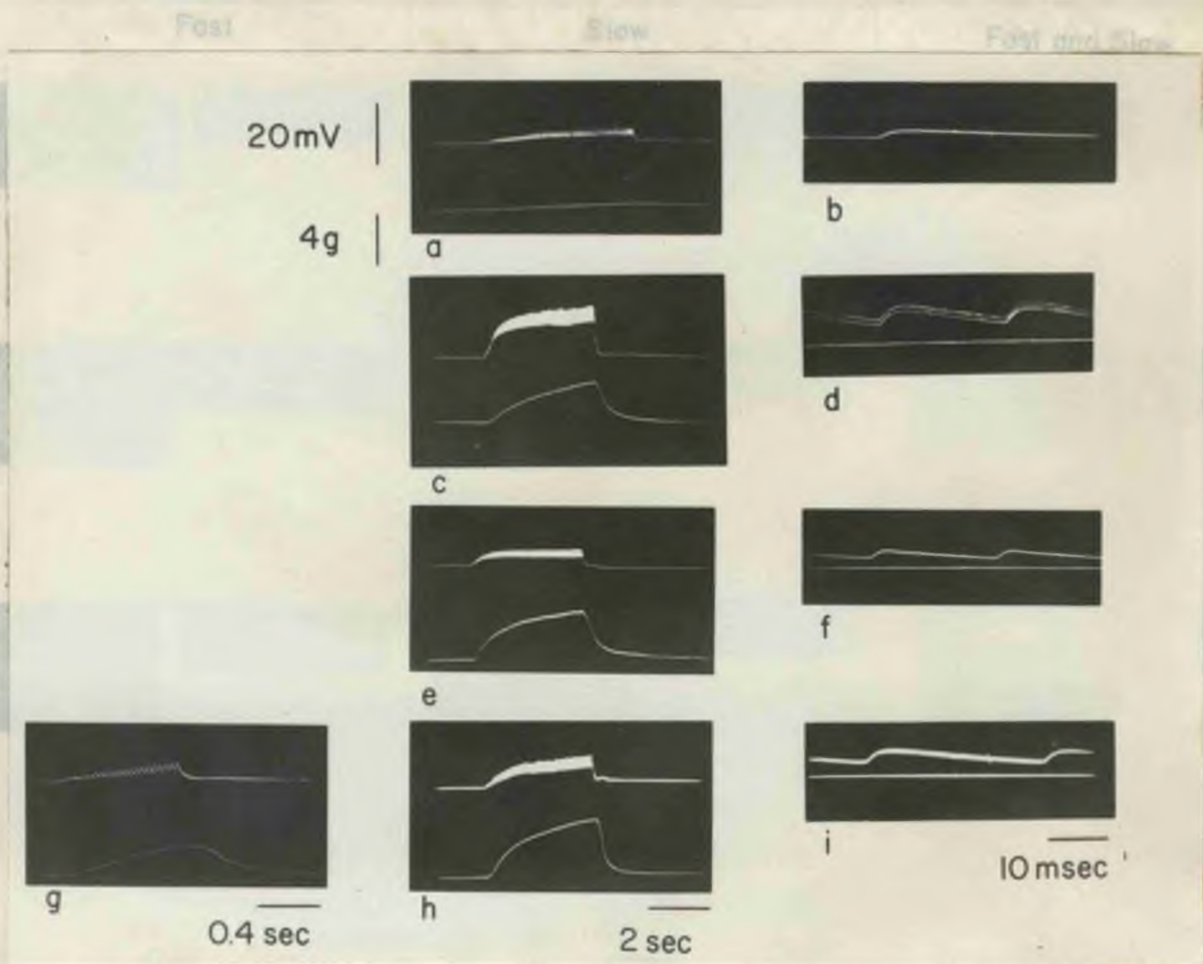


Fig. 18. Carcinus: "Slow" responses in Type C muscle fibres. (a,b,c,d) Records from a Type C fibre during stimulation of the "slow" axon at 15 per sec. (a,b) and 40 per sec. (c,d). (e,f) Records from another fibre during stimulation of the "slow" axon at 40 per sec. (g,h,i) Records from another fibre during stimulation of the "slow" axon at 30 per sec. Lower traces, mechanical activity of the whole muscle.

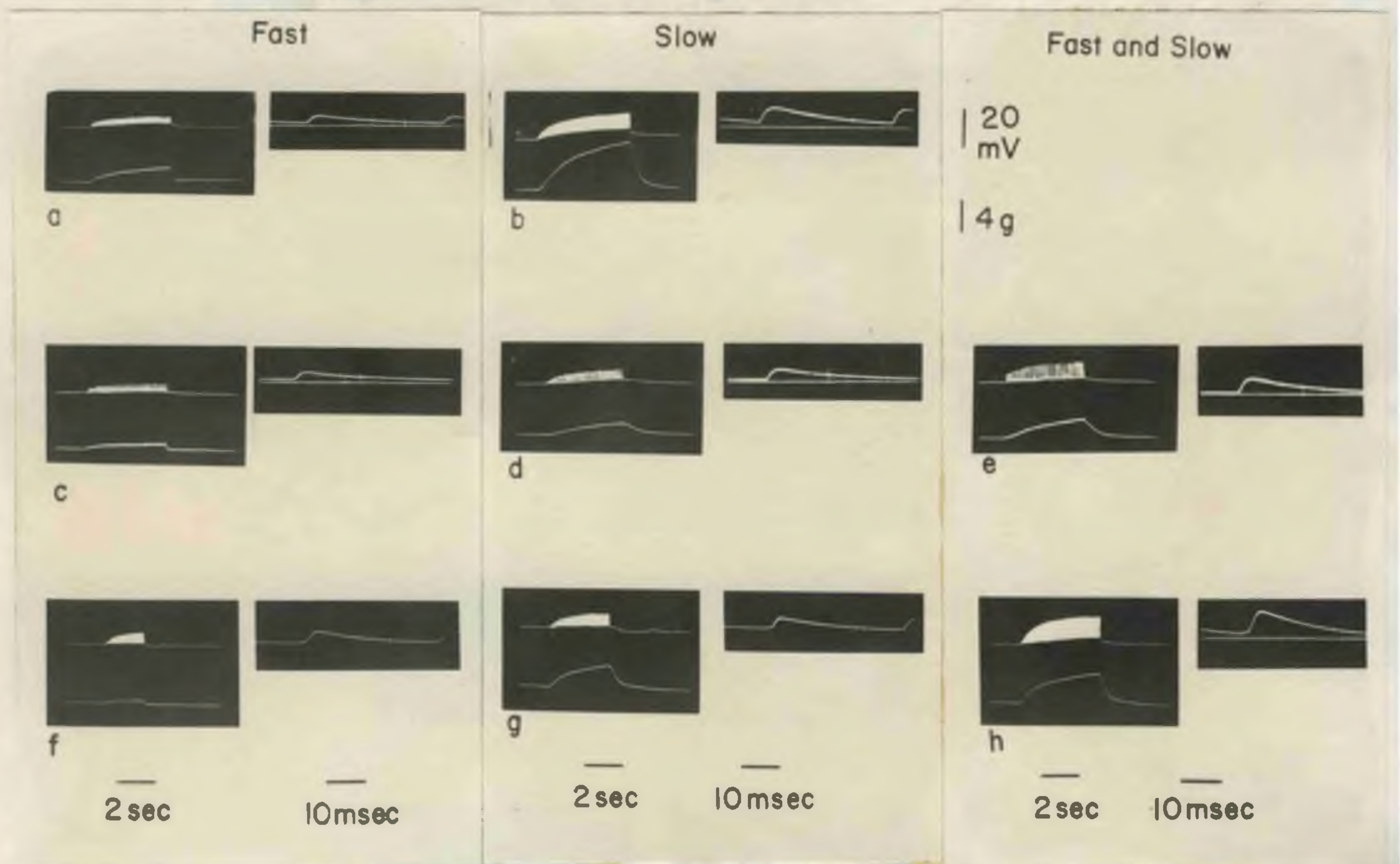


Fig. 19. Carcinus: "Fast" and "slow" responses of Type C muscle fibres. (a,b) Records from a Type C muscle fibre during stimulation of the "Fast" axon (a) and of the "slow" axon (b) at 30 per sec. (c,d,e) Records from another muscle fibre during stimulation of the "fast" axon (c), of the "slow" axon (d), and of both together (e), at 15 per sec. (f,g,h) Similar records from another fibre during stimulation at a frequency of 25 per sec. Lower traces, mechanical activity of the whole muscle.

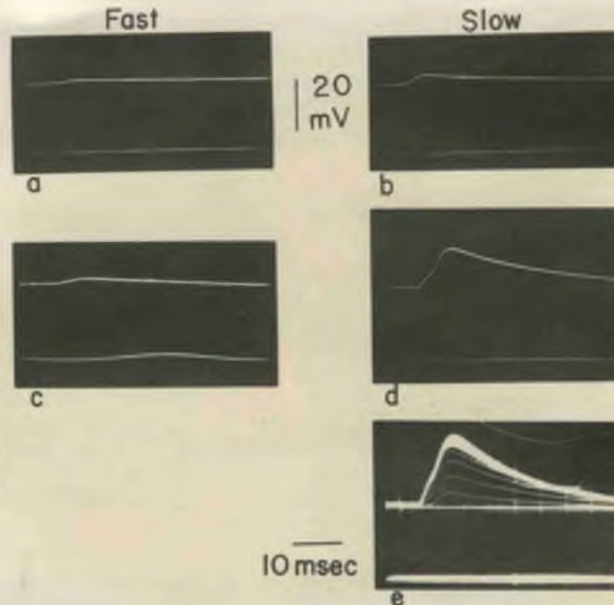


Fig. 20. Carcinus: Responses of a Type C muscle fibre to double pulses of stimulation (2.5 msec. separation) applied to the excitor axons. (a,c) Responses to "fast" axon stimulation at a frequency of 10 per sec., using single pulses (a) and double pulses (c). (b,d,e) Responses to "slow" axon stimulation at 10 per sec. (b; single pulses), 10 per sec. (d; double pulses), and 20 per sec. (e; double pulses). The electrode pulled out of the cell in (e).

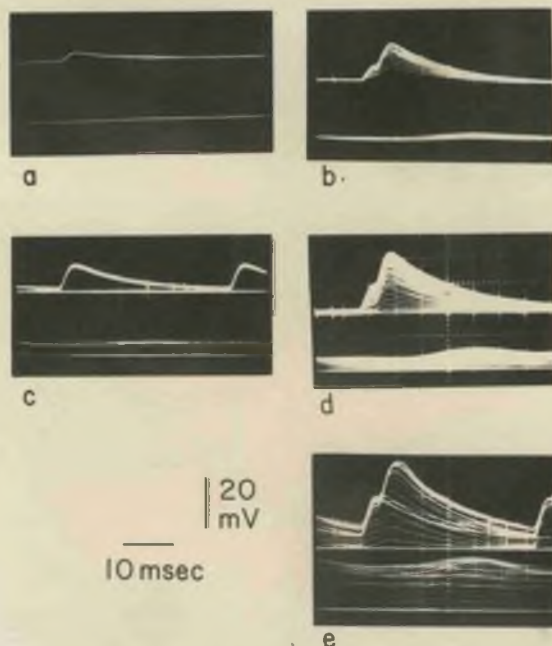


Fig. 21. Carcinus: Responses of a Type C muscle fibre to double pulses of stimulation applied to the "fast" axon. (a,c) Responses to single-pulse stimulation of the "fast" axon at 10 per sec. (a), and 30 per sec. (c); (b,d,e) Responses to double-pulse stimulation (3 msec. separation) at frequencies of 10 per sec. (e). Lower traces, mechanical response of the shole muscle.

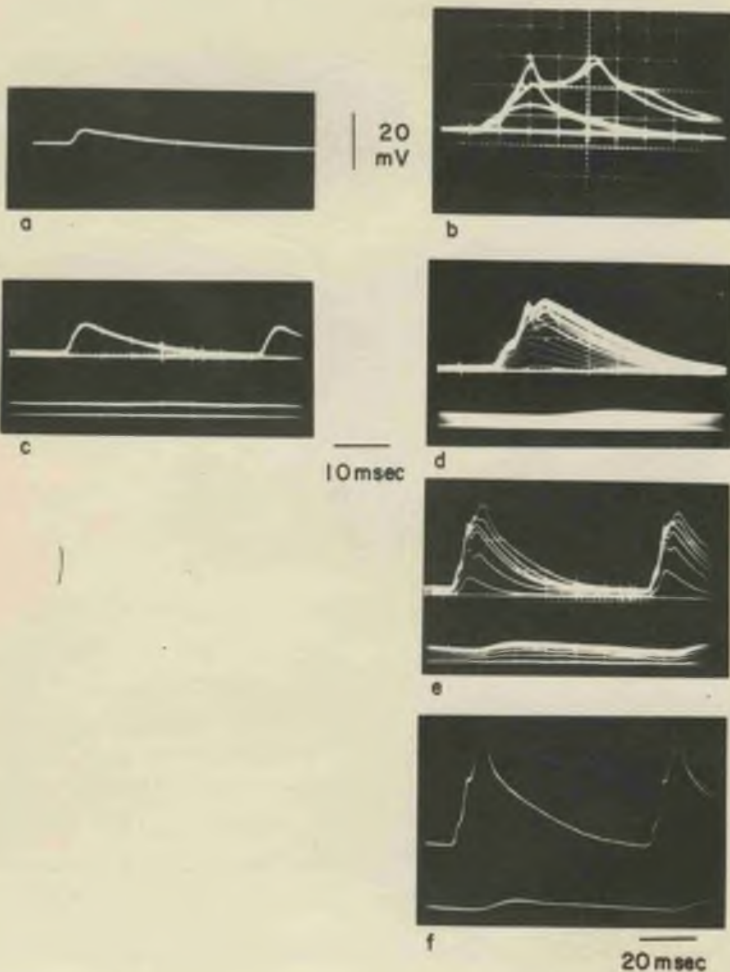


Fig. 22. Carcinus: Responses of Type C muscle fibres to double pulses of stimulation applied to the "fast" axon. (a) Response of a Type C muscle fibre to stimulation of the "fast" axon at a frequency of 4 per sec. (b) Response of the same muscle fibre to double pulses of stimulation (3 msec. separation delivered at a frequency of 4 per sec. (c) Response of another muscle fibre to stimulation of the "fast" axon at a frequency of 30 per sec. (d,e,f) Responses of the same muscle fibre to double pulses of stimulation (3 msec. separation) delivered at frequencies of 10 per sec. (d) and 17 per sec. (e, early; f, later).

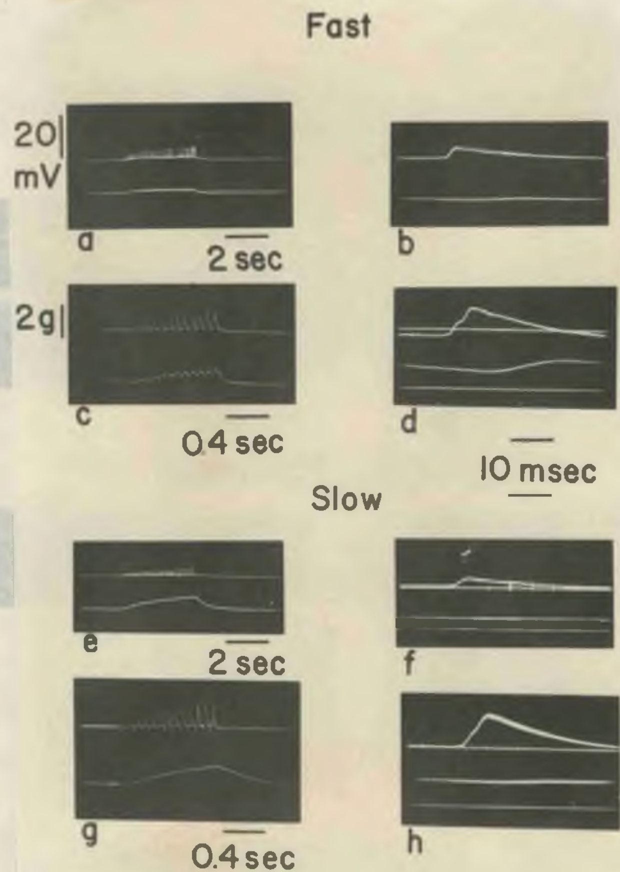


Fig. 23. Carcinus: Responses to single and double stimuli. (a,b) Responses of a Type C muscle fibre to stimulation of the "fast" axon at a frequency of 15 per sec. (c,d) Responses to the same frequency of stimulation, but using double pulses (2.5 msec. separation). (e,f) Responses to single, and (g,h) to double pulses of stimulation delivered at 15 per sec. to the "slow" axon. All records from the same muscle fibre.

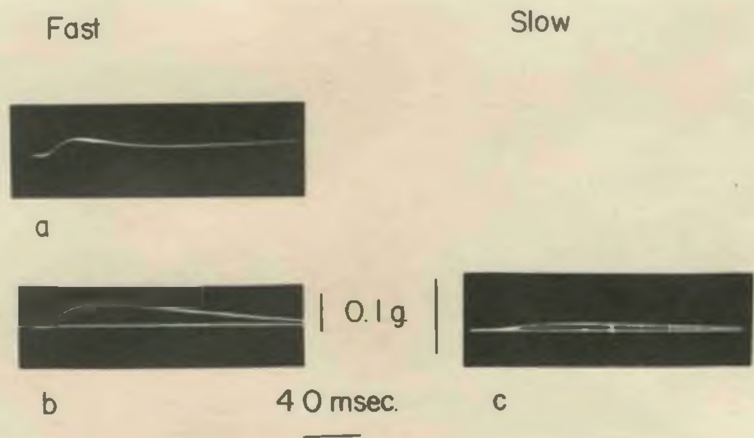


Fig. 24. Carcinus: "Fast" and "slow" mechanical responses. (a) "Fast" twitch recorded from a typical preparation in response to a single stimulus. (b) "Fast" and (c) "slow" twitches recorded from an unusual preparation in response to single stimuli delivered to the respective motor axons.

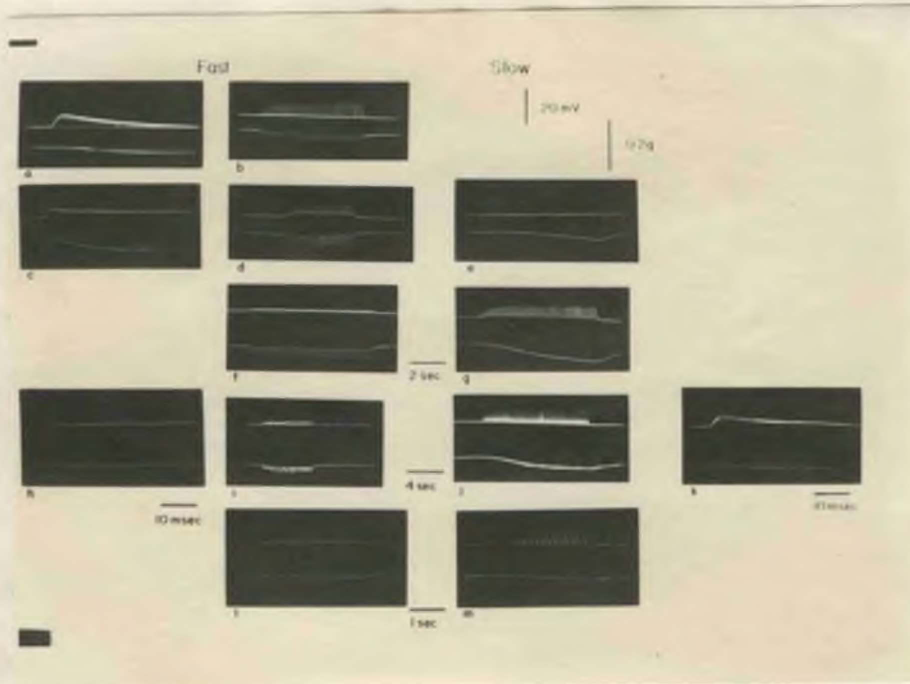


Fig. 25. Carcinus: "Fast" and "slow" electrical (Type C) and mechanical responses at low frequencies of stimulation. In each horizontal row, electrical records are from the same muscle. Stimulation was given at a frequency of 6 per sec. in all cases. In (g) the "slow" mechanical response contains minute twitches. Lower records in each case are mechanical responses of the whole muscle.

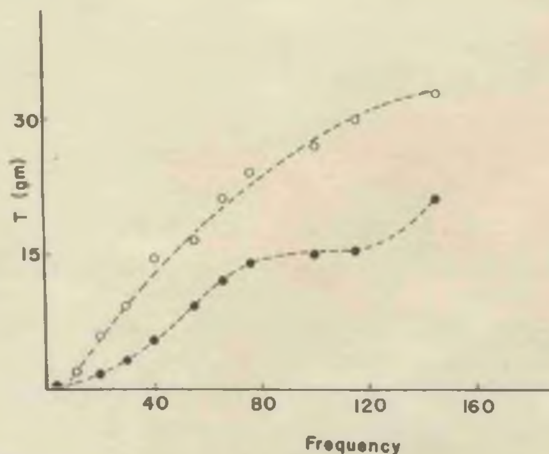
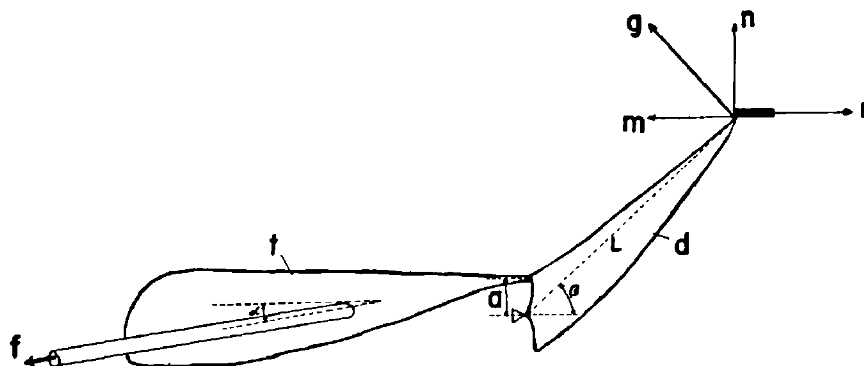
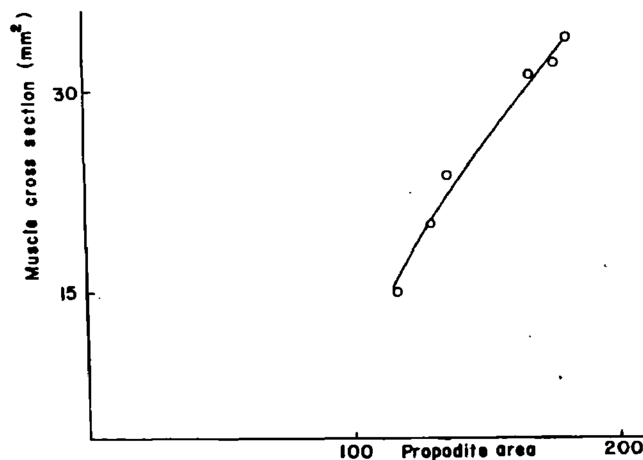


Fig. 26. Tension developed by a Carcinus muscle during three-second bursts of stimulation delivered to "slow" and "fast" motor axons at increasing frequencies. Open circles, "slow" tension; filled circles, "fast" tension. Tension (in grams) was measured at the tip of the dactyl. The lowest frequency used was 2 per sec.





<u>Fig. 26 A.</u> Upper figure: Diagram to illustrate the mechanics of the <u>Carcinus</u> closer muscle. d, dactyl; t, tendon of the closer muscle; r, recording of isometric force developed by the muscle; a, distance between tendon and pivot point of dactyl on propodite; L, distance from pivot point to dactyl tip; g, force at dactyl tip (at right angles to L); m, n, horizontal and vertical components of g; f, force developed by a single muscle fibre of the closer muscle; α , angle between muscle fibre and tendon; β , angle between L and the horizontal plane.

Lower figure: Relation between size of propodite and cross-sectional area of closer muscle. "Propodite area" is the product of the length and width of the propodite, measured at their greatest extents, in mm^2. Cross-sectional areas of six different muscles of varying size were determined.

ii) Carcinus: Opener and Stretcher Muscles

The opener and stretcher muscles are innervated by the same single motor axon (Fig. 1). In *Carcinus* a few preparations of each muscle were examined, but it was found difficult to make satisfactory preparations of these muscles without partially denervating them, so no intensive study of these muscles was made.

Results were similar for the two muscles.

Low frequencies of stimulation (1 to 5 per sec.) applied to the motor axon produced small p.s.ps. in many of the muscle fibres (Fig. 27, a). In other fibres no response was recorded at these frequencies of stimulation, and although electrical responses appeared in some of them at higher frequencies of stimulation, many remained unresponsive. This result indicated that some of the muscle fibres were denervated during exposure of the muscle.

Increase in the frequency of stimulation resulted in facilitation of individual p.s.ps. and in the appearance of depolarization "plateaus" at frequencies of stimulation of 15 to 30 per sec. (Fig. 27). In some cases the p.s.ps. had a peculiar "rounded" appearance (Fig. 27, d).

Tension in undissected preparations was observed to start at frequencies of stimulation of 5 to 10 per sec., whereas in preparations dissected for electrical recording very little

tension was evident until the frequency of stimulation was 20 to 40 per sec. This further indicates partial denervation of the muscle during dissection. Tension developed slowly and smoothly, and relaxed in about a second when stimulation was stopped.

Electrical responses obtained throughout these muscles were uniform when compared with the variation encountered in the *Carcinus* closer muscle. The responses of the opener muscle fibres resembled fairly closely the responses to "slow" axon stimulation of Type O fibres in the closer muscle. Time constants of decay of the order of 14 to 20 msec. were observed for p.s.ps. recorded from these muscles.

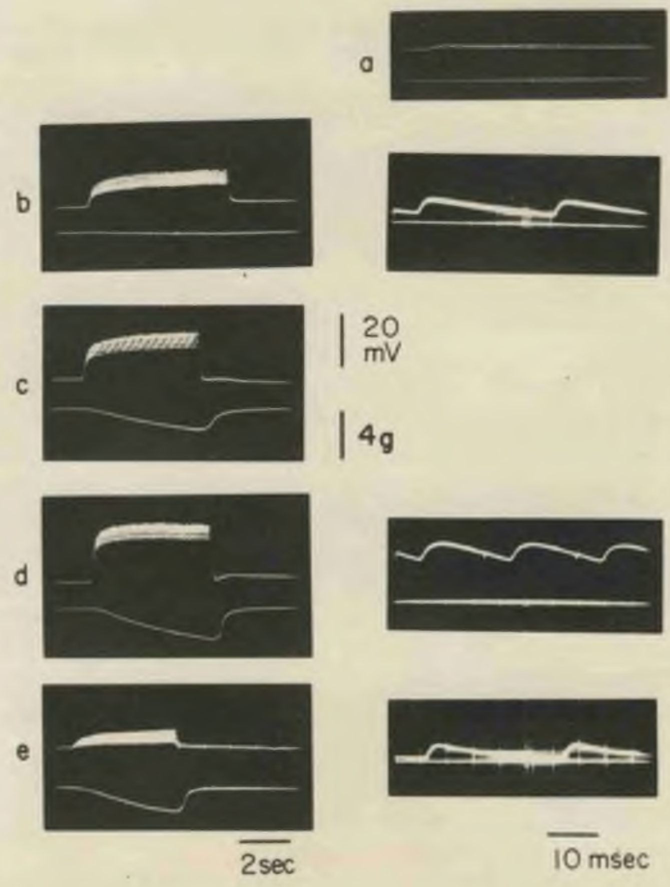


Fig. 27. Records from two Carcinus stretcher muscles. (a) P.s.p. in response to stimulation of the motor axon at a frequency of 3 per sec. (b) Electrical and mechanical responses to stimulation of the motor axon at 35 per sec.; (c) at 50 per sec.; (d) at 70 per sec. (e) Responses of another preparation during stimulation of the motor axon at 35 per sec. Tension is shown as a downward deflection in the lower traces of (b,c, d,e).

iii) Nephrops: Closer Muscle

The closer muscles of the walking legs of *Nephrops* have not been studied previously by electrophysiological methods. In the present study attention was focused on the chelate walking legs (legs 2 and 3), which could be readily perfused through the index of the propodite. The muscles were small (prepodites of these legs were usually about 1.5 cm. long and 0.4 cm. wide) and a thorough sampling of muscle fibres from different parts of the muscle was possible. (This would not have been possible with the large claw muscle, which was not studied). Altogether about fifty different preparations of this muscle were examined.

Electrical responses: Exploration of muscle fibres with a recording microelectrode revealed that electrical responses were not uniform throughout the muscle, but could be correlated with the part of the muscle recorded from. Muscle fibres at the distal end of the muscle gave small electrical responses to "fast" axon stimulation even at high frequencies (Fig. 28, a), whereas the responses to "slow" axon stimulation, though small at frequencies of stimulation below 10 per sec., became increasingly larger at higher frequencies (Fig. 28, b). In fibres located in the proximal part of the muscle, the situation was reversed. Single shocks applied to the "fast" axon pro-

duced large p.s.ps. in these fibres (Fig. 28, c). The responses to "slow" axon stimulation, on the other hand, remained small even at high frequencies of stimulation (Fig. 28, d,e). Fibres in the central part of the muscle showed responses intermediate in nature between the proximal and distal types.

The p,s.ps. recorded from proximal muscle fibres in response to single shocks applied to the "fast" axon varied in size from one or two millivolts to about 25 millivolts (Fig. 29); the usual size was about 12mV. No fibres were found to give electrically excitable membrane responses to single indirect stimuli.

At frequencies of stimulation of 3 to 8 per sec., facilitation of the electrical response occurred (Figs. 30, 33). This facilitation was much more evident in muscle fibres of partially fatigues preparations (Figs. 33,d; 34,d) than in muscle fibres of fresh preparations (e.g. Fig. 33,f). In fatigued preparations the response usually started from a smaller size initially, but could grow during repeated stimulation to a size approaching that of the "fresh" responses (Fig. 34).

The "fast" electrical response showed marked "homofacilitation" (Wiersma and van Harreveld, 1939). For several seconds after a short burst of stimulation at a frequency of 10 or 20 per sec., single stimuli produced a much larger electrical response than was the case previous to this stimulation (Fig. 31, e).

In some muscle fibres electrically excitable membrane responses were produced when frequencies of stimulation of 3 to 8 per sec. were applied to the "fast" axon (Fig. 30). These responses were graded and prolonged, and often had a rounded appearance. They usually appeared when the membrane was depolarized by about 20mV (from an initial resting potential of 65 to 75 mV). However, many muscle fibres showed no responses of this sort, even at high frequencies of stimulation and large membrane depolarizations (Fig. 31).

When two closely spaced stimuli were delivered to the "fast" axon, some of the proximal muscle fibres showed electrically excitable membrane responses of various sizes (Fig. 32). In other fibres no such responses resulted from this type of stimulation (Fig. 32, e,f).

Electrical responses to stimulation of the "slow" axon at low frequencies were always very small. "Slow" p.s.ps. recorded in proximal muscle fibres remained small even at high frequencies of stimulation. In some fibres giving large "fast" p.s.ps. there was no detectable response to "slow" axon stimulation. Muscle fibres in the distal part of the muscle showed p.s.ps. which, though small at low frequencies of stimulation, became much larger through facilitation as the frequency was increased (Fig. 35). At stimulation frequencies of 30 per second and above many distal muscle fibres showed depolarization "plateaus" (Figs. 35, 36, 37).

In some of these distal muscle fibres, electrically excitable membrane responses were observed at stimulation frequencies above 50 to 60 per sec. (Fig. 36). In others, depolarization to the same extent in response to similar stimulation frequencies produced no active responses of this sort.

Mechanical responses: The mechanical response of the muscle to a single shock applied to the "fast" axon was a weak twitch (Fig. 29,a,b,c,e,h). The twitch shown in Fig. 29(a) was exceptionally strong; the usual twitch was of the size shown in Fig. 29 (b,e,h). Muscles from different animals showed considerable variation in the mechanical responses to both "slow" and "fast" axon stimulation.

As the frequency of stimulation was increased, the mechanical response showed facilitation (Figs. 29c, 33), and at frequencies of stimulation greater than about 8 per second, tetanic contractions were built up.

As the preparations aged, the mechanical responses became smaller (Figs. 33,b,d,g; Fig. 34,b). The "twitch" response often did not appear in such preparations until a number of closely spaced "priming" stimuli had been given (Fig. 33,d).

The "slow" mechanical response was usually recordable only at frequencies of stimulation above 30 per sec. (Fig. 35). The response varied from preparation to preparation, but often

had a rapid initial rise (Fig. 35) followed by a longer period of slow growth. Unlike the smooth "slow" response most usually observed in Carcinus, and in many other crustacean muscles (Wiersma, 1961), the "slow" response in Nephrops was often seen to have small "bumps" or superimposed twitches of irregular occurrence when the stimulation frequency was higher than about 50 per sec. (Fig. 36). In preparations showing this type of tension response it was possible to record electrically excitable membrane responses from some distal fibres at the same time (Fig. 36).

The relaxation rate of the "slow" mechanical response was usually quite rapid. Tension typically returned to the base line within a second. This is in contrast to the much slower relaxation of "slow" tension in Carcinus.

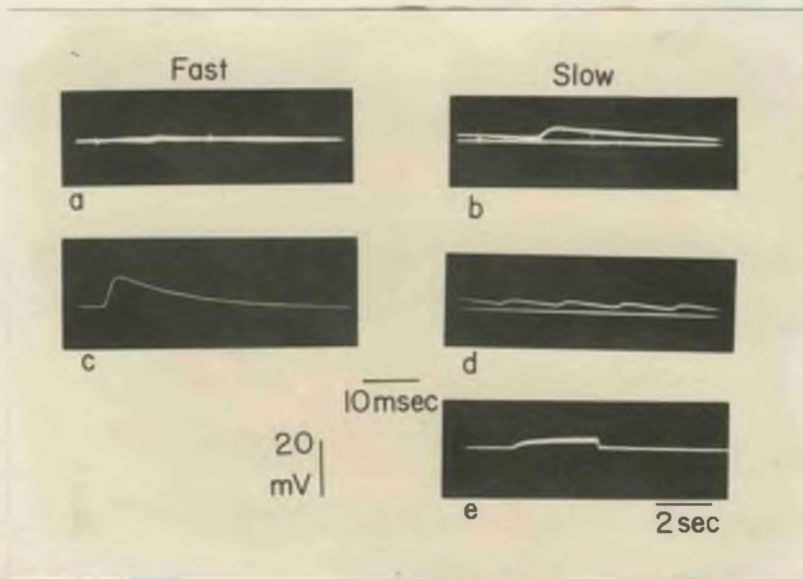


Fig. 28. Responses from distal (a,b) and proximal (c, d,e) muscle fibres of a Nephrops closer muscle. (a) Electrical response from a distal muscle fibre during stimulation of the "fast" axon at 20 per sec. (b) Response of the same fibre to stimulation of the "slow" axon at 20 per sec. (c) Response of a proximal muscle fibre to a single shock applied to the "fast" axon. (d,e) Responses of the same fibre to stimulation of the "slow" axon at 100 per sec.

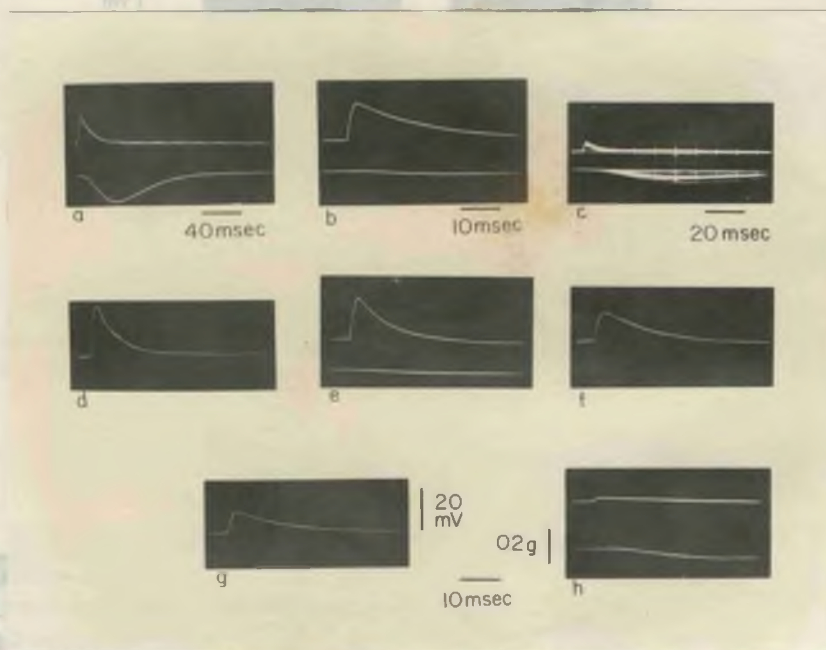


Fig. 29. Nephrops: Responses to single shocks applied to the "fast" axon. Electrical responses were recorded from proximal muscle fibres. Tension response of the whole muscle is shown in the lower traces.

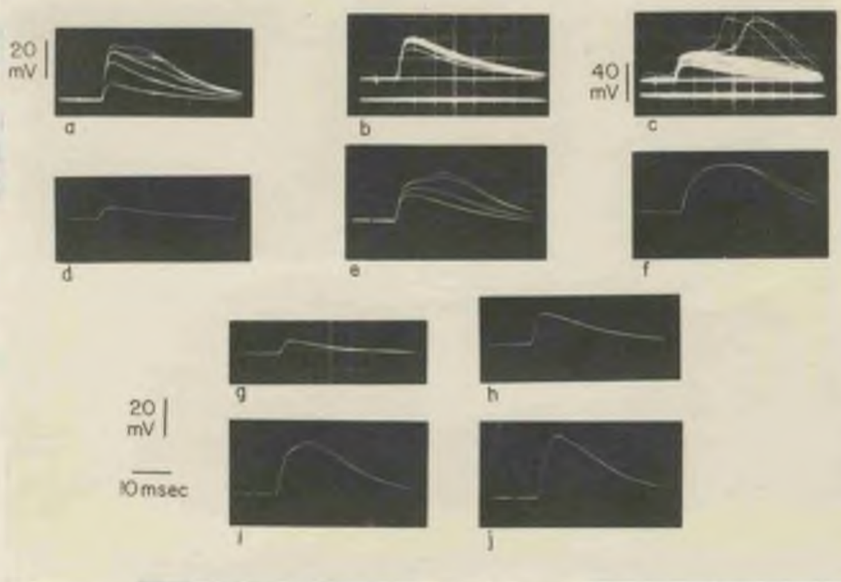


Fig. 30. Nephrops: Electrical responses of proximal muscle fibres to stimulation of the "fast" axon. (a) Response of a proximal fibre to stimulation at 10 per sec., recorded at ½ second intervals during stimulation. (b,c) Responses of another fibre to stimulation at 3 per sec. (b) and 8 per sec. (c). (d,e,f) Responses of another fibre during stimulation at 1 per sec. (d), and 10 per sec. (e,f; f later than e during stimulation). (g,h,i,j) Responses of another fibre to stimulation at 1 per sec. (g), and at 1 second intervals after start of stimulation at 10 per sec. (h,i,j).

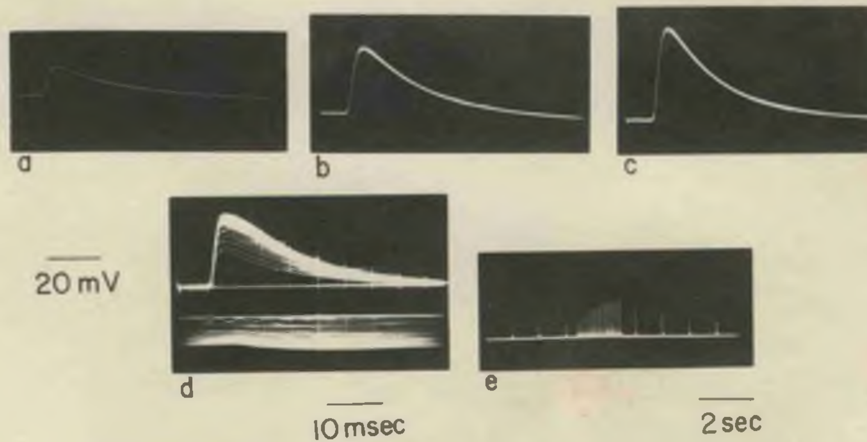


Fig. 31. Nephrops: Responses of proximal muscle fibres to stimulation of the "fast" axon. (a,b,c) Responses of a proximal muscle fibre to stimulation at 1 per sec. (a), 10 per sec. (b), and 19 per sec. (c). (d) Responses of another fibre to stimulation at 15 per sec. (lower trace, tension of the whole muscle). (e) Response of another fibre during stimulation at 1 per sec., before and after a burst of stimulation at 10 per sec.; note "homofacilitation."

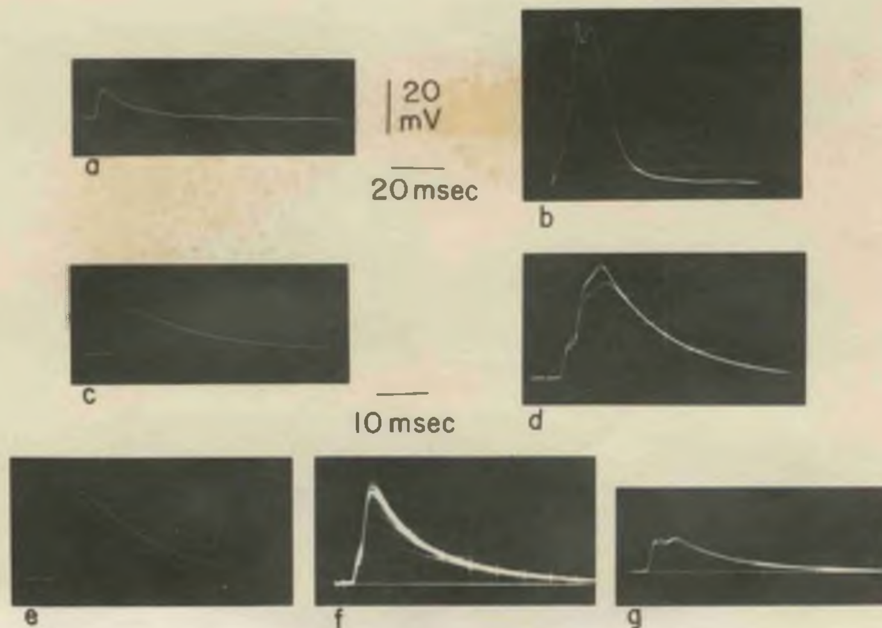


Fig. 32. Nephrops: Electrical responses of proximal muscle fibres to double shocks applied to the "fast" axon. (a,b) Response of a proximal muscle fibre to a single shock (a) and to a double shock (3 msec. separation). (c,d) Response of another fibre to a single shock (c) and to double shocks (3 msec. separation) at 1 per sec. (d). (e) Response of another fibre to a double shock (4 msec. separation). (f,g) Response of another fibre to double shocks (2.5 msec. separation) delivered at 8 per sec. (g, 40 secs. after start of stimulation).

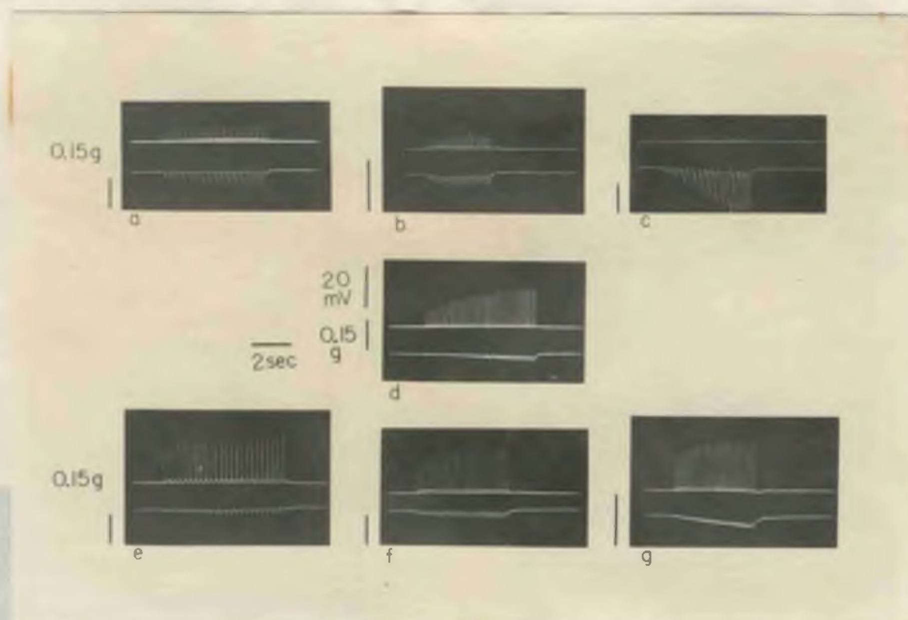


Fig. 33. Nephrops: "Fast" mechanical (lower traces) and electrical responses. (a) Responses during stimulation at 5 per sec.; electrical response from a proximal muscle fibre. (b) Responses of the same preparation after aging. (c) Responses of another preparation during stimulation at 6 per sec., showing extreme facilitation of the mechanical responses. (d) Responses of another (aged) preparation during stimulation at 8 per sec. (e,f) Responses of another preparation during stimulation at 4 per sec. (e) and 7 per sec. (f). (g) Responses of the same preparation (after aging) to stimulation at 12 per sec. All electrical responses are from proximal fibres.

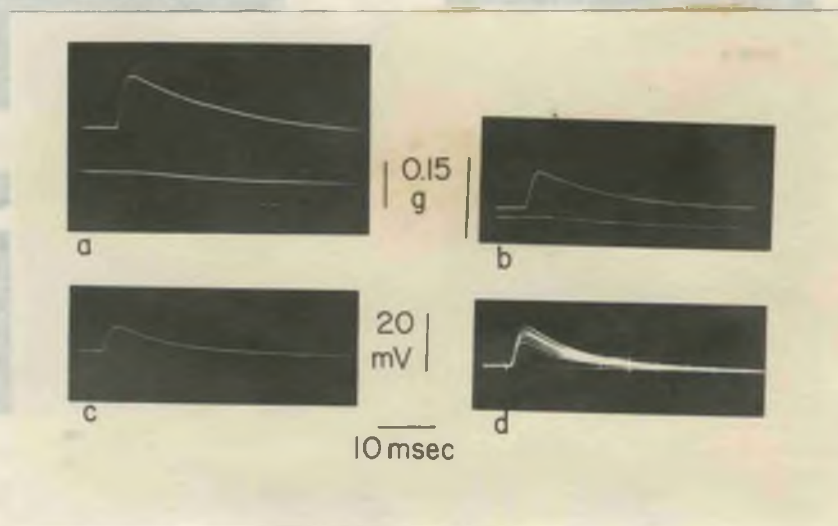


Fig. 34. Nephrops: Fatigue of "fast" electrical responses of proximal muscle fibres with aging of the preparation. (a) Responses to a single shock in a fresh preparation. (b) Responses to a single shock in the same preparation and muscle fibre after aging. (c) Response of another muscle fibre to a single shock. (d) Response of the same muscle fibre after aging, to stimulation at 6 per sec. (Lower traces in a and b, tension of the whole muscle).

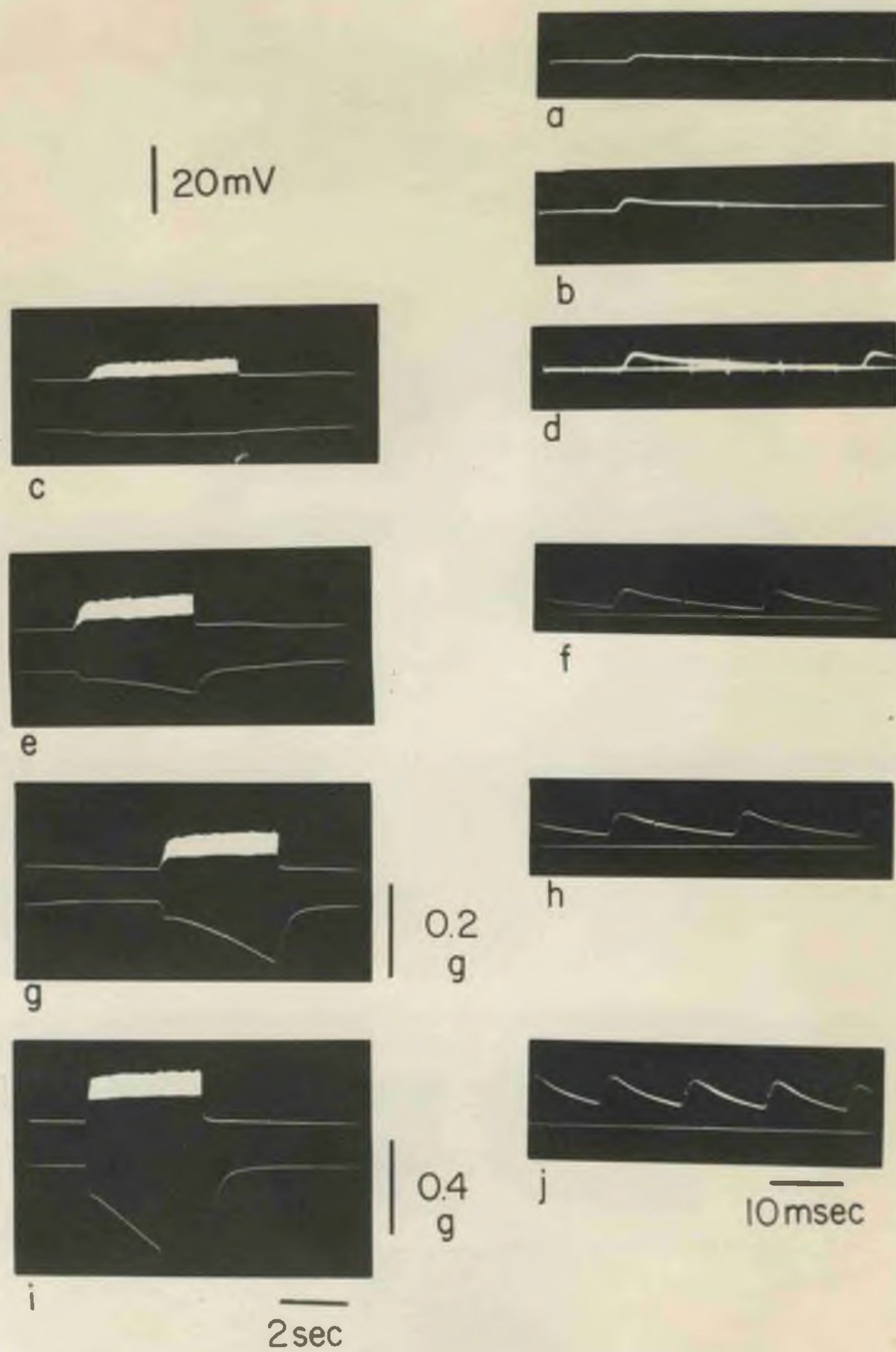


Fig. 35. Nephrops: Records from a distal muscle fibre during stimulation of the "slow" axon: (a) at 3 per sec.; (b) at 10 per sec.; (c,d) at 30 per sec.; (e,f) at 45 per sec.; (g,h) at 70 per sec.; (i,j) at 90 per sec. Lower traces (c,e, f,i) slow tension of the whole muscle.

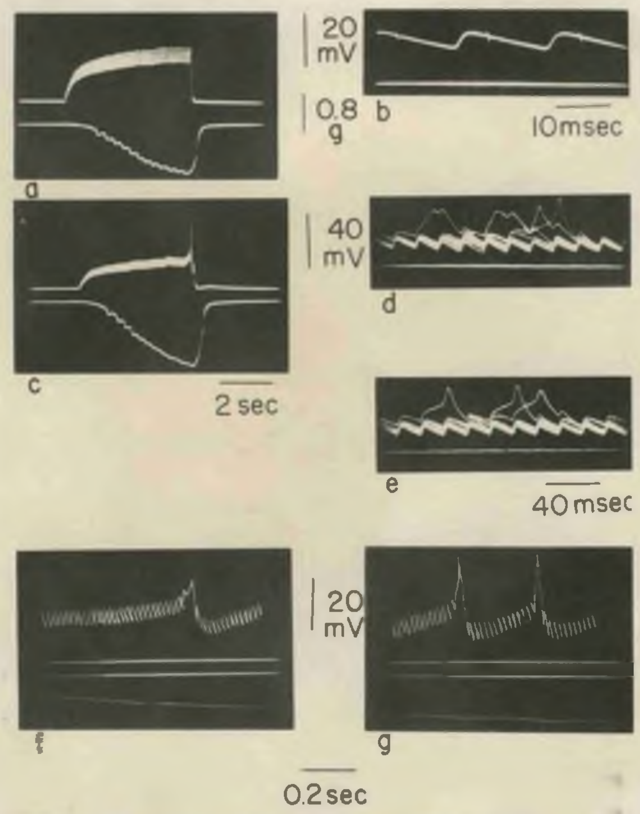


Fig. 36. Nephrops: Responses of distal muscle fibres during stimulation of the "slow" axon. (a,b) Responses to stimulation at 60 per sec. (c,d) Responses of another fibre in the same preparation to stimulation at 60 per sec. showing electrically excitable membrane responses. (e) Similar responses from another muscle fibre. (f,g) Electrically excitable responses of another muscle fibre during stimulation at 55 per sec. soon after the start of stimulation (f), and 0.1 sec. later (g). Lower traces in (a,c,f,g), tension of the whole muscle.

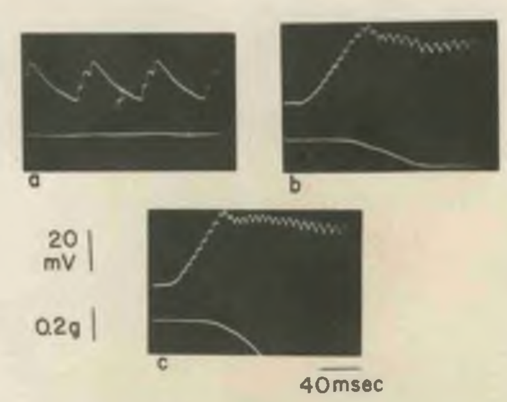


Fig. 37. Nephrops: Responses of distal muscle fibres during "slow" axon activity. (a,b,c) Activity of three muscle fibres during stimulation at 100 per sec.; in (a), three stimuli were delivered every 60 msec. Lower traces, tension of the whole muscle.

iv) Nephrops: Opener Muscle

A few preparations of the opener muscle were examined. In most respects the responses observed were similar to those seen in the closer muscle during "slow" axon stimulation.

Low frequencies of stimulation (2 to 10 per sec.) produced very small p.s.ps. in most of the muscle fibres examined (Fig. 38a). As the frequency of stimulation was increased, marked facilitation occurred, and depolarization "plateaus" were built up at frequencies of stimulation of 20 to 40 per sec. The size of these plateaus varied considerably from fibre to fibre (Fig. 38, d, e, f).

In the response of Fig. 38 (d), the depolarization "plateau" is less than that of Fig. 38(e), although the size of the individual p.s.ps., and the total magnitude of the depolarization, is greater in the former case. The explanation for this type of variation is probably to be found in the more rapid decay of the p.s.ps. of Fig. 38 (d). (See p. 40).

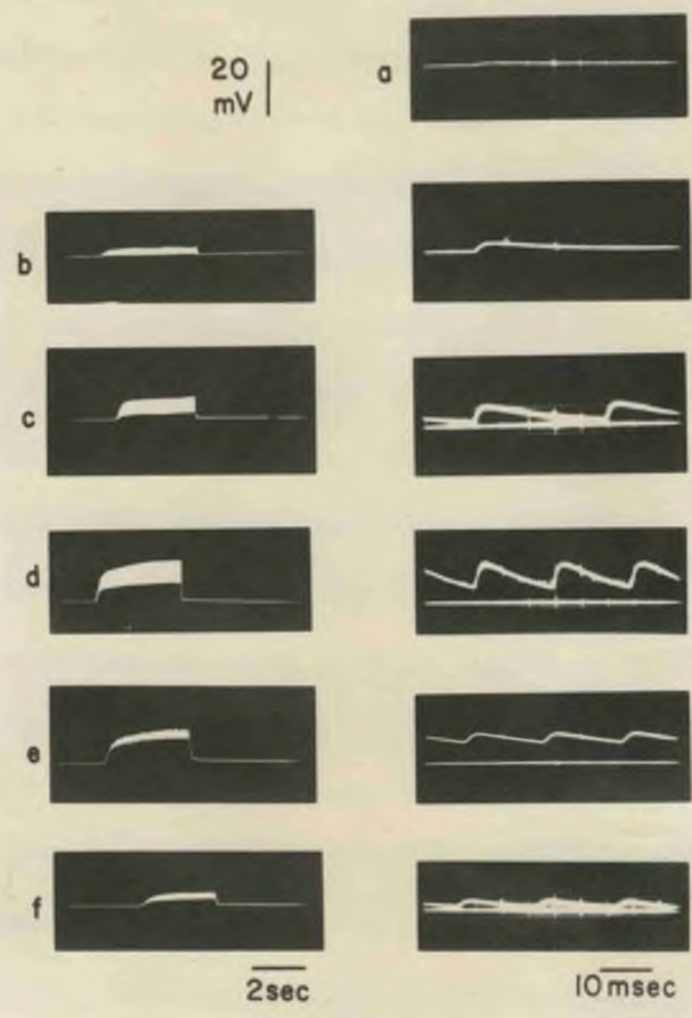


Fig. 38. Responses of muscle fibres in the opener muscle of *Nephrops*. (a to d), Responses of a muscle fibre to stimulation of the motor axon at 2 per sec. (a), 20 per sec. (b), 40 per sec. (c), 70 per sec. (d), (e,f) Responses of other muscle fibres to stimulation at 70 per sec.

v) <u>Astacus: Closer of the Claw</u>

The closer muscles of the claws of freshwater crayfish have been studied more than any other crustacean nerve-muscle preparation. They were used in the classical experiments of Richet (1879), Biedermann (1887), Lucas (1917), and van Harreveld and Wiersma (1936, 1937), and are now commonly used in classroom experiments.

The mechanical responses of these muscles to "slow" and "fast" axon simulation as they have been described in the literature, are quite distinct. A single stimulus applied to the "fast" axon gives rise to a powerful twitch contraction; higher frequencies of stimulation produce a tetanus which attains its maximum value rapidly. Stimulation of the "slow" axon gives rise to "completely smooth tetani" which rise very slowly to a final level of tension (van Harreveld and Wiersma, 1936).

The electrical responses of individual muscle fibres have been studied by Furshpan (1955) and by Hoyle and Wiersma (1958a) in the crayfish <u>Cambarus clarkii</u>. These investigators found that "slow" electrical responses consisted of very small p.s.ps. which could, in some muscle fibres, produce depolarization "plateaus." "Fast" responses were found to consist of p.s.ps. up to 20mV in magnitude; large spikes were not observed.

An interesting feature of this muscle is that it shows a "paradox" phenomenon (Hoyle and Wiersma, 1958 c). The "fast" twitch response to a single stimulus declined and vanished after repeated stimulation, although the p.s.ps. in the muscle fibres examined remained large. Low frequencies of stimulation applied to the "slow" axon gave a contraction in muscles in the "paradox" state, although recorded electrical responses were generally smaller than those resulting from "fast" axon stimulation. However, in some muscle fibres a maintained depolarization was observed during "slow" axon stimulation. This observation makes the "paradox" phenomenon in this muscle less convincing than that described for Randallia.

A great disadvantage of working with the closer muscle of the crayfish claw is the muscle's large size. It is impossible to sample the muscle fibres effectively in this muscle by microelectrode techniques. Also, it is difficult to perfuse the muscle rapidly and effectively, and degenerative changes may take place in the inner part of the muscle, though not at the exposed surface.

These drawbacks were realized soon after work was begun on this preparation. However, it was felt to be of interest to attempt to throw some light on the "paradox" phenomenon in this preparation; therefore, many muscles were studied. Sampling of muscle fibres was made as thoroughly as possible

by exposing the muscle in two ways: by removing the antagonistic opener muscle, and by clipping away part of the shell on the opposite side of the muscle. Microelectrodes were introduced as deeply as possible into the muscle on many occasions.

"Fast Responses: The electrical response of most of the muscle fibres to a single shock applied to the "fast" axon was a p.s.p. which varied in size from 2 to about 20mV in different muscle fibres (Fig. 39, a,b,c,d). No fibres giving spikes to single stimuli were seen. However, when electrical responses of the muscle were recorded with external electrodes, the response often had an irregular or notched appearance (Fig. 39,f), indicating that some fibres in the muscle were giving electrically excitable responses.

When two shocks were delivered to the nerve in quick succession, the summated response often showed a small additional electrically excitable response (Fig. 39,e). No large spikes were encountered in the fibres examined. Hoyle and Wiersma observed 20 to 30mV spikes in the closer of Cambarus when similar stimulation was given, and it is quite likely that many fibres in the closer of Astacus also give spikes in response to this stimulation, for external recordings showed additional humps and notches in the electrical response.

The mechanical response to a single stimulus was a strong twitch contraction (Fig. 39 d,f). The contraction

frequently showed notches and other irregularities (Fig. 39, d). When two closely spaced stimuli were delivered to the "fast" axon, a much stronger twitch resulted, which was also frequently characterized by humps and notches (Fig. 39,e).

When stimulation was delivered at a low frequency (up to 10 per sec.), the mechanical response showed a rapid decline in magnitude (Fig. 30). Within a few seconds, the response could decline to half its original value, or even less.

Electrical responses recorded from fibres near the surface of the muscle typically showed facilitation during this type of stimulation (Fig. 40,b,c). However, when electrodes were introduced more deeply into the muscle, the electrical responses often showed decline in magnitude along with the mechanical response (Fig. 40,d). It is quite likely that this latter behaviour is actually more typical of the muscle as a whole, although the difficulty of recording from muscle fibres other than those at the surface of the muscle may give a superficially different impression.

"Slow" responses: Single shocks to the "slow" axon produced electrical responses in the form of very small p.s.ps. in many muscle fibres (Fig. 41,a,c); in other fibres, no visible response was recorded. When double shocks were applied at low frequencies (one or two per sec.), the response showed facilitation and grew to a much larger size during successive

stimuli (Fig. 41,b). The response shown in Fig. 41 (b) showed an apparent secondary response.

As the frequency of stimulation applied to the "slow" axon was increased, facilitation of the electrical responses occurred, and depolarization "plateaus" were built up (Fig. 41,d,e,f).

Some of the muscle fibres of the crayfish claw showed responses rather different from those shown in Fig. 41. Single shocks to the "slow" axon produced no visible responses in these muscle fibres. At frequencies of 5 to 15 per sec., depolarization "plateaus" could be seen in these muscle fibres (Fig. 42), but the "slow" p.s.ps. were still extremely small and in many cases not visible. At higher frequencies of stimulation (20 to 80 per sec.), larger "plateaus" were built up; however, individual p.s.ps. were still very small, usually less than 0.5mV (Fig. 42,c). The depolarization at higher frequencies of stimulation had a more rapid rise and initial decay than that at lower frequencies of stimulation, but was not much greater in magnitude (Fig. 42,b).

The mechanical response to "slow" axon stimulation at frequencies of 5 per sec. and greater was a slowly rising contraction (Fig. 41,e,f). This contraction was not "perfectly smooth," however; small irregularities could be observed during the rising phase (Fig. 41,e). When higher amplification of tension was used, it was found that the "slow" contraction

was in fact made up of small, slow "twitches" (Fig. 43,b,c). These twitches had a slower rise and decay than "fast" twitches of the same muscle (Fig. 43,a), and they were, of course, much smaller. In most of the muscle examined, the "slow" twitches could be seen even in response to single shocks applied to the nerve (Fig. 43,d,e). At frequencies of stimulation between 0.5 and 1 per sec., the "slow" twitches showed marked facilitation (Fig. 43,d), although summation did not occur until frequencies of about 1.5 per sec. were employed.

Two shocks applied closely together in time produced a much larger "slow" twitch (Fig. 43,f).

"Slow" twitches were also observed in Carcinus, but only in a few animals. In the claw closer of Astacus they were seen in almost every preparation in which they were looked for. These animals were examined during late fall and early winter. It is not known whether muscles from animals examined during spring and summer would show the same responses. Influences of season, hormones, and thermal history may all modify the responses of a given muscle.

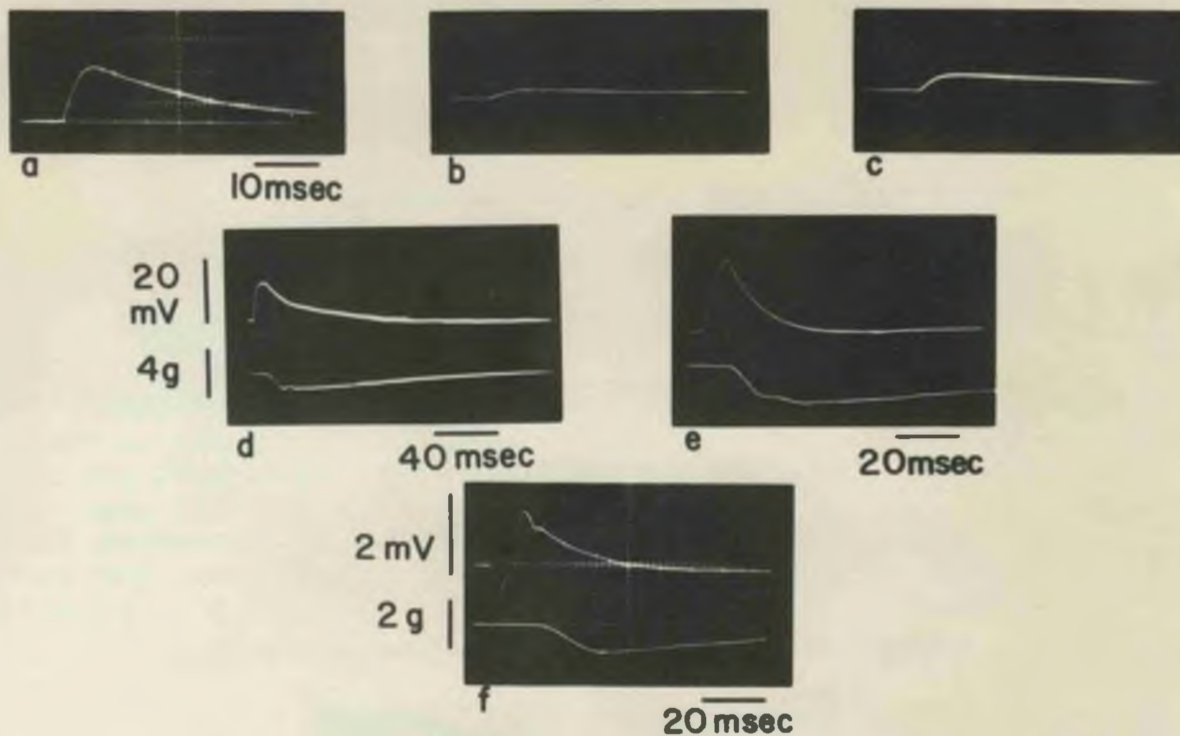


Fig. 39. "Fast" responses of the closer of the claw of Astacus. (a,b,c) P.s.ps. in three different muscle fibres responding to single shocks applied to the "fast" axon. (d,e) Response to a single shock (d) and to a double shock. (e) in the same muscle fibre. (f) Externally recorded response of the whole muscle to a single shock. (Lower traces in d,e,f, tension of the whole muscle.)

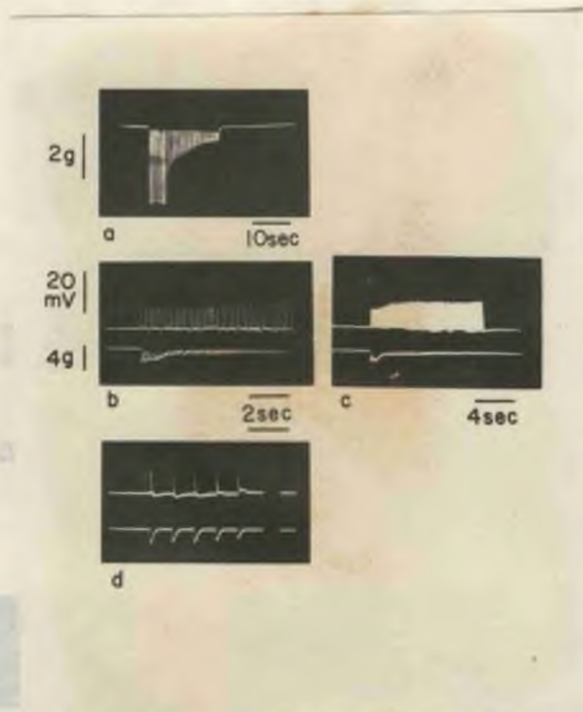


Fig. 40. Astacus: Responses of the claw closer to "fast" axon stimulation. (a) Mechanical response of a muscle to stimulation at 3 per sec. (b,c) Electrical and mechanical responses from other preparations during stimulation at 5 per sec. (b) and 8 per sec. (c). (d) Electrical and mechanical responses of another preparation during stimulation at one per sec. (Lower traces in b,c,d, tension of the whole muscle.).

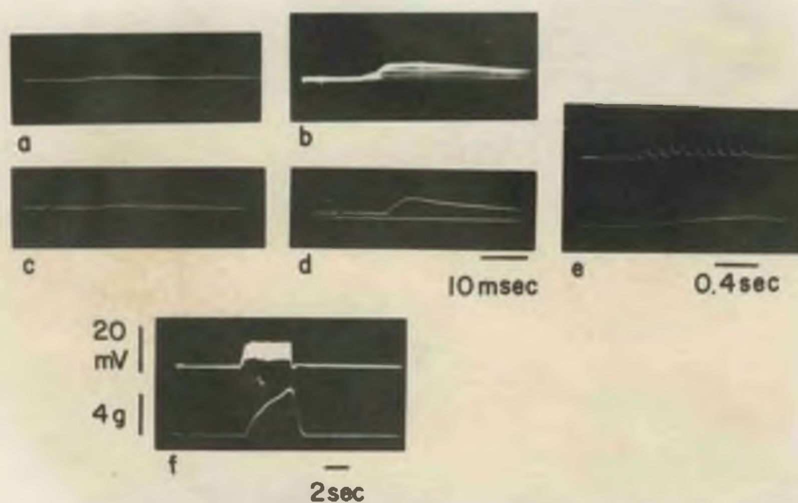


Fig. 41. "Slow" responses of the closer of the crayfish claw. (a,b) Electrical response of a muscle fibre to stimulation at 2 per sec., with single shocks (a) and with double shocks (b). (c,d,e) Responses of a muscle fibre from another preparation to stimulation at 1 per sec. (c), and 10 per sec. (d,e). (f) Responses of another preparation during stimulation at 20 per sec. (Lower traces in e,f, tension of the whole muscle).

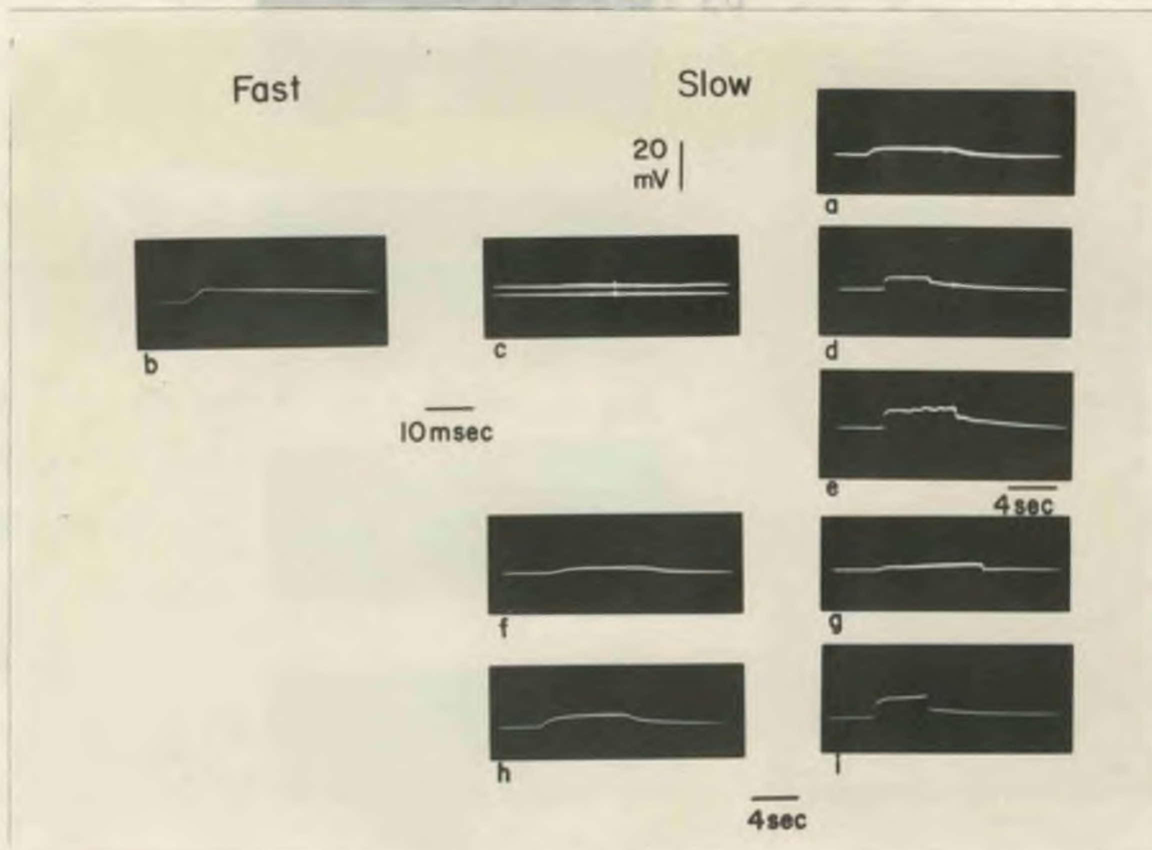
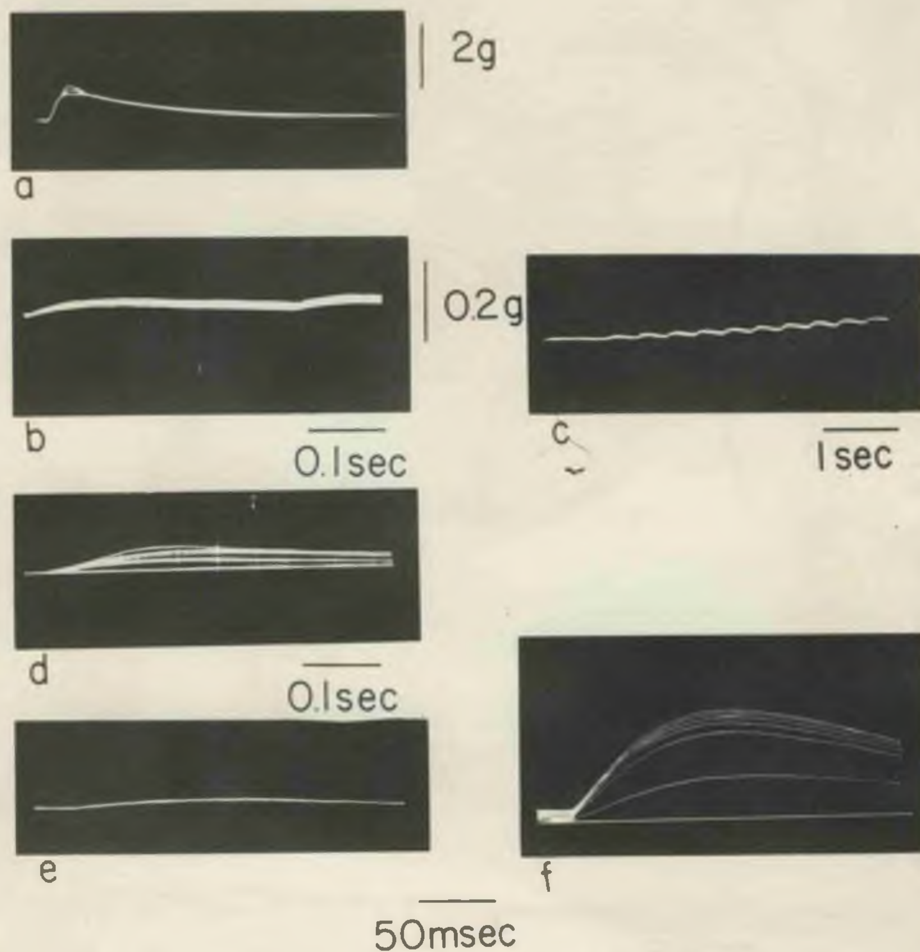


Fig. 42. Electrical responses of the closer of the crayfish claw. (a) Response of a muscle fibre to stimulation of the "slow" axon at 15 per sec. (b) Response of the same muscle fibre to a single shock applied to the "fast" axon. (c,d,e) Responses of the same muscle fibre to stimulation of the "slow" axon at 40 per sec. (c,d) and 60 per sec. (e). (f,g) Responses of another muscle fibre during stimulation of the "slow" axon at 15 per sec. (f) and 40 per sec. (g). (h,i) Similar responses from another muscle fibre.



<u>Fig. 43</u>. Mechanical responses of the closer muscle of the crayfish claw. (a) "Fast" twitches in response to stimulation at 1.5 per sec. (b,c) "Slow" responses of the same preparation in response to stimulation at 2.5 per sec. (d) Response of another preparation during stimulation at 1 per sec. (e) Response of another preparation to a single shock applied to the "slow" axon. (f) Responses of the same preparation to double shocks (2.5 msec. separation) delivered to the "slow" axon at 2 per sec.

vi) Astacus: Opener of the Claw

Microelectrode techniques have been used to investigate the electrical responses of crayfish claw opener muscles by Hoyle and Wiersma (1958a) and Boistel and Fatt (1958). The former authors reported uniform responses from all muscle fibres. The responses were small (0.5mV) p.s.ps. at 12 per sec. stimulation, and 1 to 4mV p.s.ps. at 30 per sec. stimulation, at which frequency depolarization "plateaus" were evident in some fibres. Contraction started at a stimulation frequency of about 12 per sec.

In the present study similar results were found. Single shocks to the excitor axon produced very small p.s.ps. (up to 0.5mV) in some fibres, nothing in others (Fig. 44). Growth of p.s.ps. occurred with increasing frequency of stimulation, or during a train of stimuli at frequencies greater than about 2 per sec. Depolarization "plateaus" were built up during stimulation at frequencies of 15 to 20 per sec.

Contraction was observed to occur at frequencies of excitation of 3 to 5 per sec. At 15 per sec. the contraction was quite strong (Fig. 44,d). The contraction of the opener muscle relaxed more slowly than the "slow" contraction of the closer muscle at similar frequencies of stimulation. However, decay rates of the p.s.ps. in the opener muscle were no slower than the decay rates of "slow" p.s.ps. in the closer.

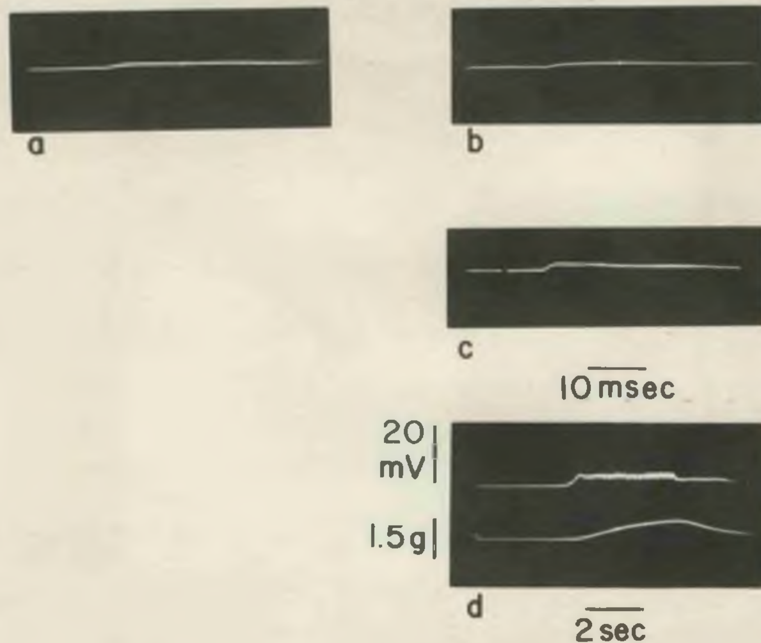


Fig. 44. Astacus: Responses of the opener muscle of the claw. (a,b) Responses to stimulation of the excitor axon at 2 per sec. (c) Response to stimulation of the excitor axon at 10 per sec. (d) Responses to stimulation at 15 per sec. (Lower trace, tension of the muscle).

vii) Astacus: Walking Leg Closer

The disadvantages inherent in the large size of the closer muscle of the crayfish claw prompted the study of the much smaller homologous muscles of the walking legs, which have not previousl been investigated. The muscle of the first walking leg was found to be the most convenient to prepare, and observations were confined to it. Perfusion was effected through the index of the propodite. Preparation of isolated "fast" and "slow" axons was more difficult than for the claw closer; often one of the axons did not function after isolation. However, numerous observations were made on both "fast" and "slow" systems.

"Fast" responses: Exploration of different fibres with a recording microelectrode revealed a variety of responses to a single shock to the "fast" axon. In some fibres the response was a small p.s.p. (Figs. 45,b, 46,b). In others, large ~~p.s.ps. (up to almost 20mV) were observed (Fig. 45,a,c; Fig.~~ Fig. 46, a), while in many fibres large spikes were recorded (Fig. 45,d,e,f). The spiking fibres were usually found deep in the muscle, in the proximal half of it.

Usually a distinct "step" could be seen in the rising phase of the spike, an indication of the height of the p.s.p. (Fatt and Katz), 1951; del Castillo, Hoyle, and Machne, 1953). A notch could often be seen on the falling phase of the spike

(Fig. 45,e). In many cases the falling phase of the spike was obscured by artefacts (Fig. 45,d).

Spike size varied from fibre to fibre. The spikes could be as large as 100mV, but averaged 65mV. If a hyperpolarizing effect of spikes from nearby fibres is assumed, the spikes may be larger by about 5 to 10mV, and in some cases overshoot the zero base level of membrane potential. (Resting potentials in these fibres averaged 77mV.)

When two shocks were applied close together in time to the "fast" axon, some of the non-spiking fibres gave rise to large spikes (Fig. 46,f), whereas others gave only a facilitated p.s.p. (Fig. 46,e). The latter sometimes showed a small electrically excitable response.

The mechanical response of the muscle to a single shock applied to the "fast" axon was a brisk twitch. The size of the response varied greatly in different muscles (Fig. 45, a,b,c,f; Fig. 46,a,b), but it was usually considerably more powerful than the twitch of the larger _Nephrops_ walking leg closer.

When the "fast" axon was excited at frequencies of 2 to 10 per sec., the mechanical response showed a small degree of facilitation, as did the electrical responses of many of the non-spiking fibres (Fig. 46,c,d). There was no rapid decrease in magnitude of the mechanical response comparable to that observed regularly in the crayfish claw.

"Slow" responses: Electrical responses to "slow" axon stimulation were present in almost all the muscle fibres examined. At frequencies of stimulation below 10 per sec. the responses were very small, often not visible. As the frequency of stimulation was increased, facilitation occurred, and depolarization "plateaus" were built up in most fibres at frequencies of stimulation greater than 20 per sec. (Fig. 47).

The "slow" mechanical response was detectable at frequencies of stimulation as low as 5 per sec. The response was a smooth tetanus, without observable twitches. It was quite powerful at 20 per sec. (Fig. 47,c), although electrical responses in the fibres examined were very small at this frequency. Again, this situation apparently contrasts with that in the *Nephrops* walking leg closer, in which the mechanical response was usually not detectable at frequencies of stimulation lower than 30 per sec., and in which the electrical responses of some of the muscle fibres were very much larger than those described here.

In general, the electrical responses observed in the closer muscle during "slow" axon stimulation were similar to those seen in the more fully studied opener of the walking leg, which is described below.

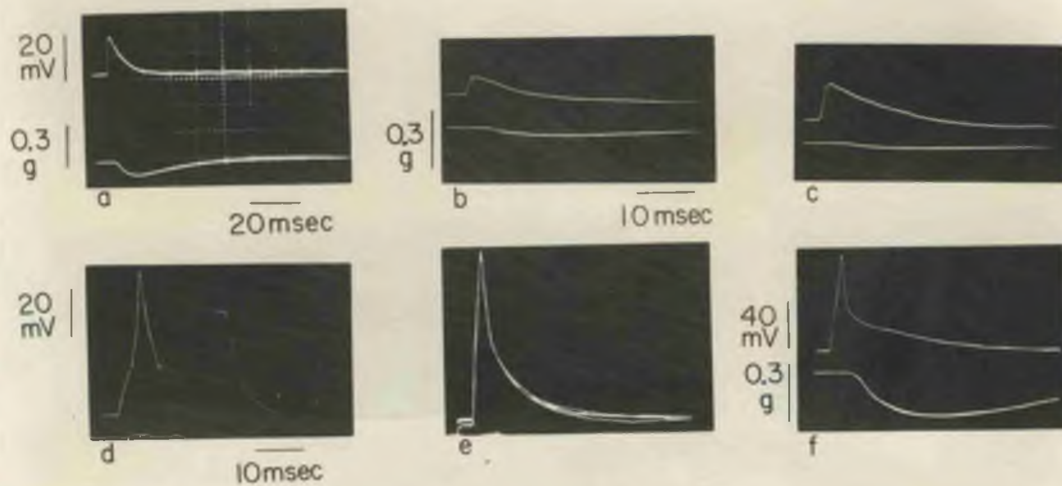


Fig. 45. Astacus: "Fast" responses in the walking leg closer. Records from different preparations showing responses to a single shock applied to the "fast" axon. (Lower traces, tension of the whole muscle.).

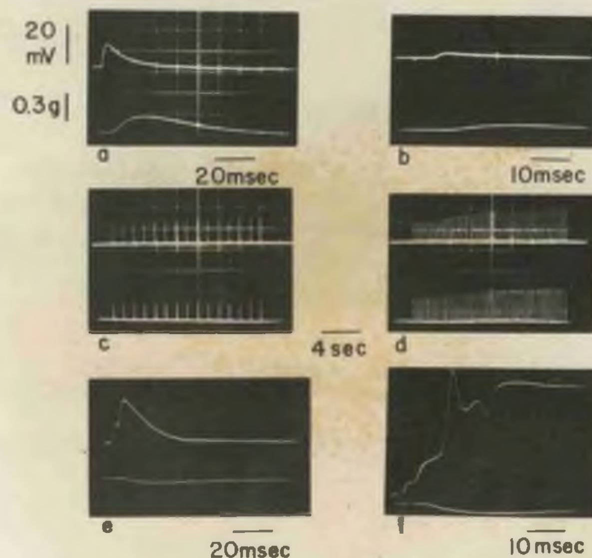


Fig. 46. Astacus: "Fast" responses in the walking leg closer. (a,b) Responses of two preparations to single shocks applied to the "fast" axon. (c,d) Responses of two preparations to excitation at 2 per sec. (c) and 6 per sec. (d). (e,f) Responses of two fibres to excitation by a double shock (3 msec. separation between stimuli). Lower traces show mechanical activity of the muscle.

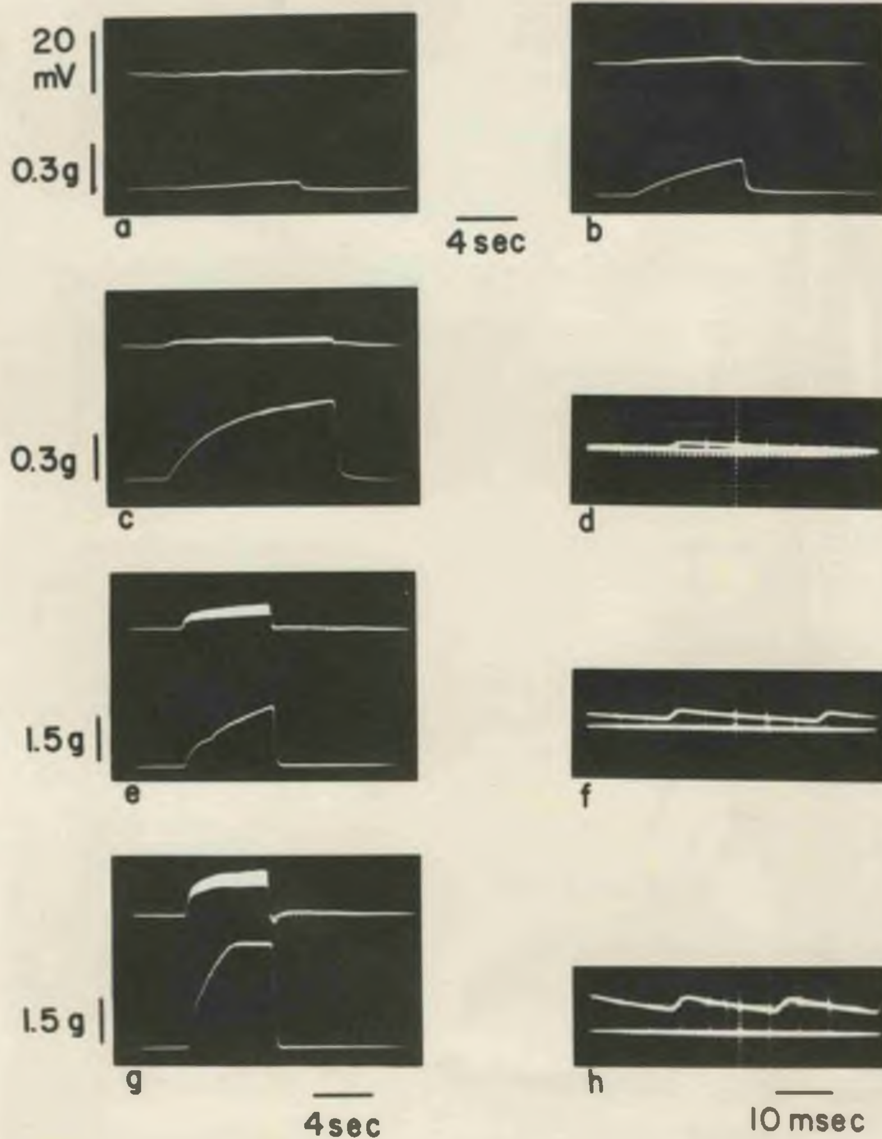


Fig. 47. Astacus: "Slow" responses of the walking leg closer. Records were made from a single muscle fibre during excitation at 8 per sec. (a), 13 per sec. (b), 20 per sec. (c,d), 40 per sec. (e,f), and 60 per sec. (g,h). Lower traces in a,b,c,e,g, tension of the whole muscle. In (g) the mechanical limit of the transducer was reached.

viii) Astacus: Walking Leg Opener

The use of the abductor muscle of the crayfish walking leg (walking leg opener) by Dudel and Kuffler (1961) in the study of crustacean transmitter release at nerve endings, suggested its use in the study of excitation and contraction. The great advantage of the muscle is its small size. A single drop of saline completely covers the muscle. There is no need to perfuse it, because it is well exposed to the saline by removal of the antagonistic closer muscle.

A single excitor axon supplies the motor innervation of this muscle. A bundle of nerve fibres containing this axon could readily be found in the walking leg nerve; however, care was necessary to avoid including the inhibitor axon in this bundle. The excitor axon also supplies innervation to the stretcher muscle. It was therefore necessary to cut the tendon of the latter muscle in order to minimize artefacts in the mechanical record caused by contraction of this muscle. Mechanical activity was recorded isometically at the tip of the dactyl, with the muscle stretched slightly past resting length.

The muscle is made up of a flat sheet of muscle fibres attached between the sides of the propodite and a central tendon. The sheet is about 3 muscle fibres thick, and contains an estimated total of 30 to 60 muscle fibres. A sampling of

responses of different muscle fibres in this muscle probably gives a better indication of events in the muscle as a whole than in any of the other muscles studied, because the sample can include a larger percentage of all the fibres.

Electrical responses: At frequencies of stimulation below 5 per sec. electrical responses in all fibres examined were less than 1mV. As the frequency of stimulation was increased above this value, facilitation of the responses occurred; at 15 per sec. total depolarizations of 2 to 7mV were observed (Fig. 48,a,e; Fig. 49,a,b). As the frequency of excitation was increased still further, depolarization "plateaus" were built up in most fibres (Figs. 49,50).

There was some variation among different fibres of the same muscle, and among different preparations. For example, the electrical response of the fibre of Fig. 49 (e) was larger than that of Fig. 49 (g), although the frequency of excitation was 30 per sec. in the former case and 50 per sec. in the latter case. Similarly, the response of Fig. 49(f) is larger than that of Fig. 49 (g) although the frequency of excitation was 50 per sec. in both cases. However, the variation encountered was not nearly as great as that found in Carcinus and Nephrops closer muscles; all the fibres appeared to have the same general properties, as judged by responses to indirect stimulation.

A representation of the variation in depolarization in

different muscle fibres at various frequencies of stimulation is given in Fig. 51.

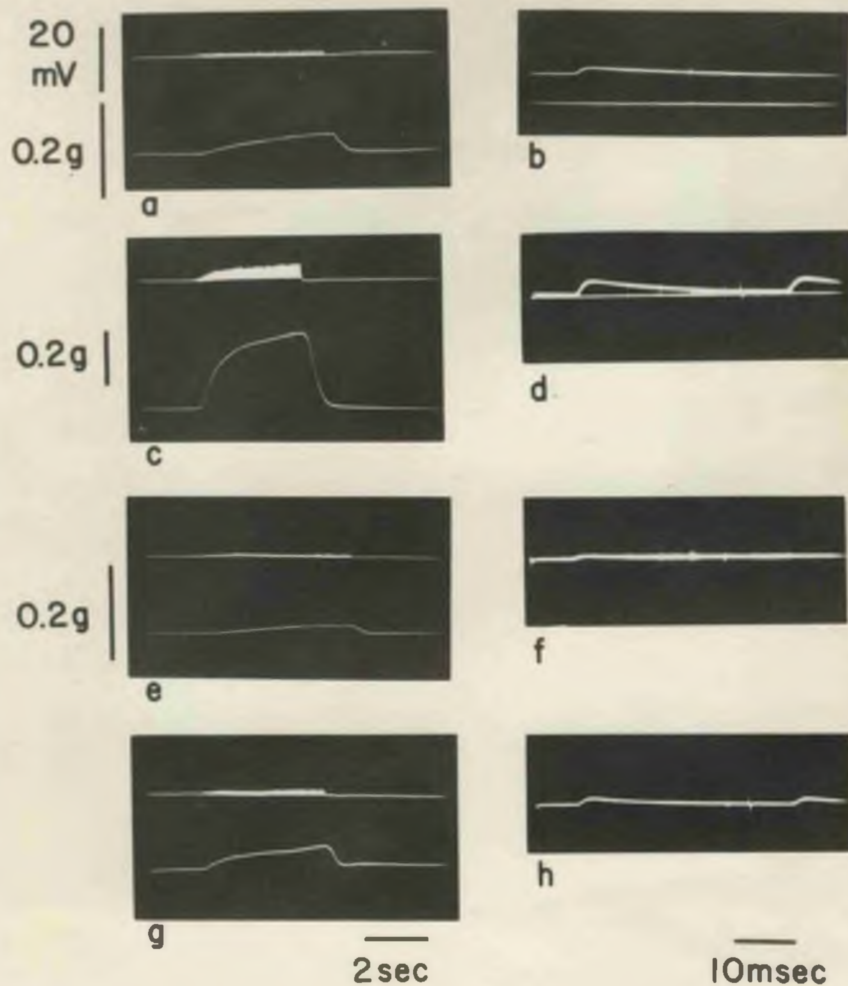
Mechanical responses: The mechanical response of the muscle was detectable at frequencies of stimulation of 5 to 10 per sec. It was a smooth slowly developing tetanus. As the frequency of excitation was increased, the rate at which the contraction developed, and its total magnitude, both increased (Figs. 48, 49, 50). At frequencies of stimulation of 70 per sec. and above the response occasionally had small irregularities superimposed on it. These were not as conspicuous as those observed in connection with the "slow" contraction of the *Nephrops* closer muscle, but may have been an indication of electrically excitable responses.

The contraction relaxed slightly more gradually than did the "slow" contraction of the walking leg closer, although no significant differences in the decay rates of the p.s.ps. in the two muscles was noted. The rate of relaxation was slower at low frequencies of excitation, although the total relaxation time remained about the same at all frequencies of stimulation.

Mechanical responses varied from muscle to muscle. Part of the variation was attributable to muscle size, but in some cases muscles of the same size from different animals gave different tension responses. The physiological state of the animal may have played a part in producing this variation.

The variation of muscle tension with average observed depolarization of the muscle fibres is shown in Fig. 52. The curve rises steeply at first, then levels off, and finally rises very steeply as the frequency of stimulation is increased above 50 per sec. However, there was considerable variation in tension in different muscles.

The most significant generalization which emerged from observations on this muscle was that appreciable contraction occurred at frequencies of stimulation of 5 to 20 per sec., when the electrical responses seen were small (2 to 7mV). This observation is in agreement with those of Hoyle and Wiersma (1958a) on several other crustacean muscles.



<u>Fig. 48</u>. <u>Astacus</u>: Responses of the walking leg opener. (a to d) Responses of a preparation to stimulation of the motor axon at a frequency of 15 per sec. (a,b) and 30 per sec. (c,d). (e to h) Responses of another preparation to stimulation of the motor axon at a frequency of 15 per sec. (e,f) and 22 per sec. (g,h). Lower traces in (a,c,e,g), mechanical responses of the whole muscle.

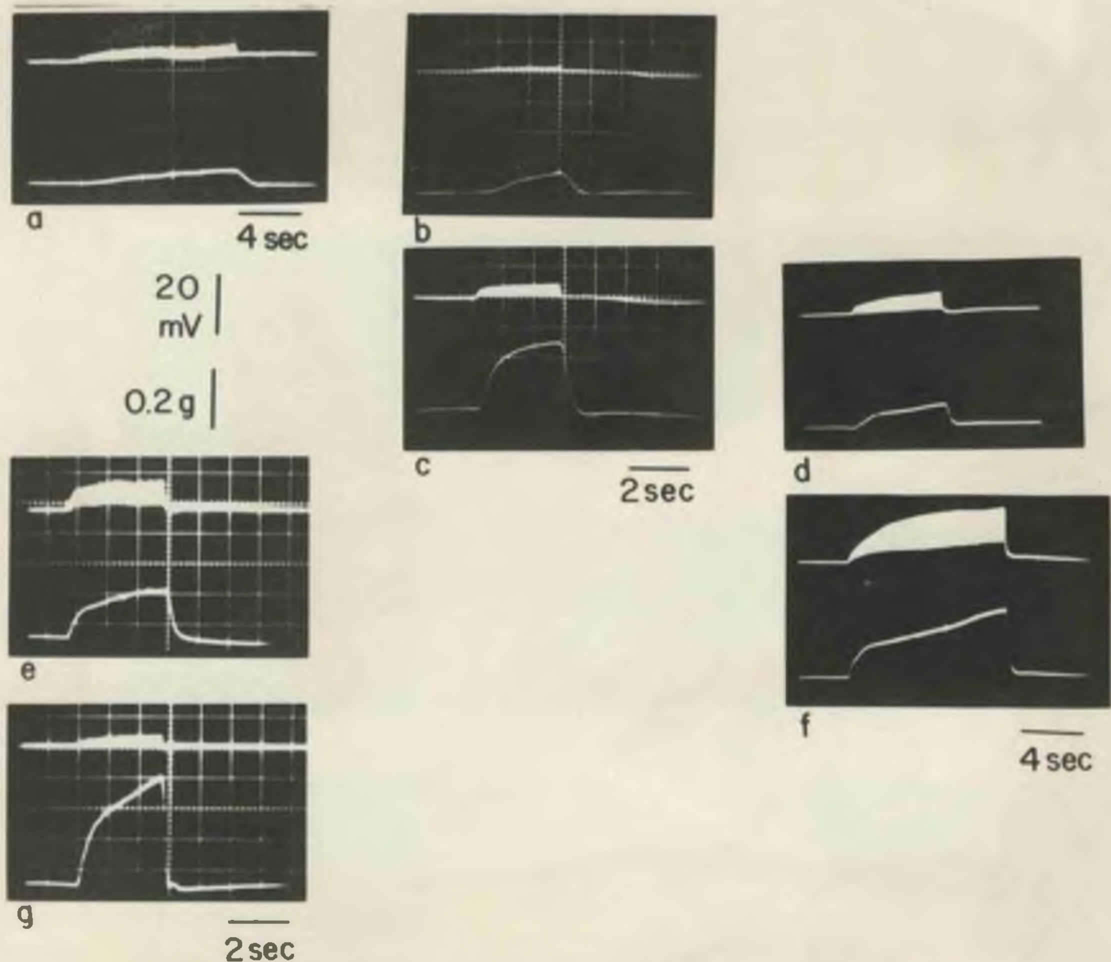


Fig. 49. Astacus: Representative responses of the walking leg opener. (a) Responses of a preparation during excitation at 15 per sec. (b,c) Responses of another preparation during excitation at 15 per sec. (b) and at 30 per sec. (c). (d,f) Responses of another preparation during excitation at per sec. (d) and 50 per sec. (f). (e) Responses of another preparation during excitation at 30 per sec. (g) Responses of another fibre of the same preparation during excitation at 50 per sec. Lower traces, mechanical activity of the whole muscle.

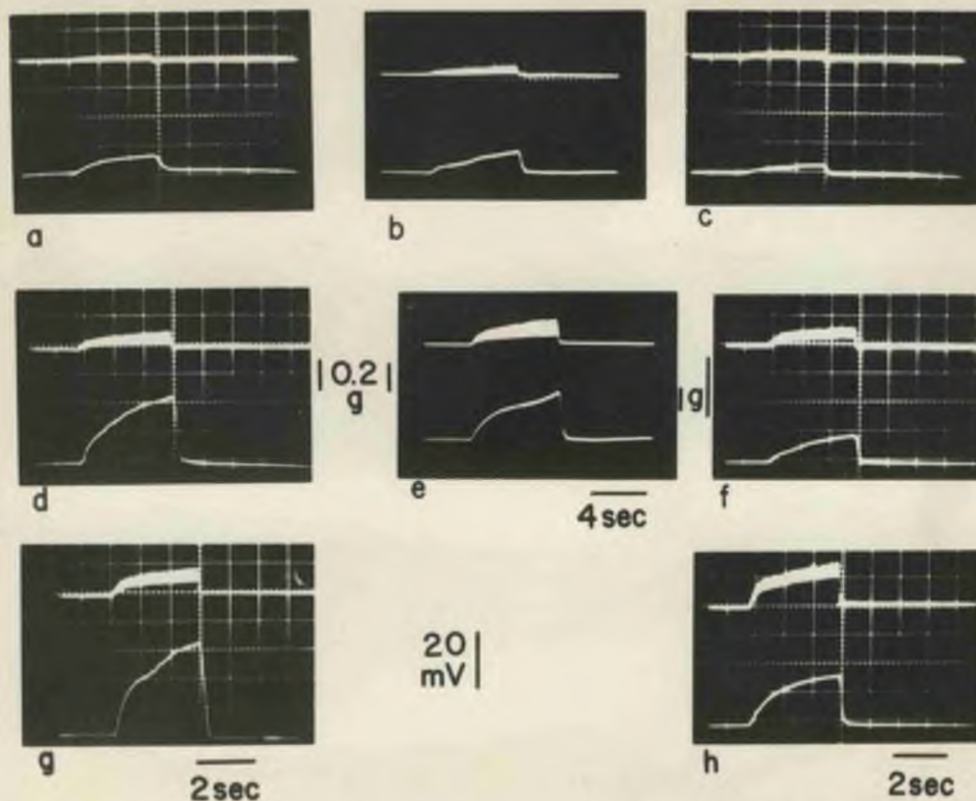


Fig. 50. Astacus: Representative responses of the walking leg opener. Records in vertical columns were recorded from the same preparation and muscle fibre. Frequencies of stimulation were: (a,b,c) 30 per sec., (d) 60 per sec., (g) 75 per sec., (h) 100 per sec. Lower traces, mechanical activity of the whole muscle.

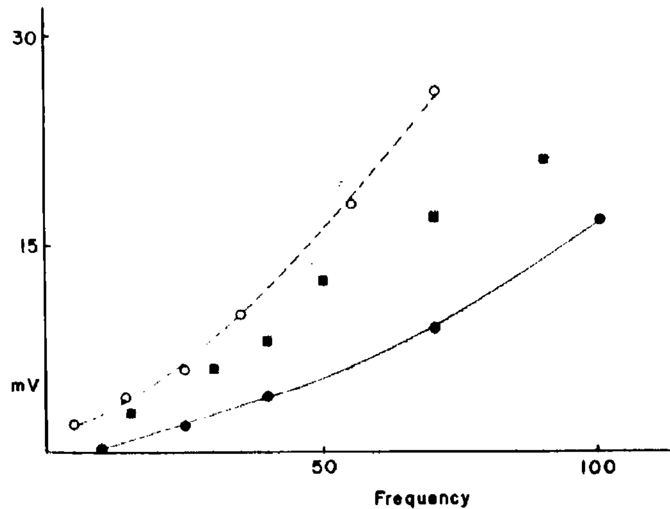


Fig. 51. *Astacus*,walking leg opener: Depolarization of muscle fibres at various frequencies of stimulation. Depolarization was measured from the base line (resting membrane potential) to the tops of the p.s.ps. Squares represent average values from twenty muscle fibres in seven preparations. Open and closed circles represent values from two individual muscle fibres close to the extreme limits of the observations. The lowest frequency used was 5 per sec.

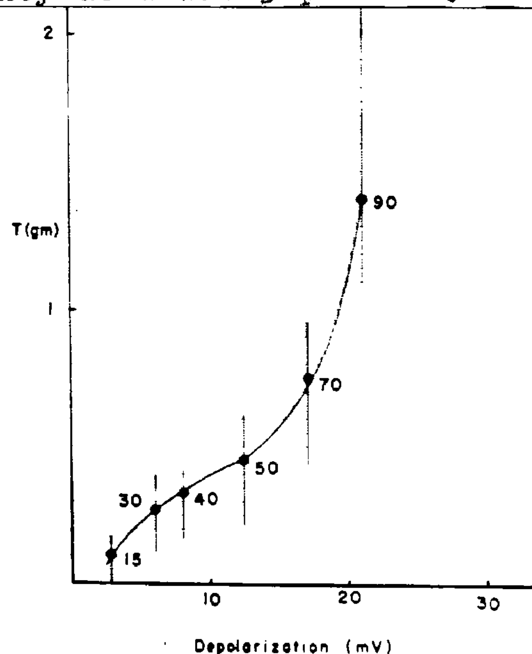


Fig. 52. *Astacus*: Walking leg opener: Average tension developed at various frequencies of stimulation (numbers by points), plotted against average observed depolarization (taken from Fig. 51). Circles represent average tension measurements from seven preparations, in which the muscles were approximately equal in size. Vertical lines indicate the range of values encountered.

ix) <u>Cancer: Stretcher Muscle</u>

This muscle was used in the preparation of single muscle fibres in later experiments, and it was of interest to obtain an idea of electrical and mechanical responses of the whole muscle. The muscle has not been studied previously.

The surface of the muscle was usually exposed by removing part of the overlying shell of the carpopodite near the attachment of the tendon of the stretcher muscle to the propodite. A large number of muscle fibres could then be sampled, although many muscle fibres remained inaccessible. However, no outstanding differences were observed in muscle fibre samples made in preparations in which the antagonistic bender muscle was removed to expose the inner surface of the stretcher muscle.

A single motor axon innervates the stretcher muscle (and also the opener). This axon could readily be isolated from the walking leg nerve in the meropodite (although great care was necessary to separate it from the closely associated inhibitor axons). Mechanical recording was made under isometric conditions at the distal end of the propodite, with the muscle stretched slightly past resting length.

<u>Electrical responses</u>: Many of the muscle fibres examined gave very small (up to 0.5mV) responses to single shocks. In others no response was detectable.

As the frequency of stimulation was increased, marked facilitation occurred. Most of the muscle fibres showed p.s.ps. of 5 to 10mV when the frequency of stimulation was 20 per sec. (Fig. 53, d,e,f,g). Small depolarization "plateaus" were built up in many of these fibres at this frequency (Fig. 53,d).

A smaller number of fibres, which were located only at the outer edges of the muscle, gave very much larger responses than those described above. Responses from two of these "edge fibres" are shown in Fig. 53 (a,b,c) and in Fig. 54. In these fibres, excitation at a frequency of 5 per sec. gave p.s.ps. which, after facilitation, were 10 to 15mV in magnitude. At slightly higher frequencies (5 to 10 per sec.) the responses became still larger, and depolarization "plateaus" were built up (Fig. 53,b,c; Fig. 54,b). At stimulation frequencies greater than 10 per sec. increasingly larger "plateaus" were established (Fig. 54,d,e).

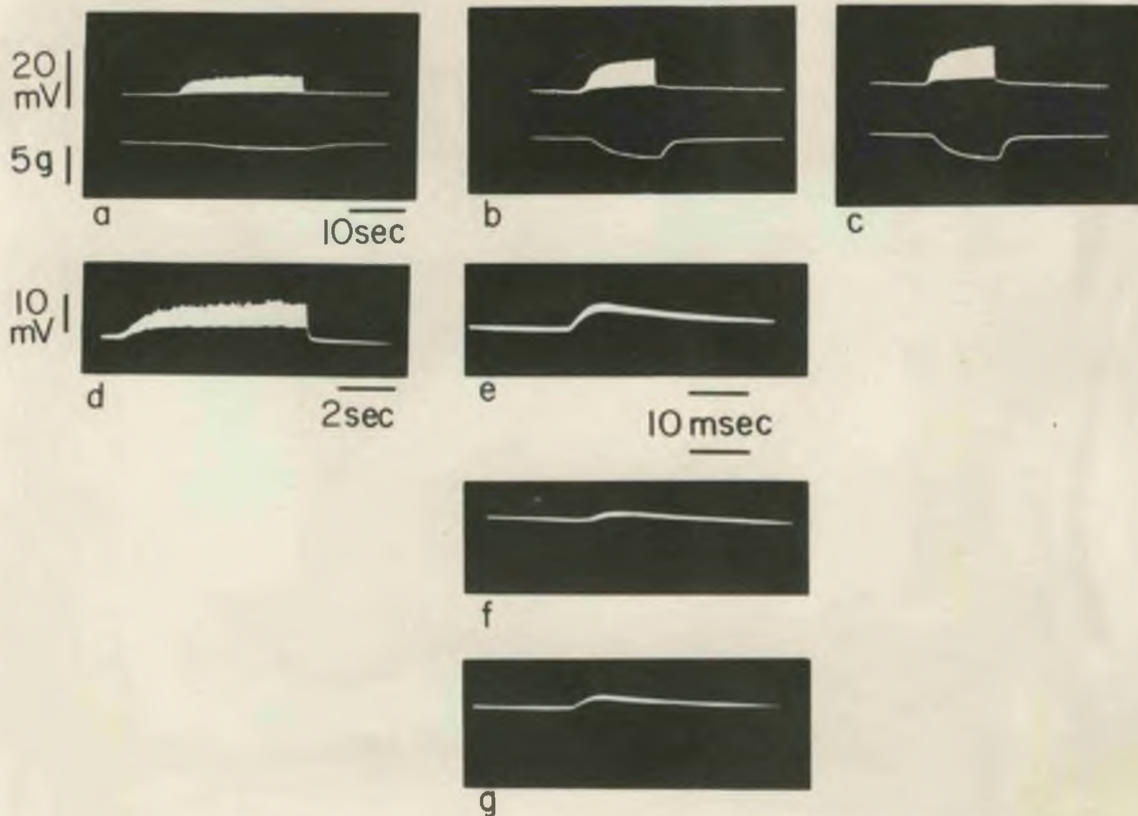
The decay rates of the p.s.ps. observed were always slow. Time constants of decay of 30 to 80 msec. were observed for different muscle fibres. The slow decay rates of the p.s.ps. may largely account for the establishment of depolarization "plateaus" at low frequencies of stimulation in many muscle fibres.

The situation described for this muscle, in which some muscle fibres give large electrical responses at low

frequencies of excitation, contrasts with that apparently existing in the crayfish claw and walking leg openers. None of the single - motor - axon innervated muscles described by Hoyle and Wiersma (1958a) had responses of this nature.

Mechanical responses: Contraction occurred at frequencies of excitation of about 4 per sec. As the frequency of excitation was increased above 5 per sec., the rate of rise and magnitude of the contraction increased markedly (Fig. 53,a,b,c). So also did the rate of relaxation. The total time required for relaxation appeared to be slightly greater at lower frequencies of excitation.

The mechanical response was always a smooth tetanus, without discernable twitches. However, the response was not studied critically at frequencies of stimulation greater than 30 per sec.



<u>Fig. 53.</u> <u>Cancer</u>, stretcher muscle: Electrical and mechanical responses. (a to c) Responses from a muscle fibre during stimulation at 5 per sec. (a), 7.5 per sec. (b), and 10 per sec. (c). (d,e) Responses from another muscle fibre during stimulation at 20 per sec. (f,g) Responses from two other muscle fibres during stimulation at 20 per sec. Lower traces in (a,b,c), tension of the whole muscle.

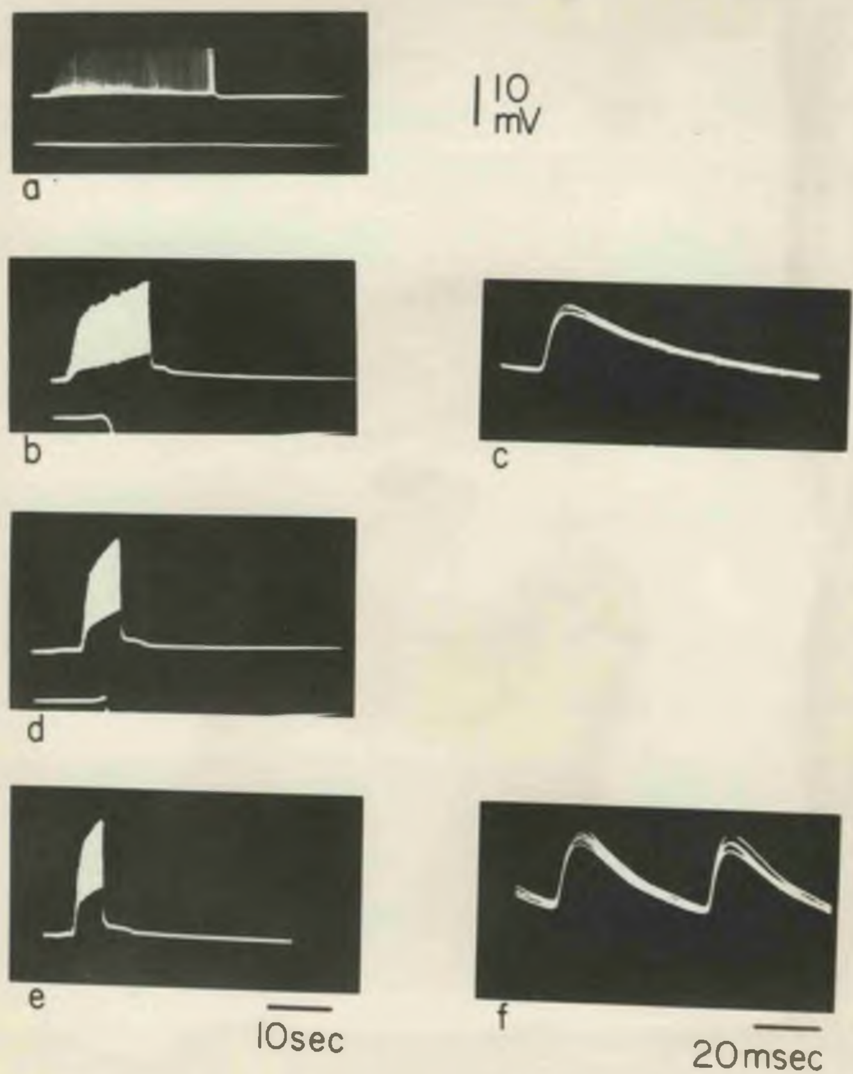


Fig. 54. Cancer, stretcher muscle. Responses from a single muscle fibre during stimulation at 5 per sec. (a), 10 per sec. (b,c), 15 per sec. (d), and 20 per sec. (e,f).

x) Pachygrapsus: Closer Muscle

The closer muscle of *Pachygrapsus* has been investigated by Furshpan (1955) and by Hoyle and Wiersma (1958a) with the aid of microelectrode techniques. The latter authors found that different muscle fibres could give very different electrical responses to stimulation of the same motor axon. Three types of muscle fibre were distinguished by them: (1) "fast" muscle fibres responding only to the fast axon and giving very large, relatively prolonged, junctional potentials;" and (3) "'general' muscle fibres innervated by both fast and slow axons, but giving only small electrical responses to slow axon stimulation and varying responses to the fast axon, each with a similar time course."*

This description of the events in the *Pachygrapsus* closer muscle coincides in many respects with that given for the *Carcinus* closer muscle in the present study. In both muscles, several distinct types of muscle fibre are present, as judged by responses to indirect stimulation. It was of interest, in the light of findings made in *Carcinus*, to re-examine the Pachygrapsus closer muscle in order to further assess similarities and differences between the muscles of the two species.

Electrical responses: Electrical recording of "fast" responses confirmed the description of Hoyle and Wiersma (1958a).

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*Parentheses indicate direct quotes from Hoyle and Wiersma (1958a).

Many fibres in the proximal part of the muscle gave large p.s.ps. in response to single stimuli applied to the "fast" axon, and a number gave spikes (Fig. 55). Elsewhere in the muscle, smaller and more slowly decaying "fast" p.s.ps. were recorded.

In many muscle fibres showing only p.s.ps. in response to a single shock, spikes could be elicited by applying closely spaced shocks (Fig. 56,c,d).

It was observed that in spiking fibres, the spike disappeared after a period of continued stimulation, leaving an abortive electrically excitable response (Fig. 56,a,b).

Responses to "slow" axon stimulation were also similar to those described by Hoyle and Wiersma. In fibres giving large "fast" responses, the "slow" responses were usually small or absent (Fig. 55,d). However, other fibres showing small, or no, "fast" responses showed very large "slow" responses, even in response to a single shock (Fig. 55,g).

Although many of the fibres showing large "slow" responses showed very small "fast" responses, in other fibres of this type quite a large "fast" response could be observed; sometimes the "fast" response to a single shock was larger than the comparable "slow" response (Fig. 57, 58). The "fast" and "slow" responses in fibres of this type always had similar decay rates. In Fig. 57, the "fast" response (Fig. 57,e) has a time constant of decay of about 50 msec.,

as does the "slow" response (Fig. 57,c). Similar time constants of decay were present in the "fast" and "slow" responses of Fig. 58(a,b).

Whereas "fast" responses in these fibres (if present) showed little facilitation on repeated stimulation, the "slow" responses usually grew to at least twice, sometimes several times, their initial size during a train of excitation (Figs. 57, 58). As the frequency of stimulation was increased to 10 per sec., depolarization "plateaus" were built up. At higher frequencies the "plateaus" were larger in size, but the p.s.ps. were not. At frequencies above 30 per sec. the p.s.ps. usually became smaller in size, as in Carcinus Type B muscle fibres (Fig. 58,i).

It is apparent that muscle fibres corresponding in many respects to Carcinus Type A and Type B muscle fibres are present in the closer of Pachygrapsus. The "general" muscle fibres of Hoyle and Wiersma are also similar in many respects to the Type C muscle fibres in Carcinus, except that the "slow" responses of these fibres are usually small even at high frequencies of stimulation.

Mechanical responses: The mechanical response of the muscle to a single shock applied to the "fast" axon was a brisk twitch of 1 to 5 gms. (measured isometrically at the tip of the dactyl). This response became smaller with repeated stimulation of the preparation, but as long as the frequency

of applied stimulation was less than 5 per sec., the response would remain for a long time.

The "slow" response was measurable at frequencies of stimulation of 5 to 10 per sec. (Fig. 57, 58). The rates of rise and decay of the response were extremely slow, although both rates increased with increasing frequency of stimulation. The strength of the response did not increase very rapidly with increasing frequency of stimulation above 20 per sec. (Fig. 59). This contrasts with the situation in the *Carcinus* closer.

There was a correlation between the curve relating tension to frequency of stimulation and that relating depolarization of the "Type B" fibres to frequency of stimulation (Fig. 59). Both curves showed a levelling off of rate of increase in magnitude with frequency of stimulation at frequencies of stimulation above 20 per sec.

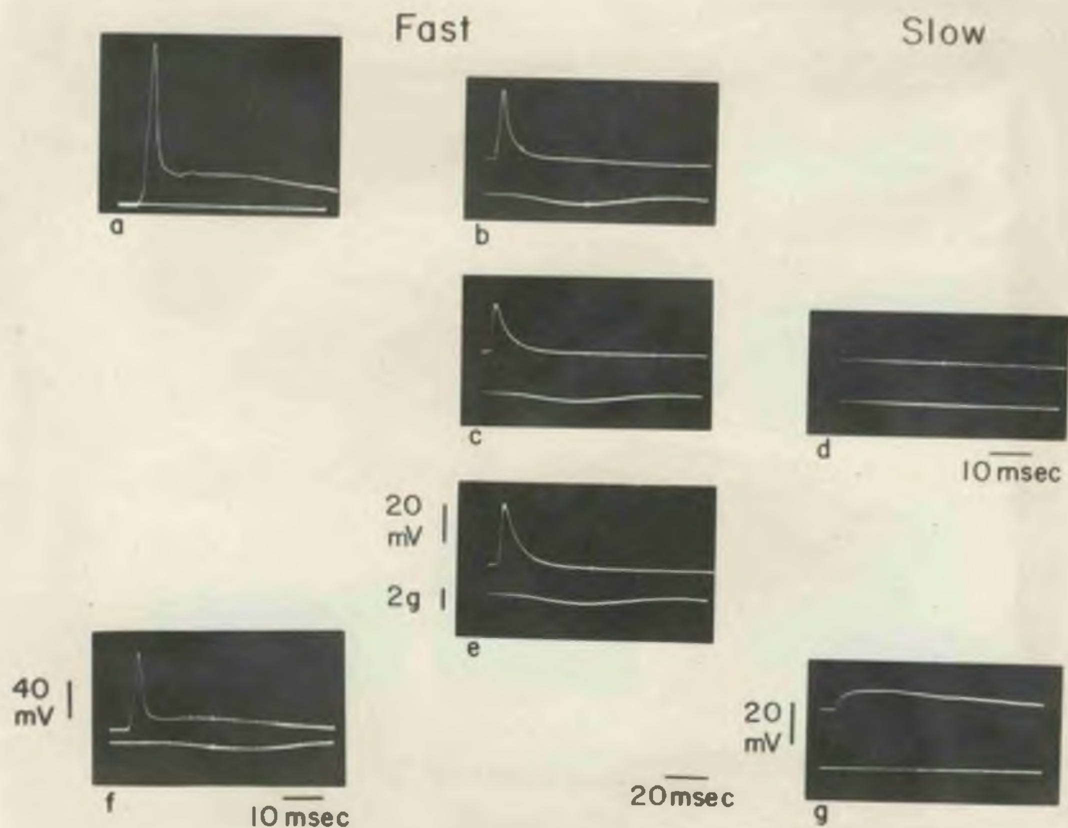


Fig. 55. Pachygrapsus, closer muscle: Comparison of "fast" and "slow" responses. (a,b,c,e,f) Representative "fast" responses from several different muscle fibres to stimulation of the "fast" axon with a single shock. (d) "Slow" response (10 per sec. excitation) from the same muscle fibre as in (c). (g) "Slow" response (single shock excitation) from a muscle fibre which had no "fast" response to a single shock, showing an extremely show decay rate. Lower traces in (b) to (g), tension of the whole muscle.

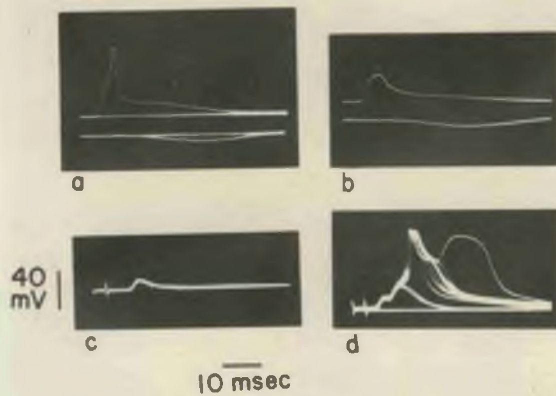


Fig. 56. Pachygrapsus, closer muscle. (a) "Fast" response to a single shock. (b) Response of the same muscle fibre after a period of stimulation. (c) "Fast" response of another muscle fibre to a single shock. (d) Responses of the same muscle fibre to closely spaced shocks (3 msec.) delivered at 5 per sec. Lower traces in (a,b), tension of the whole muscle.

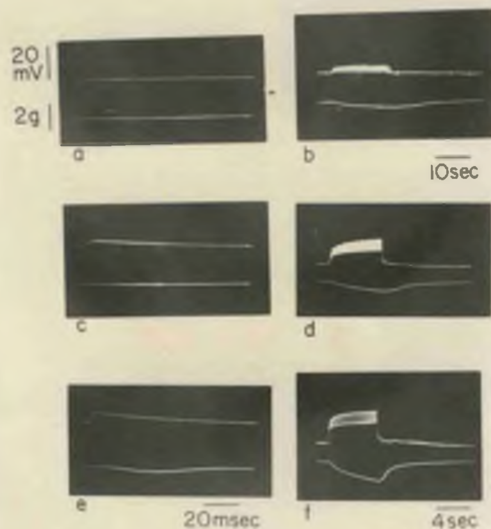


Fig. 57. Pachygrapsus, closer muscle. Responses to "fast" and "slow" axon stimulation in a single muscle fibre. (a,b,c,d,f) Responses to stimulation of the "slow" axon at frequencies of 1 per sec. (a), 7 per sec. (c), 10 per sec. (b), 25 per sec. (d), 40 per sec. (f). (e) Response to a single shock applied to the "fast" axon. Lower traces, tension of the whole muscle.

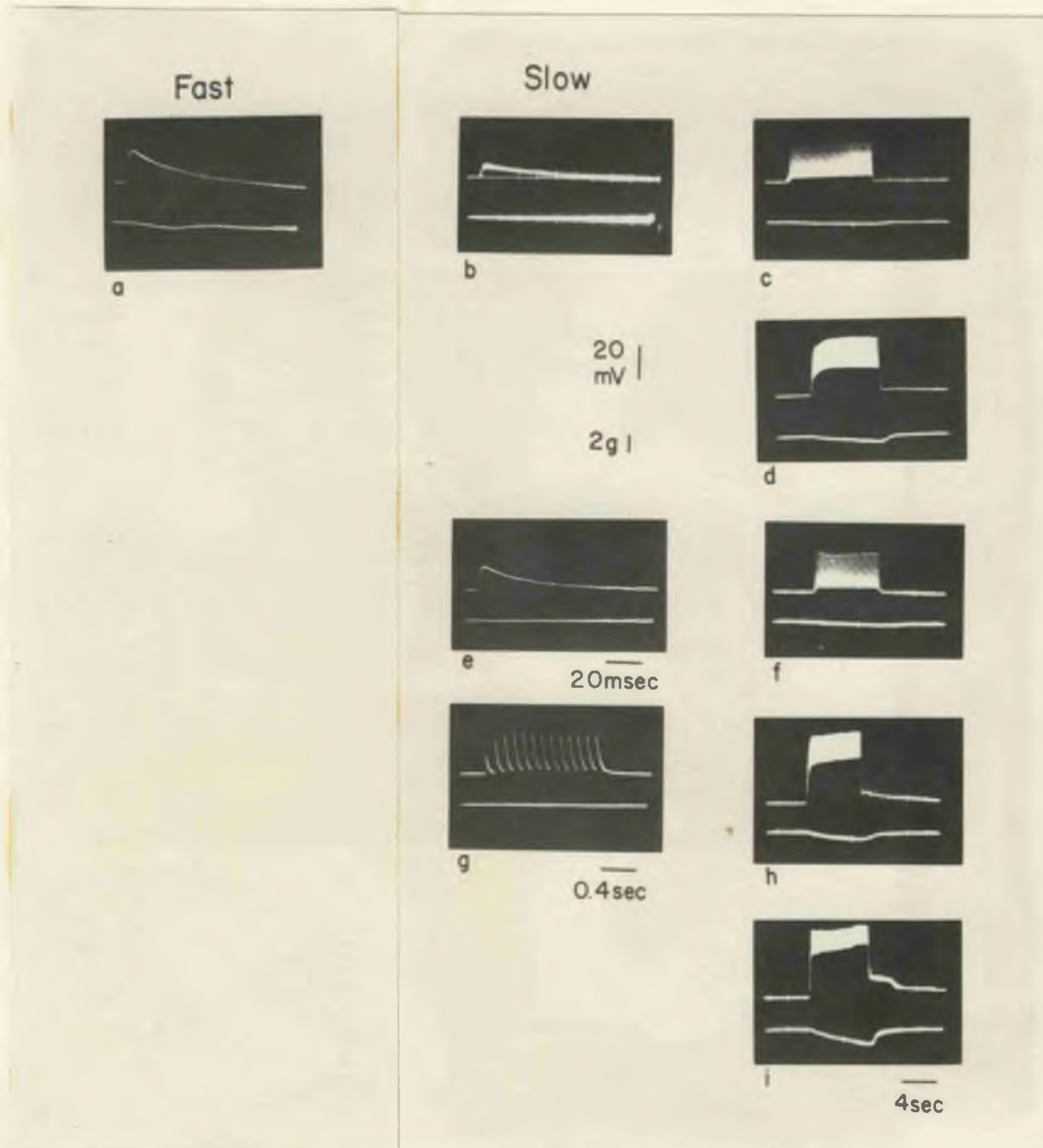


Fig. 58. Responses from muscle fibres in the *Pachygrapsus* closer muscle. (a) "Fast" response to a single shock. (b,c,d) "Slow" responses of the same muscle fibre to stimulation at 1 per sec. (b), 10 per sec. (c), and 20 per sec. (d). (e to i) Responses of another muscle fibre to stimulation of the "slow" axon with a single shock (e), at 10 per sec. (f,g), at 20 per sec. (h), and at 40 per sec. (i). No "fast" response to a single shock was seen. Lower traces, mechanical activity of the whole muscle.

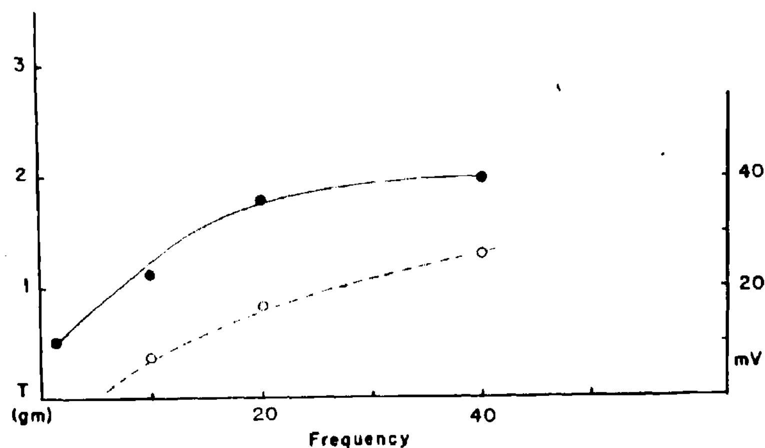


Fig. 59. "Slow" tension in the closer of Pacyhgrapsus (open circles), and depolarization in fibres giving large "slow" responses (filled circles), related to frequency of stimulation. Depolarization was measured from the base line to the tops of the p.s.ps. Tension measurements were averaged from four preparations; electrical measurements were averaged from six muscle fibres giving large "slow" responses. The lowest frequency used was 1 per sec.

c) <u>The influence of muscle fibre membrane properties on properties of indirectly produced electrical responses.</u>

It is apparent from the survey of electrical responses of crustacean muscles presented in the previous section, and from the work of Hoyle and Wiersma (1958a), that a great variety of responses can be obtained from different muscles and also from different fibres within a given muscle. This variation includes such factors as decay rates of postsynaptic potentials, excitability, patterns of facilitation, and relative magnitudes of "slow" and "fast" responses. The question arises: What factors are responsible for this variation?

Differences in the chemical nature of "slow" and "fast" transmitter substances have been invoked by Hoyle and Wiersma (1958 a, c) to explain differences between "slow" and "fast" electrical responses. Thus, such points of difference as the more rapid decay of "fast" ps.ps. in some muscle fibres, the more frequent production of spikes by "fast" axon stimulation, the more rapid fatigue of "fast" responses, etc., are associated by them with specific properties of "fast" and "slow" transmitter substances.

An alternative hypothesis is suggested by results reported for certain frog skeletal muscles, in which "slow" and "fast" muscle fibres are present. These fibres are acted on by the same transmitter substance (acetylcholine), but differences in the membrane properties and patterns of innervation of the responding muscle fibres give rise to very different electrical responses (Kuffler and Vaughan Williams, 1953a,b;

Burke and Ginsborg, 1956a,b).

At first sight this situation has little in common with that found in crustacean muscles. In the latter case, "fast" and "slow" axons commonly innervate the same muscle fibres; in the frog, the two types of muscle fibre probably do not share innervation.

However, certain observations made during the course of the study on electrical responses of different crustacean muscles suggested that these muscles may have more features in common with the frog muscles than has previously been thought, and that the nature of the responding muscle fibre could be important in determining the type of response recorded from it.

(1) Many muscles (e.g., *Carcinus*, *Nephrops*, and *Pachygrapsus* closers) possess fibres which respond primarily to stimulation of only one of the two motor axons.

(2) In the great majority of muscle fibres examined, "fast" and "slow" electrical responses were similar in appearance when equal in magnitude. This was particularly evident in the cases of *Carcinus* Type B muscle fibres and *Pachygrapsus* "slow" muscle fibres. In both cases "fast" electrical responses were often absent, but when present they showed the same slow decay rates characteristic of the "slow" p.s.ps.

(3) It was apparent that in some muscles (such as the *Carcinus* closer muscle), there was a lot of variation in both "fast" and "slow" groups of responses. The variation within the two groups was almost as great as differences between them. This situation can be more easily explained in

terms of differences in the responding muscle fibres than by a two-transmitter-substance hypothesis.

Further evidence bearing on the influence of the properties of the responding muscle on the electrical responses to indirect stimulation was obtained in the experiments described below, in which the electrical properties of various muscle fibres were determined by the use of two intracellular microelectrodes (see Methods).

Most of the experiments were performed on the closer muscle of *Carcinus*, in which three types of muscle fibre had been distinguished on the basis of responses to indirect stimulation. Some additional information was obtained from other muscles.

(i) *Carcinus: Closer Muscle*

In the Carcinus closer muscle it was possible to investigate the electrical properties of Type A, Type B and Type C muscle fibres.

The first step in this study was measurement of the membrane "cable constants" of the different muscle fibres. The methods employed for this purpose have been described previously (see Methods). In Type A muscle fibres and some Type C muscle fibres, a hyperpolarizing potential applied at one end of the fibre declined exponentially with distance along the fibre from the stimulating electrode (Fig. 60, i, c, d). However, many Type C and Type B muscle fibres had length constants of about 2 mm. and were only about 4mm. long. In these fibres the potential decline did not follow exponential laws

(Fig. 60, i. a, b), and it was necessary to employ the equations given by Weidmann (1952; see Methods) for a "cable" of finite length to compute the length constants of these fibres.

In these latter fibres it was also necessary to plot the rise and fall of total membrane charge to find the membrane time constant. An example of this type of plot for a Type B muscle fibre is given in Fig. 60 (ii). In this case the rise of membrane charge approximates an exponential curve with a time constant of 60 msec., and the fall of membrane charge has a time constant of 57 msec.

Using the methods outlined above, values for the cable constants of some Type C and Type B muscle fibres were obtained (Table 1). It was not possible to obtain meaningful values for Type A muscle fibres because in those which were examined it was found that the calculated length constants were often of the same order of magnitude as the fibre diameter. In such cases the spread of the potential would not be predictable from cable theory. However, results from one Type A fibre are included for comparison in Table 1.

The three types of muscle fibre differ in many respects. The average diameter of the Type C fibres listed is 0.21 mm (range, 0.15 mm to 0.26 mm). Type B fibres are considerably smaller (average diameter, 0.105 mm, range, 0.08 to 0.12; even smaller muscle fibres were seen) and Type A fibres were very large (0.3 to 0.8mm). In view of these differences it is not surprising that the "input resistances" (the ratio of voltage to current at the stimulating electrode; Katz and Thesleff,

1957) of Type B fibres are greater than those of Type C fibres, which in turn exceed those of the Type A fibres. But the values for the Type B fibres (average, 145 x 10^3 ohms) are considerably larger than those of Type C fibres (average, 29.8 x 10^3 ohms). The difference is greater than would be expected solely on the basis of the differences in diameter. In fibres in which other factors are constant, input resistance is inversely proportional to the 3/2 power of the fibre diameter (Katz and Thesleff 1957). Hence, taking the average diameters of Type C and Type B muscle fibres, one would expect the average input resistance of Type B fibres to be about 3 times greater than that of Type C fibres, whereas empirically it is 5 times greater. It is, of course, possible that uncertainties in the measurement of fibre diameter may have influenced the result; but the fact that the calculated myoplasmic resistances agree reasonably well in the two types of muscle fibre, and are comparable with the values obtained by Shaw (1955) and Fatt and Katz (1953a), is evidence that errors in measured fibre diameter are not important in comparing the two sets of results. It therefore appears that Type B muscle fibres have surface membranes of higher specific resistance than Type C muscle fibres.

This possibility is confirmed by calculations of the specific membrane resistance (Rm). Type B muscle fibres have membrane resistances outside the range of values for Type C muscle fibres. This difference in specific membrane resistance is reflected also in the higher length constants of Type B muscle fibres, and in their larger time constants.

It is interesting that although the time constants of

Type B muscle fibres are larger than those of Type C muscle fibres, the calculated membrane capacitances are generally less, and it was found that the means of the two samples are highly significantly different ($t = 3.74$). It is possible that a result of this sort could be brought about by different degrees of folding of the membrane in the two types of fibre, but it is also possible that a more fundamental difference in membrane structures exists.

It is of interest to compare the membrane time constants with the time constants of decay of "fast" and "slow" p.s.ps. recorded in the same muscle fibre (Fig. 62, a to d; Fig. 63, a to g: FigS. 64, 65). A number of such comparisons are made in Table 2. In general, there is close agreement between the time constant of decay of "slow" p.s.ps. and the membrane time constant. This holds true also for "fast" p.s.ps. in some cases, but in others the "fast" p.s.ps. decayed more rapidly than would be expected from the membrane time constant. When some of these fibres were examined along their length with a recording microelectrode it was found that the "fast" p.s.p. decayed more rapidly at one end of the fibre than at the other (Fig. 63, f, g; Figs. 64, 65) and that its size was greatest at the point of maximum rate of decay. In some instances the decay of the "fast" p.s.p. in some parts of the muscle fibre was slower than that of the "slow" p.s.p. in the same fibre (Fig. 65). It seemed likely in such cases that part of the fibre was not innervated by the "fast" axon. If this was true, the results could be readily explained, since a potential change affecting only part of the fibre would

decay at its point of application more rapidly than one affecting the whole fibre (Burke and Ginsborg, 1956a). Whether such a situation is normal or induced accidentally during the experiment could not be determined. In any case no evidence was found in this muscle for "lingering transmitter action" (Hoyle and Wiersma 1958a). Instead it appeared that the transmitter action does not outlast the rising phase of the p.s.p. Although differences in decay rates of "fast" and "slow" p.s.ps. have been observed in this muscle and in other crustacean muscles (Fatt and Katz, 1935b; Hoyle and Wiersma 1958a) no evidence was found in the present study that such differences were not the result of incomplete innervation or of the influence of fields produced by nearby muscle fibres. More study is needed to clarify this point.

Observations were made on the behavior of muscle fibres subjected to depolarizing pulses applied through the stimulating microelectrode. Typical responses from Type C muscle fibres are shown in Fig. 65 (h to l). In most cases a "hump" appears during the initial phase of the response at depolarizations of about 20 mV (Fig. 63, i; Fig. 66, (i)) and a repolarization follows. (Occasionally, strong hyperpolarizing pulses gave rise to the same phenomenon.) When increasingly strong currents were applied, the response showed an increased rate of rise, and small oscillations usually appeared at depolarizations of 30 to 40 mV. (Fig. 63; j, k, l). The first of these oscillations sometimes resembled a small (5 to 10 mV) spike (Fig. 63,1). The responses were similar to those described for a crayfish opener muscle (Fatt and Ginsborg 1958). There

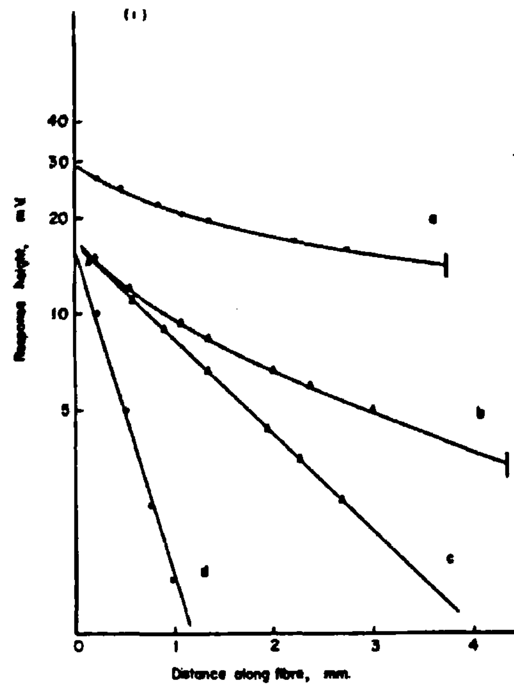
is apparently a tendency to produce electrically excitable responses in Type C fibres but strong depolarization is necessary to evoke them, and they are invariably small.

In Type B fibres, the response was limited to a slow "hump" followed by repolarization (Fig. 62.f, g). Oscillations were not observed. The total response, including the "hump" showed marked rectification even at small depolarizations (Fig. 66,ii). In addition, the time constants of rise and fall of charge became much less than for hyperpolarizing pulses. These changes indicate that the membrane resistance is much less during depolarization than at the membrane resting potential level, probably due to an increased potassium conductance, as in other systems (Burke and Ginsborg 1956; Jenerick, 1959; Werman, McCann and Grundfest 1961). The lower membrane resistance during depolarization accounts in part for the fact that the "slow" p.s.ps. become much smaller as a depolarization "plateau" is built up (See p.37). In Type C fibres, rectification often does not occur to any great extent until fairly large membrane depolarizations are attained (Fig. 66, (i)) and as a result the p.s.ps. show little attenuation due to decreased membrane resistance (cf. Dudel and Kuffler, 1960).

Examination of the deeply buried Type A fibres by means of two electrode techniques was not usually possible. However, in those which were examined (Fig. 61), it was found that graded electrically exitable membrane responses could usually be produced by depolarization (Fig. 61, d). Even the fibre of Fig. 7 (a,b) and Fig. 61(c) showed a small degree of

electrical excitability, for the voltage changes produced by depolarizing current exceeded those produced by equal hyperpolarizing currents (Fig. 66 (iii)).

It is evident from the data presented above that the categories of muscle fibre established for the Carcinus closer muscle on the basis of responses to indirect stimulation are associated with significant differences in the membrane properties of these fibres. Type A muscle fibres have electrically excitable membranes, low membrane resistances and small time constants; Type B muscle fibres have electrically inexcitable membranes, high membrane resistances and large time constants; and Type C muscle fibres have intermediate properties. However, to explain the electrical responses to nervous stimulation it is also necessary to assume differences in the density of "fast" axon innervation for the different muscle fibre types because the size of the "fast" p.s.ps., unlike that of the "slow" p.s.ps., is inversely related to the "input resistance" of the muscle fibre. Type A muscle fibres must be assumed to possess a much denser "fast" axon innervation than Type C or Type B muscle fibres. If such an assumption is made, however, and if it is also assumed that "fast" and "slow" nerve endings may differ in rate of transmitter substance release and in facilitation, most of the varieties of indirect responses of this muscle can be explained without postulating two excitatory transmitter substances.



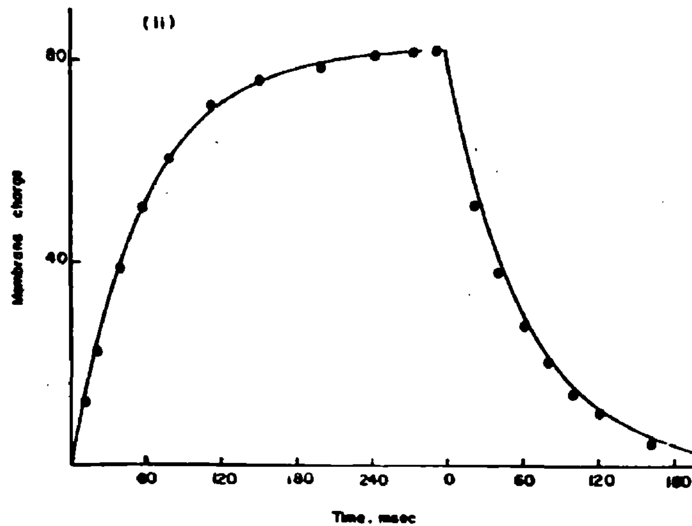


Fig. 60. (i) Examples of the decay of applied hyperpolarizing pulses along the length of the muscle fibre. (a) Type B, (b,c) Type C, (d) Type A muscle fibres. Total lengths and length constants were: (a) 3.5 mm and 2.4 mm, (b) 4.3 mm and 1.75 mm and 0.4 mm.

(ii) Rise and fall of total membrane charge (arbitrary units) in a Type B muscle fibre. The solid lines are exponentials with time constant 60 msec; the points were obtained experimentally. Membrane charge rises with a time constant of 60 msec. and falls with a time constant of 57 msec.

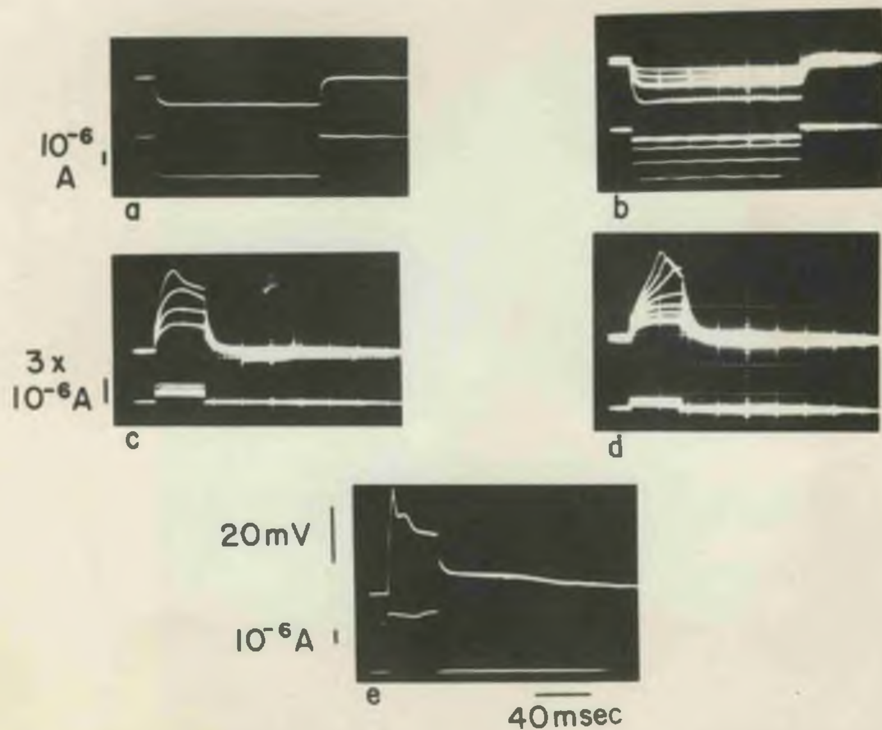


Fig. 61. Responses of Carcinus Type A muscle fibres to direct stimulation. (a,c) Responses to hyperpolarization (a) and to depolarization (c) of the fibre of Fig. 7 (a,b). (b,d) Responses to hyperpolarization (b) and depolarization (d) of another Type A fibre. (e) Response to depolarization of another Type A fibre. Note that Type A fibres have short time constants and low input resistances.

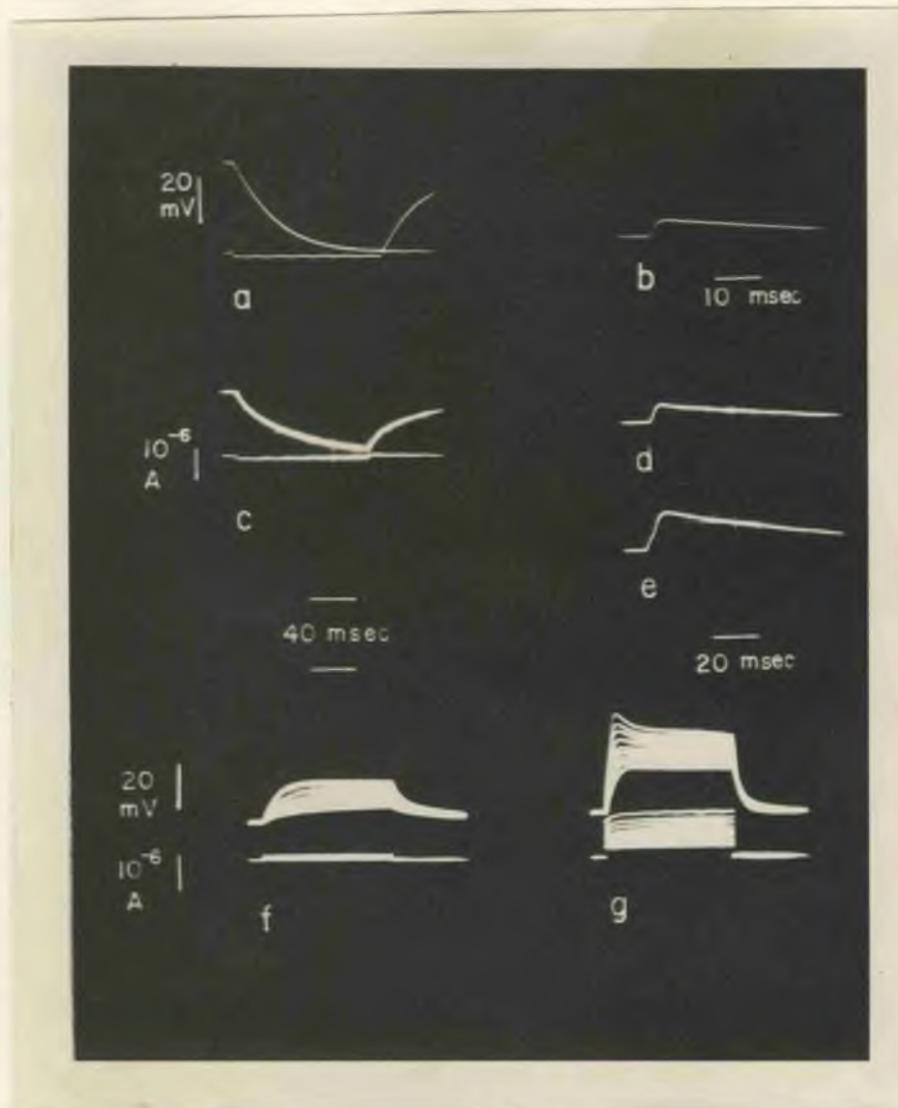


Fig. 62. Properties of Type B muscle fibres. (a) Response of a Type B muscle fibre to a hyperpolarizing pulse (electrode separation, 0.2 mm) illustrating the large time constant and high input resistance compared with Type C fibres. (b) "Slow" p.s.p. from the same muscle fibre (time constant of decay, 35 msec.; stimulation frequency, 2 per second). (c) Response of another Type B muscle fibre to a hyperpolarizing pulse (electrode separation, 0.1 mm). (d) Single "slow" p.s.p. from the same fibre (time constant of decay, 64 msec.). (e) Response of the same fibre to two stimuli separated by 3 msec. (f,g) Responses of the same fibre to depolarizing pulses.

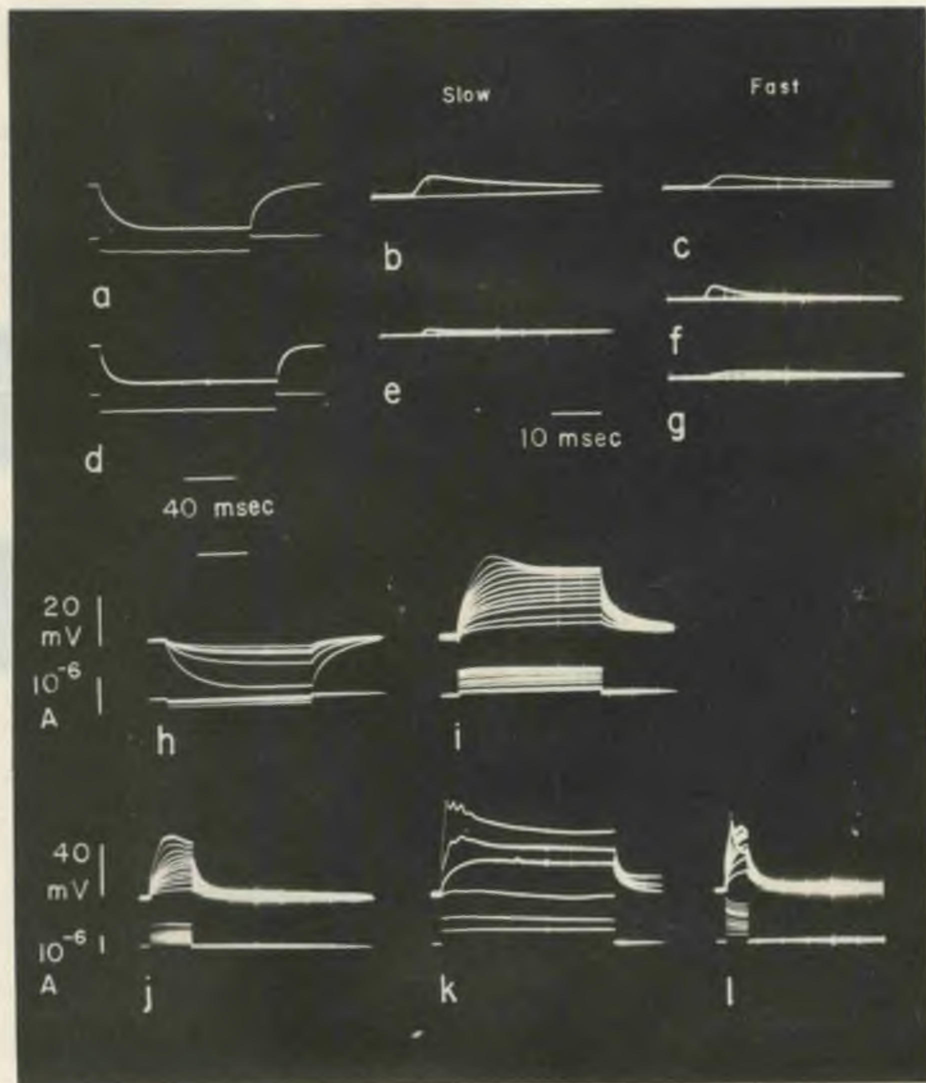
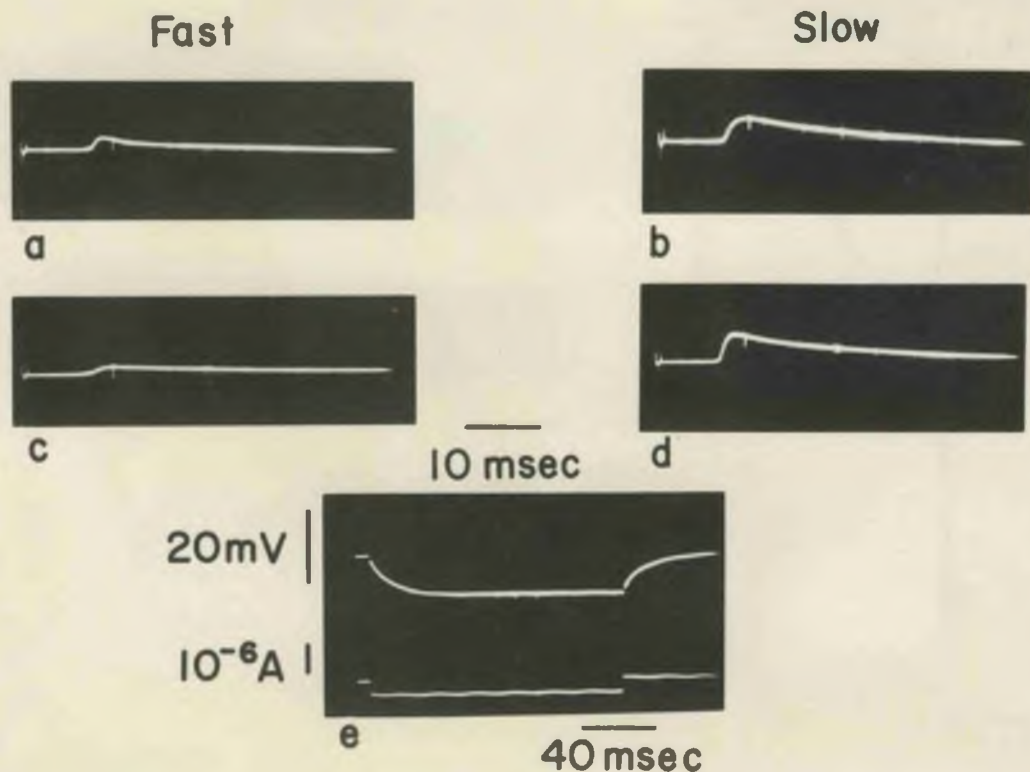


Fig. 63. Properties of Type C muscle fibres. (a) Response of a Type C fibre to a hyperpolarizing pulse,; electrode separation, 0.12 mm; estimated membrane time constant, 22 msec. (b) "Slow" and (c) "fast" p.s.ps. from the same muscle fibre, with time constants of decay of 22 and 21 msec. respectively (stimulation frequency, 6 per second). (d) Hyperpolarizing pulse in another fibre; electrode separation 0.09 mm; estimated time constant, 10.5 msec. (e) "slow p.s.p. (decay time constant 11 msec) and (f,g) "fast" p.s.ps. recorded near opposite ends of the fibre, with decay time constants of 6.5 and 15 msec. (h, i) Responses to hyperpolarizing and depolarizing stimuli of a Type C fibre. (j,k,l) Other types of response to depolarization in Type C muscle fibres.



<u>Fig. 64</u>. A further example of "fast" and "slow" p.s.ps. in a Type C muscle fibre. (a,c) "Fast" p.s.ps. recorded at 0.5 mm. from the shell end of the fibre (a), and 3.0 mm from the shell end (c); time constants of decay were approximately 10 msec. (a), and 16 msec. (c). (b,d) "Slow" p.s.ps. recorded at the same places, with time constants of decay of 18 msec. (b) and 17 msec. (d). (c) Membrane response during application of a hyperpolarizing pulse; time constant, 21 msec; electrode separation, 0.09 mm.

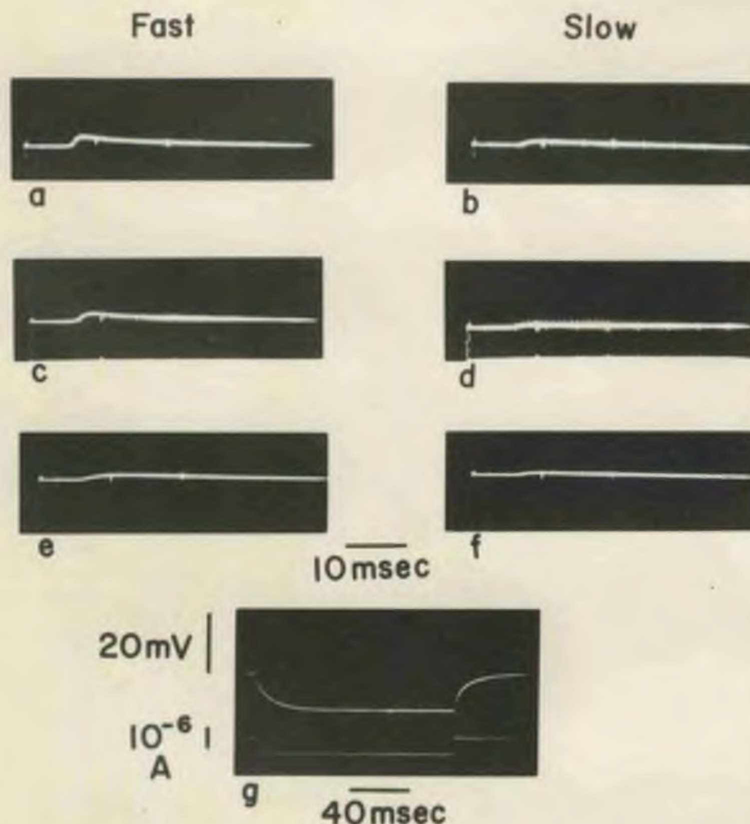
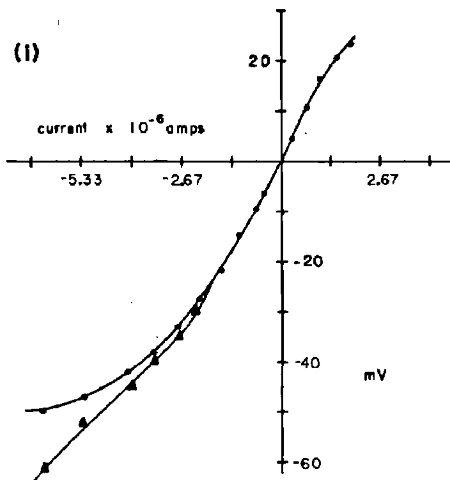
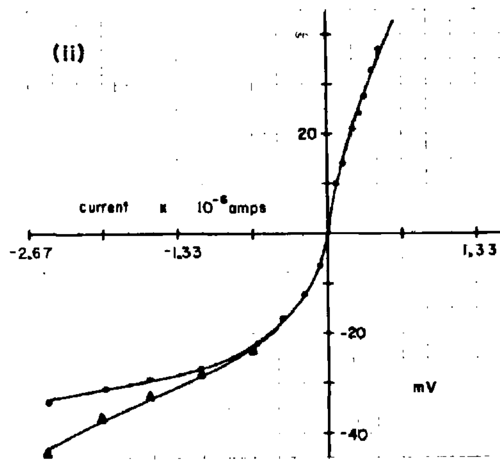


Fig. 65. A further example of "fast" and "slow" p.s.ps. in a Type C muscle fibre. (a,c,e) "Fast" p.s.ps. recorded at 0.5 mm. from the shell (a), close to the tendon (e), and about half-way between these two points (c). In (a) the time constant of decay was 18 msec., in (c) it was 27 msec. (b,d,f) "Slow" p.s.ps. recorded at the same locations as (a,c,e). In (b) the time constant of decay was 22 msec., in (f) it was 18 msec. (g) Membrane response during application of a hyperpolarizing pulse (electrode separation, 0.06 mm); time constant was 19 msec.





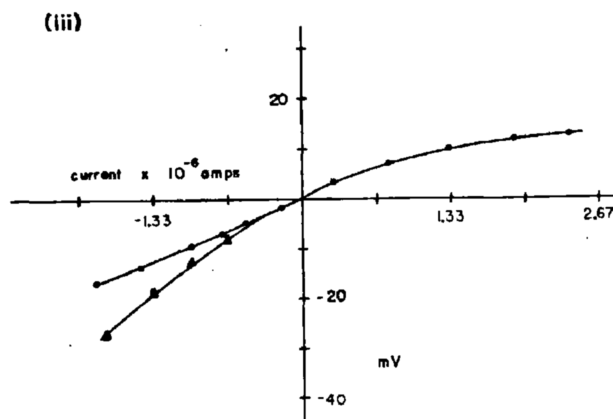


Fig. 66. Current - voltage relationships in (i) a Type C muscle fibre (ii) a Type B muscle fibre and (iii) the Type A muscle fibre of Fig. 7 (a,b). Closed circles represent the final level of membrane potential, triangles represent the height of the initial response to depolarization. Depolarization is taken as negative.

Table 1. Membrane properties of muscle fibres in the Carcinus closer muscle.

Fibre (type)	Diameter (mm)	Input Resistance $\times 10^3 \Omega$	Length Constant (mm)	R_i* (Ω cm)	R_m* (Ω cm)	Time Constant msec	C_m* $\mu F/cm^2$
1.A	0.5	13.9	0.4	650	88.5	4	45
2.B	0.12	115	2.45	51	1050	39	37
3.B	0.10	205	2.40	66.5	1550	60	39
4.B	0.105	114	2.65	54	1420	42	30
5.B	0.12	100	2.6	44	980	37	38
6.B	0.08	190	1.78	52	850	29	34
Mean	0.105	1459	2.39	53	1170	41.4	36
7.C	0.19	40	1.4	81	334	16	48
8.C	0.17	36.5	1.6	53	317	20	63
9.C	0.22	35	1.8	74	435	16	37
10.C	0.25	19.8	1.15	84	179	11	61
11.C	0.16	37.5	1.95	39	367	20	54.5
12.C	0.175	38	1.5	61	313	18	57.5
13.C	0.25	21.7	1.75	61	298	20	67
14.C	0.23	19.5	1.7	47	246	16	65
15.C	0.215	21.4	2.0	39	287	15	52
16.C	0.25	17	1.1	76	147	10	68
17.C	0.26	20.8	1.45	76	245	10.5	43
18.C	0.15	46	1.23	66	217	8	37
Mean	0.210	29.8	1.55	63	282	15	54.5

* R_i - specific resistance of the myoplasm; R_m - resistance of unit area of the membrane; C_m - capacitance of unit area of the membrane.

Table 2. Comparison of membrane time constants with decay times of fast and slow post-synaptic potentials.

Fibre Type	Time Constant (msec)	Decay of P.S.P. to 37% Slow	Fast
1. A	5*		7.5
2. B	39	32	
3. B	60	64	
4. B	39	37	
5. B	42	36	
6. B	34	32	
7. C	16	15	17
8. C	21	21	
9. C	16	19	
10. C	12	11	6.5**
11. C	18.5	16.5	8.5 **
12. C	17	22	24
13. C	21	22	21
14. C	22	22.5	
15. C	20	17	

* The time constants of Type A fibres are probably underestimated by the direct method, due to the relatively large fibre diameters.

** Measured at the point of maximum size of the fast p.s.p. (Elsewhere in the fibre the fast p.s.p. was smaller and decayed more slowly, indicating limited fast axon innervation).

(ii) Nephrops: Closer Muscle.

Muscle fibres in several closer muscles of Nephrops walking legs were examined in order to compare responses from proximal fibres (giving large "fast" electrical responses) with those from distal muscle fibres (giving small "fast" responses but large "slow" responses).

When proximal muscle fibres were stimulated by depolarizing pulses applied through the current-passing microelectrode, several different types of response were observed. In some fibres large "spikes" were produced, often at a critical depolarization level (Fig. 67a). In other cases smaller, graded electrically excitable responses of various types were produced (Fig. 67, b, c). It is apparent that a considerable range in membrane excitability occurs in these muscle fibres.

No prolonged graded responses of the type sometimes produced by indirect stimulation (Fig. 30) were encountered in directly stimulated fibres. However, the fibres giving these prolonged responses were almost always fairly deep in the muscle; only fibres close to the surface of the muscle could be examined by techniques of direct stimulation.

Responses of distal muscle fibres to direct stimulation were also varied. In some relatively inexcitable fibres the responses were limited to small oscillations (Fig. 67, d), whereas in other fibres larger electrically excitable responses could be produced (Fig. 67, e). It thus appears that in both proximal and distal parts of the muscle, fibres having the same general properties of electrical excitability can be found.

In both groups, some fibres give rise to small graded electrically excitable responses while others show larger responses, often with a threshold.

No extensive measurement of membrane "cable constants" was made in Nephrops muscle fibres, but the input resistances and time constants of those fibres which were examined could be estimated. In eight proximal muscle fibres, the average input resistance was 28 x 10^3 ohms (range, 12 x 10^3 to 50 x 10^3 ohms) and the average time constant was 13 msec. (range, 4 to 17 msec.) In five distal muscle fibres the average input resistance was 40 x 10^3 ohms (range, 20 x 10^3 to 62 x 10^3 ohms) and the average time constant was 20 msec. (range, 12 to 25 msec). Values for distal muscle fibres have a higher average than those for proximal muscle fibres, but the range of values is not as wide as that found in Carcinus, and could be determined largely by fibre diameter as in frog muscles (Katz and Thesleff, 1957). Compared with Carcinus muscle, Nephrops muscles are much more homogeneous with respect to the electrical properties of the constituent muscle fibres. It was observed also that "fast" and "slow" p.s.ps. had fairly uniform decay rates throughout the muscle.

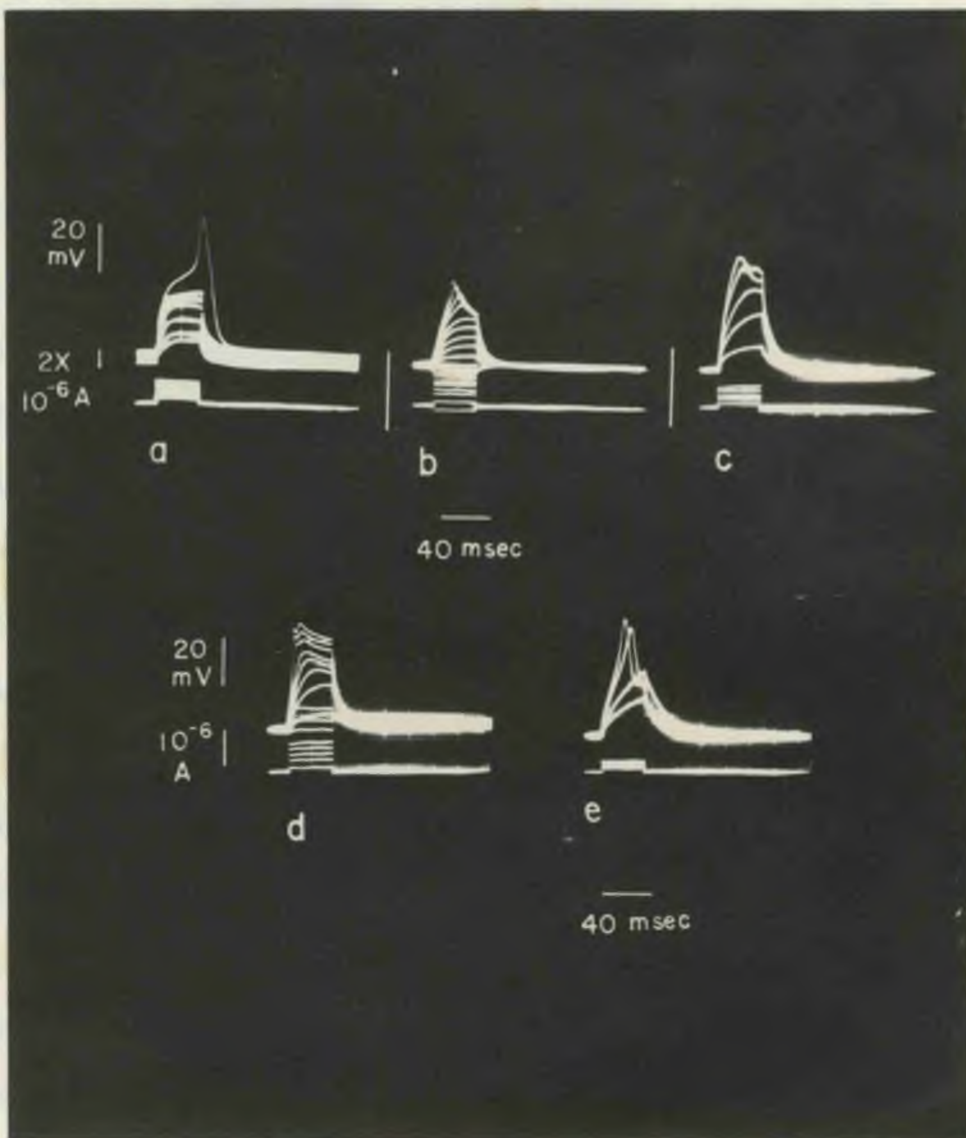


Fig. 67. Responses of Nephrops muscle fibres to direct depolarization. (a,b, c) Responses of three proximal fibres. (d,e) Responses of two distal fibres.

(iii) Cancer: Stretcher Muscle

Using the methods described for Carcinus, determinations of the membrane constants of some fibres in the stretcher muscle of Cancer were made. The data is given in Table 3.

The Cancer muscle fibres appeared from this sample to be more homogenous than were the Carcinus muscle fibres. Three fibres from the edge of a muscle, and four fibres from the central region, were selected. Although the sample is not large enough to form a basis for firm statistical conclusions, the data indicates that the "edge" fibres are smaller in diameter, shorter in length, and have higher input resistances, in general, than do fibres remote from the edge. No significant differences in any of the other membrane properties are apparent in the two groups. It is possible, therefore, that the smaller sizes of the "edge" fibres may be partly responsible for the larger electrical responses recorded during indirect stimulation. Smaller diameters mean larger input resistances (Katz and Thesleff, 1957), and shorter lengths mean less decay of applied potential over the length of the fibre.

Compared with Carcinus Type C muscle fibres, the Cancer fibres have higher internal resistances, higher membrane resistances, and larger length and time constants. It is possible that the larger diameters of the stretcher muscle fibres may have introduced errors in determining membrane constants (see Fatt and Katz, 1953a). It is also possible that errors in measurement of the fibre diameters played a part. However, the fact remains that Cancer muscle fibres have much larger length and time constants; therefore it is reasonable to

expect that Rm is truly larger in Cancer fibres than in most Carcinus fibres.

Membrane capacitances of Cancer fibres averaged less than those of Carcinus Type C fibres, but in view of possible errors (mentioned above), this difference may not be significant.

The larger time constants of Cancer fibres when compared with Carcinus Type C fibres accounts for the slower decay rates of p.s.ps. in the former fibres. In Table 4, comparisons of membrane time constant and time constant of decay of p.s.p. are made for Cancer fibres. As in Carcinus fibres, there is reasonable agreement between the two sets of values, and the means of the two samples are about the same.

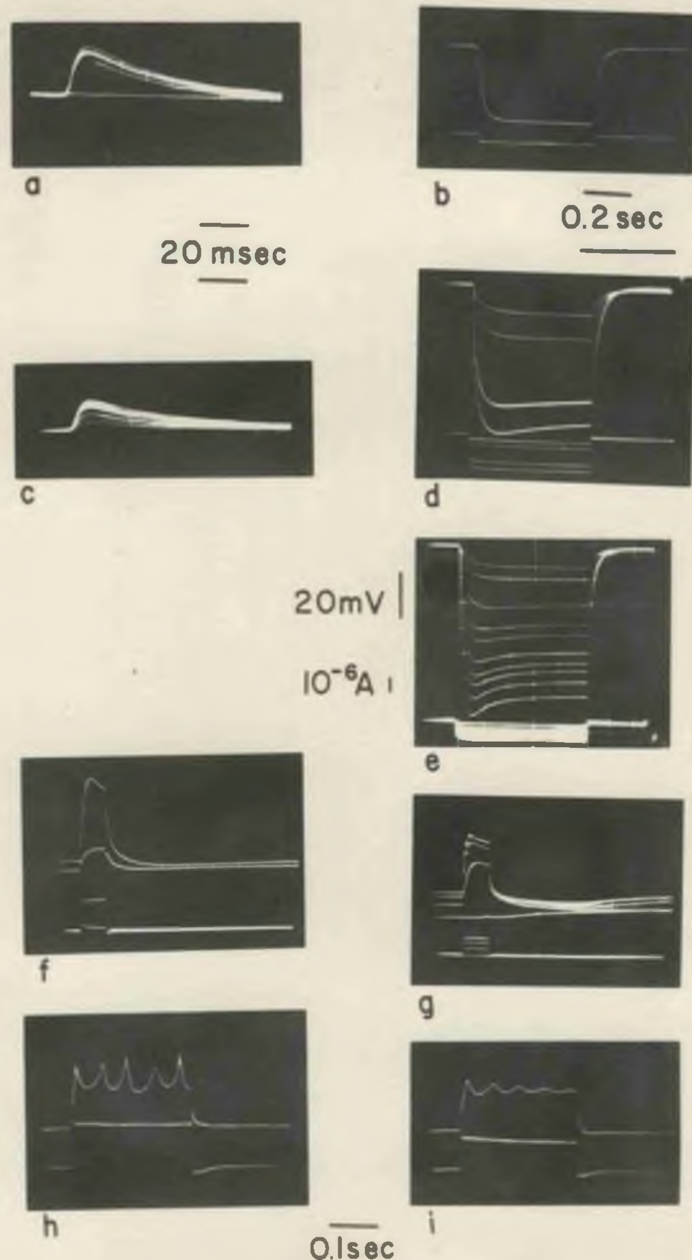
Examples of p.s.ps. and hyperpolarizing pulses recorded from the same muscle fibre are given in Fig. 68 (a to d), to illustrate the approximate agreement between decay rate of p.s.p. and membrane time constant.

Almost all of the stretcher muscle fibres studied showed "rectification" in response to strong hyperpolarizing currents. The initial phase of the response was a pronounced "hump" which was succeeded by a gradual decline in magnitude of the hyperpolarization to a steady level (Fig. 68, d, e). The initial sharp rise to a "hump" may represent an initial phase of potassium inactivation, which is gradually reduced. A similar explanation has been advanced by Reuben, Werman, and Grundfest (1961) to explain the somewhat different "hyperpolarizing responses" of lobster muscle fibres. On the other hand, the

hyperpolarizing "rectification" may indicate a delayed increase in chloride conductance. Chloride "rectification" has been reported for crayfish muscle fibres (Reuben, Girardier and Grundfest, 1962). Voltage-current curves for Cancer fibres (e.g., Fig. 69) show that the initial phase of the hyperpolarization at high currents swings above the line drawn through points obtained with lower currents. This observation indicates that the first of the two mechanisms mentioned above may operate in Cancer fibres.

Responses to strong depolarizing currents in these muscle fibres commonly showed a "hump" followed by rectification (Fig. 68 f, g; Fig. 69). Sometimes small oscillations could be seen initially (Fig. 68, g). It is probable that responses to depolarization can be explained mainly in terms of delayed increase in potassium conductance (Werman, McCann, and Grundfest, 1961; see p. 85).

In some fibres and in certain circumstances, active responses could be obtained. The fibre of Fig. 68 (h, i) had a low resting potential (55 mV) which may have been due to damage or to potassium leakage from nearby muscle fibres. When a strong depolarizing current was passed through the stimulating electrode, a series of small spikes resulted (Fig. 68 h). A slightly weaker current gave rise to smaller oscillations (Fig. 68 i). Occasionally other fibres were encountered which gave spike-like responses (Fig. 94). In general, however, fibres in the Cancer stretcher muscle appeared to be comparatively electrically inexcitable.



<u>Fig. 68.</u> Properties of muscle fibres in the stretcher muscle of <u>Cancer</u>. (a,c) P.s.ps. from two muscle fibres during stimulation of the motor axon at 7 per sec.; decay time constants were 40 msec. (a), and 45 msec. (c). (b,d) Responses to hyperpolarizing pulses in the same two fibres; membrane time constants were estimated to be 45 msec. (b) and 52 msec. (d). (e) Responses to hyperpolarizing stimulation in another muscle fibre. (f,g) Responses to depolarizing stimulation in other muscle fibres. (h,i) Responses to depolarizing stimulation of a partly depolarized muscle fibre.

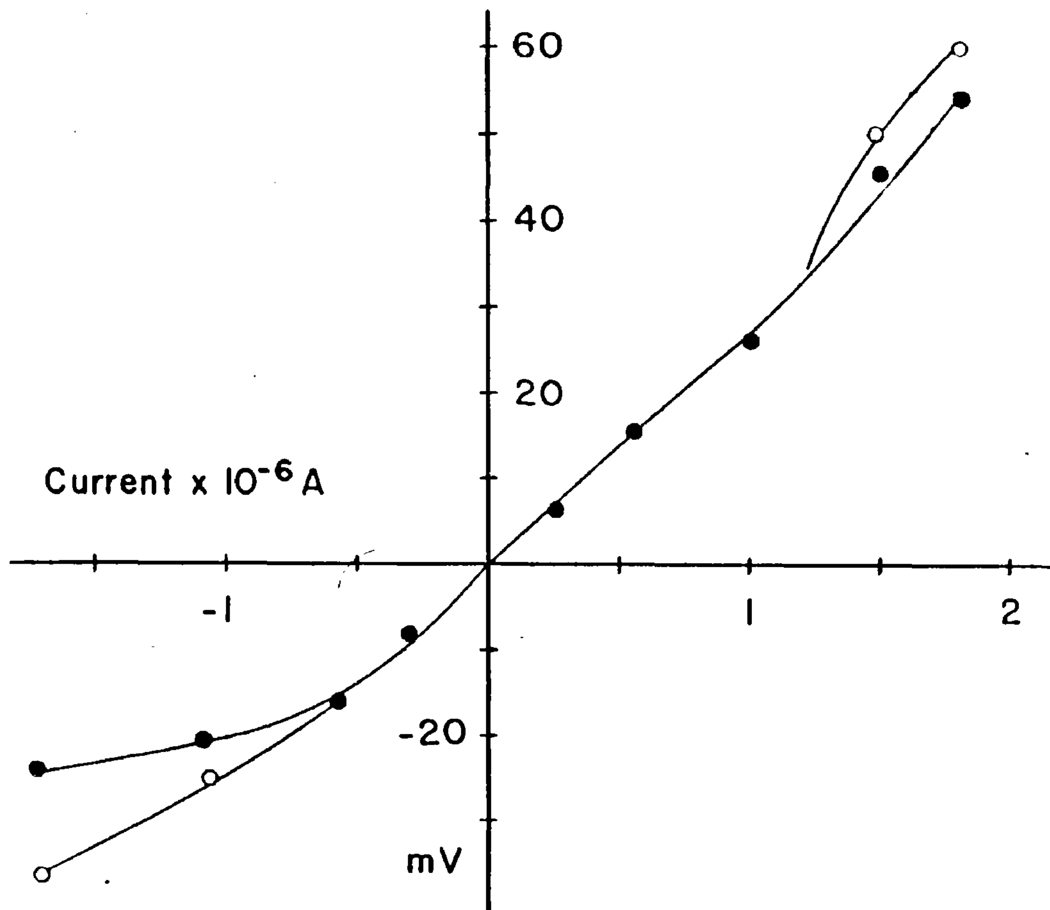


Fig. 69. Voltage - current graph for a fibre of the *Cancer* stretcher muscle. Open circles represent the initial response to the applied current; filled circles represent the final steady potential level. Depolarization is taken as negative.

Table 3. Membrane properties of muscle fibres in the *Cancer* stretcher muscle.

Fibre	Diameter (mm)	Input Resistance $\times 10^3 \Omega$	Length Constant (mm)	R_i (Ω cm)	R_m (Ω cm)	Time Constant msec	C_m $\mu F/cm^2$	Length mm
1*	0.157	115	2.16	103	1220	55	45	5.0
2*	0.230	88	1.95	187	1240	44	36	5.5
3*	0.275	100	2.2	265	1880	60	27	5.5
4	0.235	60	2.0	130	890	55	62	6.0
5	0.250	72	2.5	140	1400	58	42	6.6
6	0.304	73	2.0	200	1395	60	43	6.0
7	0.350	44.5	2.5	170	1220	40	33	6.5
Mean	0.262	79	2.19	174	1320	53	41	

*Fibres located at the edge of the muscle: the other fibres were located in the central region of the muscle.

Table 4. Comparison of membrane time constants with decay times of fast and slow postsynaptic potentials in the *Cancer* stretcher muscle.

Fibre	Time Constant (msec)	Time constant of decay of p.s.p. (msec)
a	40	38
b	44	40
c	65	50
d	45	50
e	45	35
f	45	45
g	45	55
Mean	47	45

(iv) Pachygrapsus: Closer Muscle

A few fibres in the Pachygrapsus closer muscle were examined by two-electrode techniques. Unfortunately the fibres giving large "slow" responses, and also those giving rise to spikes in response to single stimuli applied to the "fast" axon, were not accessible at the surface of the muscle, and could not be studied by these methods. Most of the fibres examined gave large p.s.ps. (without spikes) in response to single stimuli applied to the "fast" axon, and very small "slow" p.s.ps. These fibres were in the proximal part of the muscle. A few fibres in the more distal region of the muscle, giving smaller responses to "fast" stimuli and larger responses to "slow" stimuli, were also examined.

The proximal fibres were found to have short time constants (varying from 2 msec. to 8 msec., averaging 4 msec.), and very low input resistances (ranging from 5 x 10^3 ohms to 15 x 10^3 ohms, averaging 8 x 10^3 ohms; Fig. 70). These fibres were not large in absolute size (most were between 0.1 and 0.2 mm in diameter), but they had short length constants (0.3 to 0.6 mm), therefore it is likely that any attempt to measure "cable constants" would be subject to serious errors.

Depolarization of these fibres did not give rise to electrically excitable responses until the membrane potential was lowered by about 30 mV (Fig. 70, c, d, e). An electrically excitable membrane response then appeared; its size could be varied by altering the strength of the stimulus. At lower depolarizations a small initial "hump" was sometimes seen (Fig. 71) before the spike-like response appeared with increased

stimulus intensity.

The indirectly produced "fast" electrical responses in these muscle fibres were large (10 to 25 mV) and rapidly decaying p.s.ps. Time constants of decay were usually about 5 msec. Spikes were not seen in these fibres at low frequencies of stimulation; the spiking fibres were usually located one or two layers in from the surface of the muscle. It appears that the fibres examined at the surface of the muscle by two-electrode techniques were probably much less electrically excitable than the deeper spiking fibres.

The distal muscle fibres which were examined proved to have higher input resistances (20×10^3 to 40×10^3 ohms) and larger time constants (10 to 20 msec.). Correspondingly, the indirectly produced electrical responses showed slower decay rates than those in the proximal part of the muscle. "Fast" p.s.ps. were observed to have time constants of decay of 8 to 15 msec.

The observations on Pachygrapsus muscle fibres, though incomplete, indicate a pattern similar to that found in Carcinus. Fibres responding primarily to "fast" axon stimulation have lower membrane resistances than those responding to both "fast" and "slow" axon stimulation. Among the fibres responding primarily to "fast" axon stimulation, some appear to have membranes of less electrical excitability than others.

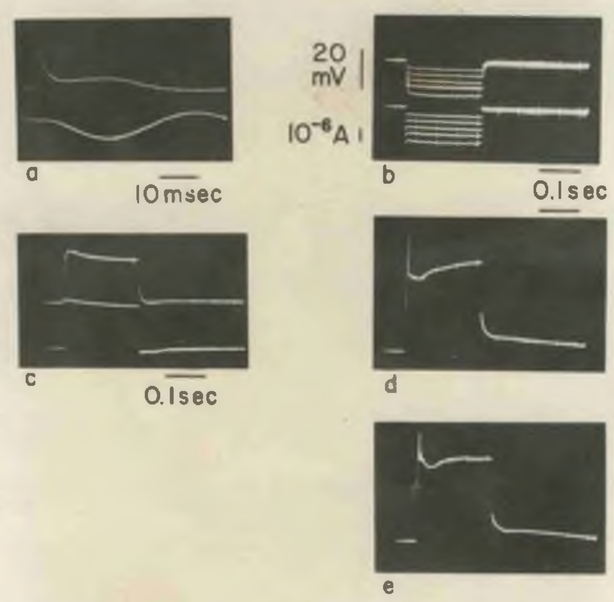


Fig. 70. Properties of a proximal fibre in the Pachygrapsus closer muscle. (a) "Fast" p.s.p. in response to a single stimulus (lower trace, mechanical response of the muscle). (b) Responses of the same muscle fibre to hyperpolarizing current, showing the short time constant and low input resistance. (c,d) Responses to depolarizing current; a spike of about 20 mV. arises with strong depolarization (d). (e) Response of another proximal fibre to depolarization.

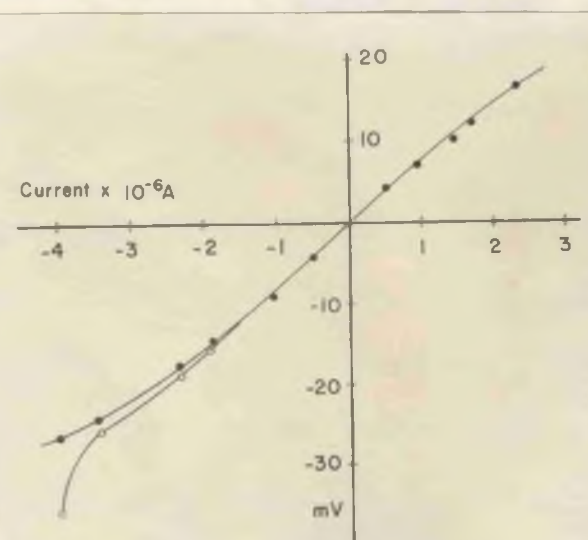


Fig. 71. Voltage-current plot for a proximal muscle fibre in the Pachygrapsus closer muscle. Open circles, initial response; full circles, final potential level. Hyperpolarization is taken as positive.

(v) <u>Other properties of crustacean muscle fibres</u>.

Data on the resting potentials and sarcomere lengths of fibres of some of the muscles studied are presented in Table 5.

Average resting potentials were obtained from samples of 30 muscle fibres in each case. Only fibres which appeared to be normal and undamaged were used. The main point of interest which emerges from these measurements is that *Carcinus* Type B fibres and *Pachygrapsus* "slow" fibres have lower average resting potentials than other fibres in the same muscle. These differences were statistically significant. However, a few Type B fibres were found which had relatively high resting potentials (75 mV). The crustacean "slow" fibres are similar to frog "slow" fibres with respect to their relatively low resting potentials (Kuffler and Vanghan Williams, 1953a).

Crayfish muscle fibres were found to have higher resting potentials than did crab muscle fibres. The values measured in these animals are similar to those obtained by Orkand (1962) in muscles of *Orconectes virilis* and *Cambarus clarkii*. The values obtained by Hoyle and Wiersma (1958a) for the claw opener of *Cambarus clarkii* were much lower (average, 62 mV). No ready explanation for this wide variation is apparent, although it is possible that species-specific and seasonal factors may be important, as well as the condition of individual animals (see p. 115).

An examination of *Carcinus* muscle fibres in fixed material (muscles fixed at slightly greater than resting length) revealed differences in appearance of fibres from different parts of the muscle. In those parts of the muscle in which Type C and

Type B muscle fibres had been located by microelectrode examination, the fixed muscle fibres had sarcomere lengths averaging 9.0 μ (range, 6.0 to 11 μ). In the part of the muscle in which Type A muscle fibres had been found, most of the fixed muscle fibres had shorter sarcomere lengths, averaging 4.7 μ (range, 4.3 to 5.5 μ), although some fibres having the larger sarcomere lengths characteristic of the rest of the muscle were also found. It seems likely, therefore, that Type A muscle fibres may have structural differences, as well as differences in membrane properties, further distinguishing them from Type B and Type C muscle fibres.

In the Carcinus stretcher muscle, the fibres examined did not show as wide a range in sarcomere length; values were restricted to a comparatively narrow range. This may indicate greater uniformity of muscle fibre properties in the stretcher.

In the Nephrops closer muscle, however, it was observed that proximal muscle fibres had shorter average sarcomere lengths than distal fibres. In the proximal part of the muscle, fibres having short sarcomere lengths (4.0 μ) were found in juxtaposition with fibres having longer sarcomere lengths. This was not the case in the distal part of the muscle.

In the Pachygrapsus closer muscle, a wide range of fibre sarcomere lengths was found throughout the muscle. Fibres having short sarcomere lengths were often found beside those having long sarcomere lengths.

In general, it seems probable that morphologically different types of muscle fibre can occur in the same muscle

in crustaceans and that the morphological differences may be correlated with the differences in membrane properties already described. Further corroborative work on this point would be desirable, employing techniques to mark the fibre recorded from with microelectrodes so that the same fibre could be examined to determine its anatomical characteristics.

Table 5. Data on resting potentials and sacromere lengths of crustacean muscle fibres.

Muscle	Average Resting Potential (mV) ± S.E.	Range	Average Sarcomere Length (μ)*	Range
Carcinus closer				
Type A fibres	70±0.6	65–82	4.7	4.3–5.5
Type B fibres	63±1.1	50–75	9.0	6.0–11.0
Type C fibres	69±1.9	64–80		
Carcinus stretcher	67±1.0	63–74	6.0	5.0–6.6
Nephrops closer				
proximal fibres	72±1.2	66–85	4.8	4.0–7.3
distal fibres	69±0.9	65–80	6.4	5.1–7.7
Astacus				
leg closer	80±1.0	65–87		
leg opener	79±1.0	65–90		
Cancer stretcher	67±1.7	63–75		
Pachygrapsus closer				
"fast" fibres	75±0.5	68–85	6.4	4.9–8.3
"slow" fibres	62±1.5	55–68		
"intermediate" fibres	71±1.2	63–75		

* Values were obtained from 20 fibres in each case.

d) Potassium Contracture in Crustacean Muscles

In what has been described thus far, attention has been focused on the electrical events of the muscles studied and on the way in which these events may be modified in different muscle fibres by variations in membrane properties, density of innervation by "fast" and "slow" axons, etc. Muscular contraction is associated with the electrical events, and the nature of the relationship between the two activities is of interest. Do the electrical events bring about contraction by virtue of their depolarizing action on the muscle fibre membrane, or does contraction result from some other action not dependent on depolarization *per se*?

One step in attempting to answer this question is to determine the membrane potential levels at which contraction is initiated by different methods of depolarizing the muscle fibre, such as stimulation of the motor nerve, addition of chemical agents to the muscle, application of depolarizing current etc. The same result obtained by different methods would support the hypothesis that depolarization of the muscle fibre surface membrane is an essential link in the chain of events connecting transmitter substance release with contraction. Different results obtained by different methods would indicate other possibilities, such as: (1) the transmitter substance brings about contraction by a method not dependent on membrane depolarization (Hoyle and Wiersma, 1958 b, c; Hoyle, 1962); (2) different methods of

producing depolarization affect the excitation-contraction coupling sequence in different ways, so that the threshold of depolarization and/or the slope of the depolarization-tension curve is altered, depending on the method used to produce depolarization.

One method of producing depolarization is to add depolarizing chemicals, particularly potassium chloride, to the muscle. This method of studying contraction has been employed very frequently in studies on frog muscle, but not at all in studies on crustacean muscle until recently (Atwood, 1962; Hoyle and Smyth, 1963). Apparently the work described below, which was done in 1961 and 1962, is the first study on potassium contracture to be made on crustacean muscles.

In using high potassium solutions to produce contracture of the whole muscle, it must be borne in mind that depolarization of fibres deep in the muscle may be less than those at the surface (Frank, 1960b). This difficulty can be minimized by: (1) use of a very small muscle, or (2) use of a muscle with a very loose structure which can be rapidly perfused with the depolarizing solution. In the present study, use was made of the opener muscle of the crayfish walking leg, a very small muscle, and of the Carcinus closer muscle, which in many animals had a very loose structure. However, it is likely that even in these muscles depolarization may not have been completely uniform; therefore the measurements relating tension of the whole muscle to membrane

potential must be regarded as relative rather than absolute.

A further possible difficulty in the use of potassium depolarization to study tension is that various types of muscle fibre in the same muscle may behave differently. In the *Carcinus* closer muscle, where several different types of muscle fibre were distinguished, the problem of non-uniform behaviour may be serious. However, most of the muscle is made up of Type C muscle fibres, so it is probable that the behaviour of the muscle as a whole is determined largely by their responses.

In the crayfish walking leg opener muscle, the muscle fibres appeared to have similar characteristics, as judged by their responses to indirect stimulation. On *a priori* grounds the responses of these fibres to depolarization can be assumed to be fairly uniform. However, this assumption can be confirmed only by work with single muscle fibres.

Although the use of potassium contracture of whole muscles as a tool to study excitation-contraction coupling is evidently of limited value, it was felt that the technique could be used advantageously in mapping out some of the characteristics of the responses of crustacean muscles to depolarization.

(i) *Carcinus*: *Closer* *Muscle*

The responses of the *Carcinus* closer muscle to potassium depolarization were studied by injecting experimental solutions into the muscle through needles inserted through the

shell near the muscle or into the dactyl. The solutions were injected rapidly by means of a hypodermic syringe to insure speedy distribution of the solutions to various parts of the muscle. Usually the injection rate was 50 to 80 cc. per minute. (The volume of the propodite containing the muscle averaged 0.9cc.)

In some experiments only the mechanical response of the muscle was recorded; in other cases both electrical and mechanical measurements were made. Electrical measurements usually involved sampling the membrane potentials of six to ten exposed muscle fibres (Type C) in quick succession.

High potassium solutions for use in these experiments were made by substituting the desired amount of potassium for an equivalent amount of sodium which was omitted from the solution.

The mechanical response. Preliminary experiments were performed to ascertain the mechanical response to solutions containing high potassium concentrations. In these experiments recording of tension was made from the tip of the dactyl, using an isometric spring and a kymograph.

No contraction of the muscle was measured until potassium concentrations of 30 to 40 mM were applied. At potassium concentrations of 30 to 50 mM, a weak contraction of the muscle was observed (Fig. 72, a_1, b, d). This contraction lasted for periods of at least 10 minutes, provided perfusion was maintained. In many cases the contraction

slowly increased in magnitude during such prolonged perfusion. The contracture relaxed rapidly and completely when a solution containing the normal 10 mM KCl was perfused into the muscle. If the same raised concentration of KCl was applied soon after the first application, a contraction again occurred, and usually the second contraction was slightly stronger than the first (Fig. 72, b_1, 2). After further repetitions of the process, the contraction remained constant in height (Fig. 72, b).

As the amount of KCl in the experimental solution was increased, the strength of the contraction and its rate of rise to a final level also increased (Fig. 72 a, d). At KCl concentrations of 150 m M the contraction was maximal; raising the KCl concentration further did not give a stronger contraction (Fig. 72, a 3, 6).

Prolonged perfusion with a solution containing a KCl concentration of greater than about 50 mM resulted in a decline in magnitude of the response with time (Fig. 72, a 7, c 1, d 2). However, the tension remained above the base level for at least 10 minutes. When high concentrations of KCl (above 150 mM) were used, perfusion with normal saline after a prolonged stay in the high KCl solution failed to lower the tension to base level (Fig. 72, a 8, 9). When lower KCl concentrations (70 to 100 m M) were used, the tension returned to base level with application of normal saline after a prolonged exposure. However, subsequent

contractions evoked soon afterwards by application of the same KCl solution were less powerful than before (Fig. 72, c 1 to 4). This latter effect was even more marked when higher KCl concentrations were employed (Fig. 72 d, 2 to 4).

The observations indicate that potassium contracture in crustacean muscles has much in common with the same process in frog muscles. In both cases the response declines with time in high KCl concentrations, suggesting exhaustion of an activating agent (Hodgkin and Horowicz, 1960a), or development of an inhibition (see Discussion). However, the tension response of crustacean muscles declines more slowly. In time course of relaxation, the contracture of crustacean muscles is intermediate between frog "twitch" and "slow" fibres (Kuffler and Vaughan Williams, 1953b).

In many muscles the contractural response was a smooth, slowly rising tension which soon reached a plateau phase. However, in some muscles the response (to 60 to 100 mM KCl) was different in appearance, and consisted of a rapidly rising initial tension which decayed very soon after reaching its peak to a lower plateau (Fig. 72, e, f). Successive applications of a given KCl solution resulted in a decrease in the initial rapid phase of tension and an increase in height of the subsequent plateau (Fig. 72, f).

This observation suggests that two components of the contractile response are present in these muscles: a rapidly rising and declining initial phase, and a slower

secondary phase. It is possible that the first phase corresponds to activation of the excitable Type A fibres, and that the secondary phase corresponds to activation of Type B and Type C fibres. The motor nerves were found to be silent during responses of this type; therefore, the activity is myogenic. Spikes were never observed in fibres of this muscle during application of high KCl solutions. It is possible, therefore, that the two phases of the contractural response represent activity of two types of fibres with different time courses of response, as is the case in some frog muscles (Kuffler and Vaughan Williams, 1953b). It is also possible that all fibres have a similar response characterized by a rapid initial phase of tension. The former alternative seems more likely in view of the fact that the closer muscle is known to contain muscle fibres with different membrane properties and excitabilities.

In several muscles the tension developed by maximal stimulation of the motor axons was compared with the tension produced by perfusion of the muscle with a solution containing a strong KCl solution (150 to 250 mM). The results showed that activation of contraction by strong KCl was as effective as stimulation of the motor nerves.* In Fig. 72, g 1, stimulation of the "fast" axon produced a contraction which rapidly declined with continued stimulation. Stimulation of the "slow" axon (Fig. 72, g2) produced

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*The observation is evidence that the perfusion of the muscle is effective, and that KCl solutions depolarize the entire muscle.

a stronger contraction which was maintained for a longer time, but also declined rapidly. Subsequent stimulation of the axons gave smaller and briefer contractions. Application of a strong KCl solution gave a contraction which was more powerful and longer-lasting than the contractions produced by the motor axons (Fig. 72, g5).

In another muscle (Fig. 72, h) the contraction produced by simultaneous stimulation of "fast" and "slow" axons was similar in size and duration to that produced by application of a strong KCl solution.

Values for the maximum tension developed per unit cross-sectional area of the muscle were calculated, using the information about the mechanics of the muscle presented previously (see p. 46). It was estimated that the Carcinus closer muscle could develop a tension of 2300 to 4200 gm/cm^2 with maximum activation (five determinations). These values compare with 2500 to 4000 gm/cm^2 for single frog muscle fibres activated by maximum nerve stimulation, and 3000 to 4400 gm/cm^2 for the same fibres giving maximum contracture tension (Hodgkin and Horowicz, 1960b).

Effects of divalent cations. The work of Frank (1958, 1960) has shown that potassium contracture does not occur in frog skeletal muscle unless external calcium ions, or certain other divalent or multivalent cations, are available. In the case of an insect spiracular muscle,

Hoyle (1961) has found that potassium contracture occurs in the absence of external calcium, and is inhibited by a small excess of magnesium ions. It is of interest to know how crustacean muscle behaves when the external concentrations of divalent cations are altered.

When a solution containing no calcium and high KCl is perfused into a closer muscle, the normal contracture is abolished, provided that the muscle has previously been perfused for about two minutes with a calcium-free, low KCl solution (Fig. 73, a). When normal saline is applied, the muscle can again give a KCl contracture (Fig. 73, a6).

If a solution containing no calcium and high KCl is given without prior washing in calcium-free saline, the contracture was often greater than that produced in normal calcium (Fig. 73, b1, 2). However, after a number of contractures had occurred in normal calcium or in low calcium, the contracture of the muscle in zero calcium without prior washing in calcium-free solution was much reduced (Fig. 73, b).

It was found that a short period of perfusion in calcium-free solution, followed by return to normal saline, did not reduce the strength of a subsequent KCl contracture below that of a contracture induced previous to the treatment with zero calcium (Fig. 73, d,e). However, after prolonged perfusion with zero calcium, a marked reduction in subsequent contracture strength in normal calcium was produced (Fig. 73, e). If a high KCl solution containing

no calcium, or a small amount, was applied during a short period of perfusion with calcium-free saline, a marked reduction in the strength of a subsequent contracture was observed (Fig. 73, c,d). This reduction was much greater than that produced by several intervening contractures in a solution containing normal calcium.

The amount of calcium present in the solution influenced the initial rate of rise of the contracture tension as well as its strength. As the amount of calcium in the solution was increased, the response showed a slower rate of rise and a slower relaxation (Fig. 74,a). The size of the response was lower at high calcium concentrations, provided the KCl concentration was the same.

At calcium concentrations of less than 10 mM, the size of the contracture response rapidly became less with repeated application of KCl (Fig. 74,b). However, after a period of perfusion in high calcium solution, the response increased in size (Fig. 74, b8). At calcium concentrations greater than 10 mM, the size of the contracture declined relatively slowly with repeated application of high KCl (Fig. 74, b8, 9, 10).

It was found that the concentration of magnesium ions in the saline had relatively little effect on the size of the contracture. At high magnesium concentrations (above 35 mM), both size and rate of rise of the response were reduced slightly, as in high calcium solutions.

As has been reported by Frank (1961) for frog muscle, the presence of strontium ions in calcium-free saline permits the muscle to develop tension during application of high KCl (Fig. 75, a).* However, barium ions did not permit tension development in calcium-free solutions (Fig. 75, b, c). If a small amount of calcium was added to a barium saline, contracture in response to high KCl could occur (Fig. 75, b, c). Frank (1962) also found that barium could not substitute for calcium in frog muscle.

The experiments with divalent cations indicate that calcium (or a chemically similar cation such as strontium) is necessary for potassium contracture. Depletion of calcium (by prolonged washing in calcium-free saline, for example) inhibits tension development during potassium contracture. Application of high KCl during perfusion with calcium-free saline apparently exhausts or inhibits some step in the excitation-contraction coupling process, even though no tension is developed by this treatment. Raising the calcium concentration above normal decreases the excitability of the muscle (presumably by making KCl less effective as a depolarizing agent; see Werman and Grundfest, 1961). The reverse process, reduction of calcium, apparently has the effect of increasing the depolarizing effectiveness of KCl, since the contractions in low calcium were initially

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*Since this work was completed, it has been reported by others (Zacharova et al., 1962) that strontium permits tension development in crayfish muscle fibres during direct depolarization with intracellular electrodes.

stronger than those in normal saline when the muscle had not been previously perfused with a "conditioning" low-calcium, low-potassium solution.

<u>Contractions in low-chloride solutions</u>. Hodgkin and Horowicz (1959) observed that alteration of the chloride concentration of the solution surrounding a frog muscle fibre produced a transient change in membrane potential. With a lowering of chloride concentration, transient depolarization was produced, which disappeared within a few minutes.

No observations were made by these authors on contractile activity associated with depolarization. In the present study it was found that when a solution with over a third of the sodium chloride replaced with osmotically equivalent sodium sulphate was perfused into the cell, a transient contraction, lasting about 3 seconds, was produced. About one minute after this transient depolarization had subsided, a gradually developing, powerful contraction of the muscle occurred, which was slowly reversible with perfusion of normal saline. This latter contraction was unlike the usual potassium contracture in that the tension developed could be maintained at a high level for a much longer time than the tension of a potassium contracture.

Immediately after the termination of a sulphate-induced contracture lasting 5 to 10 minutes, a normal-sized potassium contracture could be produced.

A solution containing a high sulphate concentration undoubtedly reduces the concentration of ionized calcium present in the perfusion medium (Hill and Howarth, 1957; Hodgkin and Horowicz, 1959). This would tend to reduce the tension obtained during a potassium contracture. It is evident therefore, that the action of sulphate is quite different from that of KCl.

A prolonged, slowly reversible contraction could be produced by substituting sucrose for sodium chloride. It has already been shown by Fatt and Katz (1953a) that sucrose solutions depolarize crab muscles.

The sulphate and sucrose contractures were not investigated further in the present study. The initial rapid phase is probably due to the "chloride depolarization" of Hodgkin and Horowicz. The later prolonged contractures probably are similar to those described in frog "slow" fibres by Swift et al. (1960) and by van der Kloot (1961). Further study of this phenomenon would be of interest.

Membrane potential and KCl. It has been shown that *Carcinus* closer muscles develop contracture in solutions containing KCl concentrations greater than about 30 mM. It is of importance to determine the membrane potential at which contraction occurs in fibres of this muscle. This value can only be found by approximation in whole muscle preparations, but it was of value to have this approximation.

Measurements of membrane potential were confined to Type C muscle fibres, since they made up most of the muscle and were the only fibres which could be easily impaled without laborious searching. When a series of measurements was made on one muscle, the same muscle fibres were used in all measurements. Solutions were changed by rapid perfusion of the muscle (p. 100).

When the KCl concentration of the perfusion medium was altered, the membrane potentials of the muscle fibres rapidly assumed a new value, but if the new solution was maintained for any length of time, a further slow drift in membrane potential occurred. The changes were much slower than comparable ones in frog muscle fibres (Hodgkin and Horowicz, 1959, 1960a). It was found that about half an hour was needed before a final steady value of membrane potential was reached at a new KCl concentration (cf. Werman et al., 1961). Provided this length of time was allowed, the plot of membrane potential against external potassium concentration had a slope approximating that which would be expected from the Nernst equation (Table 6; Fig. 76). The slope of the line in the example shown departs from theoretical behaviour at KCl concentrations less than 20 mM and greater than 70 mM. The former deviation may be due to decreased potassium conductance at higher membrane potentials (Hoyle, 1957; Girardier et al., 1961); the latter deviation can probably be attributed to progressive loading of the

muscle with KCl during prolonged soaking in increasingly greater KCl concentrations.

In the experiment quoted above, membrane potentials were measured as the KCl concentration was progressively increased. It is well known that when muscles soaked in high KCl concentration, the membrane potential does not return immediately to its former level, but approaches it very slowly (Fatt and Katz, 1953a; Adrian, 1960; Hodgkin and Horowicz, 1959, 1960a). In the closer muscle of *Carcinus* this phenomenon was quite marked. One experiment illustrating this point is given in Table 7. It can be seen that repeated application of a high KCl solution at short intervals drives the membrane potential downwards. This phenomenon is an adequate explanation for the fact that KCl contractures increase in size during successive applications of 30 to 50 mM KCl solutions.

Because of "membrane hysterisis", almost all of the measurements described below were made with progressive increase in KCl concentration.

Hodgkin and Horowicz (1960 a) observed that frog muscle fibres with large diameters repolarized more slowly than fibres with small diameters when the external potassium concentration was lowered. They accounted for the asymmetry between "on" and "off" effects of potassium by postulating a special region of the muscle fibre in which potassium ions are retained for a short time. This suggestion has recently been further elaborated by Adrian and Freygang (1962, a, b).

There is electron microscope evidence for the existence of a large extracellular space within Carcinus muscle fibres (Peachey, 1959). If the hypothesis of the Cambridge workers is correct, and applicable at least in part to crab muscles, the large diameters of the Carcinus muscle fibres together with their extensive extracellular spaces may account for the slowness of the electrical responses to changes in external potassium ion concentration.

It would have been of interest to perform experiments with solutions of constant [K] - [Cl] product (Hodgkin and Horowicz, 1959) on crab muscle. However, a satisfactory impermeant anion which could be substituted for chloride was not readily available*, so experiments were limited to observations of the effect of changing external potassium at constant chloride concentration.

It has been shown (Fig. 76) that the slope of the membrane potential-Ko relation is similar to that predicted by the Nernst equation, provided sufficient time is allowed in each KCl solution used. When membrane potentials are measured very soon after application of a new KCl solution, the slope of the graph is much less than that expected on the basis of the Nernst equation (Fig. 77, 78). Care must be taken, therefore, to distinguish between "immediate" and "delayed" effects of KCl application. The "immediate" KCl effect was of more interest in connection with potassium

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*Boistel and Fatt (1958) have used pyroglutamate and acetylglycine.

contracture, because contracture tension typically reaches its maximum value within half a minute of perfusion of the muscle with a high KCl solution.

A number of muscles were perfused for short periods with salines of gradually increasing KCl concentration, in order to find the KCl concentration and corresponding membrane potential at which the muscle develops tension. At each KCl concentration, membrane potential measurements from 5 to 10 fibres were obtained within two minutes; then the next KCl concentration was applied.

Typical results of such experiments are shown in Figs. 77 and 78. In Fig. 77, two muscles were examined. Membrane potentials from one muscle, averaged slightly less than those from the other muscle, but in both cases tension appeared when the average membrane potential was less than about 55 mV, and increased greatly in magnitude when the membrane potential was lowered still further. No tension was developed when average membrane potentials were 55 to 60 mV in magnitude.

Experiments done on other muscles (e.g., Fig. 78) led to the same result: that tension is developed when the average membrane potential is lower than 55 mV. Results from fifteen muscles gave an average "threshold" membrane potential for tension development of 55.3 ± 0.7 mV (range, 58 to 53.5 mV).

It is possible that this value is too high, because: (1) The fibres with lower than average membrane potentials may give rise to all of the observed tension in the "threshold" region; (2) Type B fibres have lower average membrane potentials than Type C fibres at normal KCl concentrations and may give rise to tension in the "threshold" region at lower membrane potentials than those observed in Type C muscle fibres. The rapid rise in tension with small increments of depolarization suggests, however, that Type C fibres, which make up most of the muscle, are probably activated as the membrane potential is lowered past 55 mV.

It is also possible that the various types of muscle fibre have different "threshold" membrane potentials. Such a possibility could only be explored by isolation of single muscle fibres.

Inhibition of contracture. It was observed that stimulation of the inhibitor axon could produce complete or partial relaxation of KCl contracture.* The phenomenon is illustrated in Fig. 79. Relaxation of tension was rapid and complete when the inhibitor axon was stimulated at high frequencies, but gradual and incomplete at low frequencies. After cessation of stimulation, the tension gradually approached its original level. Rapid perfusion with fresh KCl solution speeded the return of tension to a level usually somewhat less than the original (Fig. 79a)

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*It was necessary to keep the KCl concentration below 50 mM to avoid blocking nerve conduction.

During stimulation of the inhibitor axon, hyperpolarization of the membranes of muscle fibres examined with intracellular electrodes was observed (Fig. 79b). This phenomenon has previously been thoroughly studied by Boistel and Fatt (1958) and by Hoyle and Wiersma (1958b). It is probable that this membrane hyperpolarization is mainly responsible for relaxation of tension. However, studies on innervated single muscle fibres would be necessary to determine this point more precisely.

Effect of thermal history of the animal on potassium chloride contracture. It was found that the muscles of animals kept for a short period in the cold showed different responses to high KCl solutions than muscles of animals maintained at a higher temperature.

The discovery was originally accidental. A change in Glasgow weather during the Fall of 1961 gave rise to a sudden cold spell. Animals kept in an unheated aquarium room were exposed to a lower temperature than they had experienced previously. When muscles of these animals were perfused with solutions containing raised KCl concentrations, they were found to develop tension at lower KCl concentrations than did muscles from the same animals before exposure to cold. An example is shown in Fig. 80.

The shift in the tension-potassium curve in these muscles was accompanied by a shift in the membrane potential-potassium curve. Membrane potentials at a given KCl

concentration were lower in animals exposed to cold (Fig. 80).

A series of observations was made on muscles of two groups of animals. One group was kept at 15° C. for two weeks, and the other at 5° C. for two weeks. Muscles from each group were perfused with solutions of increasing KCl concentration, and measurements were made of "immediate" membrane potential and tension responses. Muscles of the same size were chosen to allow valid comparison of the sizes of the tension responses. Only muscles of loose structure which could be easily perfused, were used.

The results are summarized in Fig. 81. Both tension and membrane potential plots show the shifts of Fig. 80. These shifts were significant. All of the values for tension development in the muscles from cold-acclimated animals fell above the range of values for the control group at 40 and 70 mM KCl. The average membrane potentials for cold-acclimated muscles were all lower than the means for muscles of the control group at 40 and 70 mM KCl.

In both groups of muscles the "threshold" membrane potential for tension development was about 55 mV. It appears, therefore, that the shift in the tension-KCl curve is dependent on the shift of the membrane potential-KCl curve.

What causes the latter shift?

An hypothesis which comes readily to mind is that muscles from cold-acclimated animals contain less potassium.

This would produce the observed result: that depolarization is greater at a given KCl concentration in muscles from cold-acclimated animals. Also, it is known that muscles of certain animals lose potassium ions and gain sodium ions when exposed to cold (Hashish, 1958; Eliassen and Leivestad, 1961).

A test of this hypothesis was made by determining the potassium contents of muscles from animals kept at 15° C. and at 5° C. for two weeks. The muscle used for analysis was the extensor of the claw. The muscles were rapidly dissected, washed for 30 seconds in isosmotic sucrose solution (Shaw, 1955), blotted, and oven-dried to constant weight to determine water content. The dried muscles were ground in 5% trichloracetic acid (cf. Robertson, 1960b), and then extracted in this solution for two days. The potassium contents of the extracts were determined by means of an Eel flame photometer.*

The results of these analyses are given in Table 8. It was found that results for paired muscles from the same animal were similar. Therefore, the method employed was probably adequate for comparative measurements, although perhaps not for absolute measurements.

The difference between the means of muscles from the two groups was significant at the 5% level ($t = 1.95$).

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*The author is indebted to Dr. G. Leaf of the Biochemistry Department, University of Glasgow, for permission to use this instrument.

Therefore, the conclusion can be drawn that muscles from cold-acclimated animals have less potassium.

Potassium loss in cooled muscles would be expected from the lowering of resting potential with lowered temperature which is known to occur in this muscle (Fatt and Katz, 1953a). A lowered resting potential would result in a loss of potassium by the muscle. Less efficient extrusion of sodium probably also plays a part.

If muscles of animals exposed to cold lose potassium, the blood of these animals should gain potassium. A test of this notion was made by determining blood potassium concentrations of animals exposed to various temperature conditions. Potassium determinations were made on 0.2 ml. samples of cell-free serum. About 0.5 ml. of blood was drawn for each analysis and centrifuged to separate the cells from the serum.

The animals to be tested were all males taken from the same shipment of crabs.* Prior to the experiment, nine animals were kept at 17° C. for two weeks and nine animals were kept at 5° C. for two weeks. Blood samples were drawn from all animals at the end of this period. Then five of the animals that had been kept at 17° C. were transferred to 5° C. (Group II, Table 9); the remaining four were kept at 17° C. (Group I, Table 9). Five of the animals kept at 5° C. were transferred to 17° C. (Group IV, Table 9); the others were left at 5° C. (Group III, Table 9).

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*The animals were not fed, because it is known that food intake causes increase in blood potassium levels (Robertson, 1960a).

The results (Table 9, Fig. 82) show: (1) that the animals kept for two weeks at 17° C. have higher blood potassium levels than those kept at 5° C. for two weeks (at the start of the experiment); (2) that the animals transferred from 17° C. to 5° C. showed a transient increase in blood potassium, followed by a drop in blood potassium to the level of the cold-acclimated group; (3) that the animals transferred from 5° C. to 17° C. showed a gradual increase in blood potassium to the level of the animals kept at 17° C.; (4) that the two control groups (I and III) showed fluctuations in measured blood potassium levels, but no maintained increase or decrease (although a slight increase may have been present in Group III).

The sudden increase in blood potassium in Group II animals probably reflects a loss of potassium from the muscles immediately following transfer to the lower temperature. It is more difficult to account for the very rapid subsequent fall in blood potassium levels. Possible explanations are: (1) interaction with regulation of other blood ions; (2) active excretion of potassium; (3) uptake of potassium by other types of cell.

Whatever the mechanisms which control blood potassium levels in these unfed animals, it is evident that the blood potassium levels move in the same direction as those of the

muscles. This factor may act to maintain a constant muscle membrane potential in animals at different temperatures, and may be of value in enabling the animal to adapt its activity to the new environmental conditions. (cf. Hoyle, 1954).

The observations on muscle and blood potassium changes are of interest in the present study because they indicate how one source of variation in measurements of muscle resting potentials can originate. Other factors such as season, stage in the moulting cycle, etc., are probably important as well.

Fig. 72. Responses of *Carcinus* closer muscles to potassium depolarization. Each letter indicates a different preparation. The numbers indicate application of experimental solutions as described below. Breaks in the base line indicate time gaps. Contraction is downwards. Horizontal calibrations, 5 min.; vertical calibration, 30 gm.

a) 1. Application of 50 mM KCl solution, followed by return to normal saline (relaxation).
2. Application and withdrawal of 100 mM KCl.
3. Application and withdrawal of 180 mM KCl.
4, 5. Application and withdrawal of 70 mM KCl.
6. Application and removal of 250 mM KCl.
7. Application of 250 mM. KCl; continued perfusion.
8. Application of normal (10 mM KCl) saline.
9. Disconnection of the muscle from the recoding lever after 5 mins.

b) 1. 2. 3. 4. Application and removal of 40 mM KCl; (4) was done 10 mins. after (3).

c) 1. Application of 70 mM KCl, and removal after 5 mins.
2. Application and removal of 40 mM KCl.
3. Application and removal of 70 mM KCl.
4. Application and removal of 40 mM KCl.

d) 1. Successive application of 30 mM, 35 mM, 45 mM, and 50 mM KCl, followed by return to normal saline.
2. Perfusion for 1 min. with 200 mM KCl.
3. Perfusion with 200 mM KCl after 10 min.; return to normal saline.
4. Perfusion with 200 mM KCl after 10 min.

e) 1,2. Responses of a preparation to brief application of 90 mM KCl.

f) 1 to 5. Responses of a preparation to successive brief applications of 90 mM KCl.

g) 1. Stimulation of the "fast" axon at 150 per sec. for one min.
2. Stimulation of the "slow" axon at 150 per sec. for three min.
3. Stimulation of the "fast" axon at 150 per sec. for one min.
4. Stimulation of the "slow" axon at 150 per sec. for one min.
5. Application of 180 mM KCl.

h) 1. Stimulation of the "fast" and "slow" axons at 180 per sec. for two min.
2. Application of 180 mM. KCl.

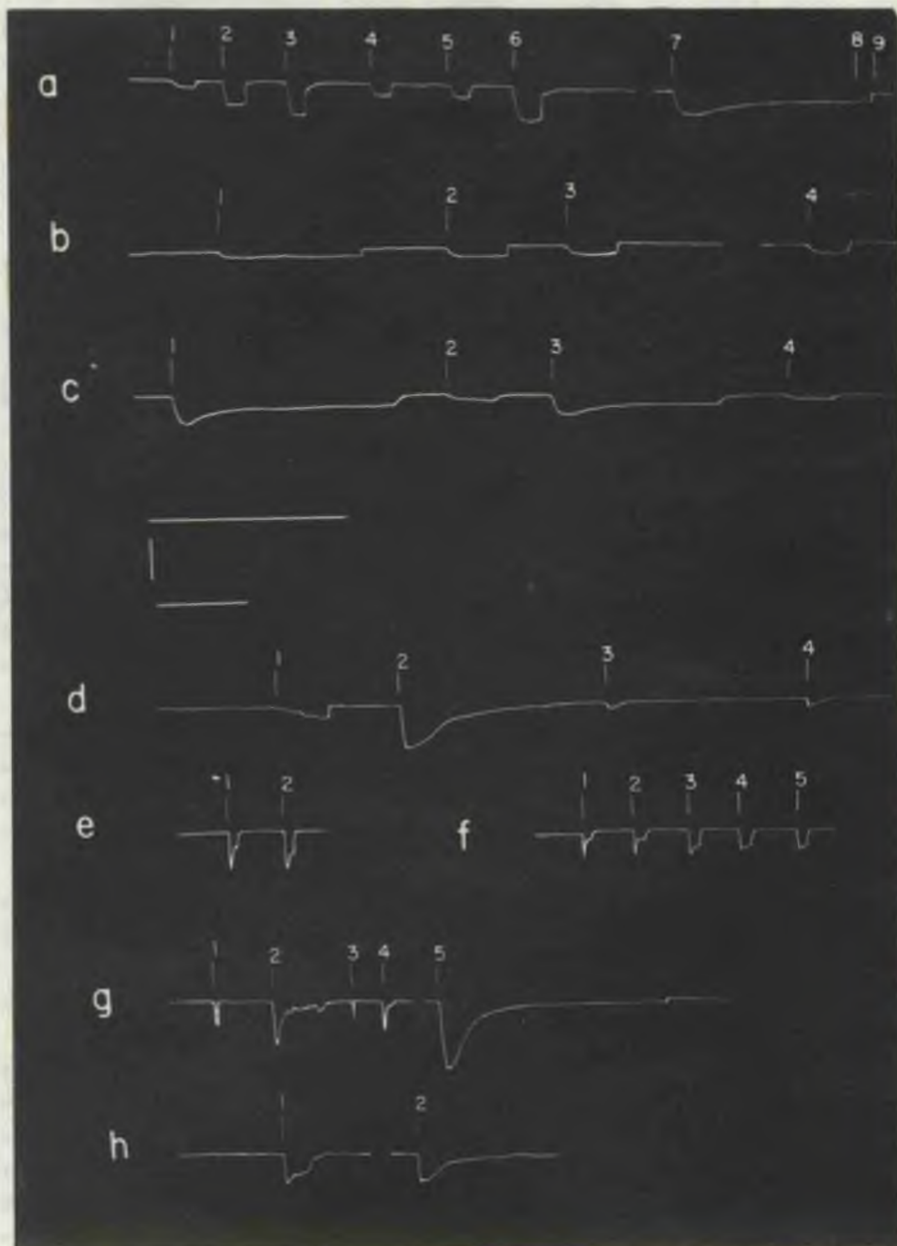
a
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Fig. 73. Potassium contracture in *Carcinus* closer muscles as influenced by calcium ion concentration. Horizontal calibration, 5 min.; vertical calibration, 30 gm. Letters and numbers as in Fig. 72.

a) 1,2. Application and removal of 60 mM. KCl solution; normal Ca (15 mM).
3. Application of solution containing 0 Ca, 10 mM KCl.
4. Application of solution containing 0 Ca, 60 mM KCl.
5. Application of normal saline (15 mM Ca, 10 mM KCl.)
6. Application and removal of 60 mM KCl.

b) 1. Application and removal of 80 mM KCl.
2. Application of 0 Ca, 80 mM KCl and removal with normal saline.
3. Application of 0 Ca, 10 mM KCl.
4. Application of 0 Ca, 100 mM KCl, followed by return to 0 Ca, 10 mM KCl after ½ minute.
5. Application of normal saline.
6. Application of 15 mM Ca, 80 mM KCl. and removal with normal saline.
7. Application and removal of 0 Ca, 80 mM KCl.
8. Application and removal of 15 mM Ca, 80 mM KCl.

c) 1. Application and removal of 15 mM Ca, 80 mM KCl.
2. " " " " 10 mM Ca, 80 mM KCl.
3. Application of 2 mM Ca, 10 mM KCl.
4. Application of 2 mM Ca, 80 mM KCl, and removal with 2 mM Ca, 10 mM KCl.
5. Application of normal saline.
6,7. Application and removal of 15 mM Ca, 80 mM KCl.

d) 1. Application and removal of a solution containing 15 mM Ca, 80 mM KCl.
2. Application of 0 mM Ca, 10 mM KCl.
3. Return to normal saline.
4. As in 1.
5. As in 2.
6. As in 3.
7. As in 1.
8. As in 2.
9. Application of 0 Ca, 80 mM KCl, and removal with 0 Ca, 10 mM KCl after ½ min.
10. Return to normal saline.
11. Application and removal of 15 mM Ca, 80 mM KCl after 3 min. in normal saline.

e) 1. Application and removal of 15 mM Ca, 80 mM KCl.
2. Application of 0 Ca, 10 mM KCl.
3. Return to normal saline.
4. As in 1.
5. As in 2.
6. As in 3.
7. Application of 15 mM Ca, 80 mM KCl after 3 min. in normal saline.

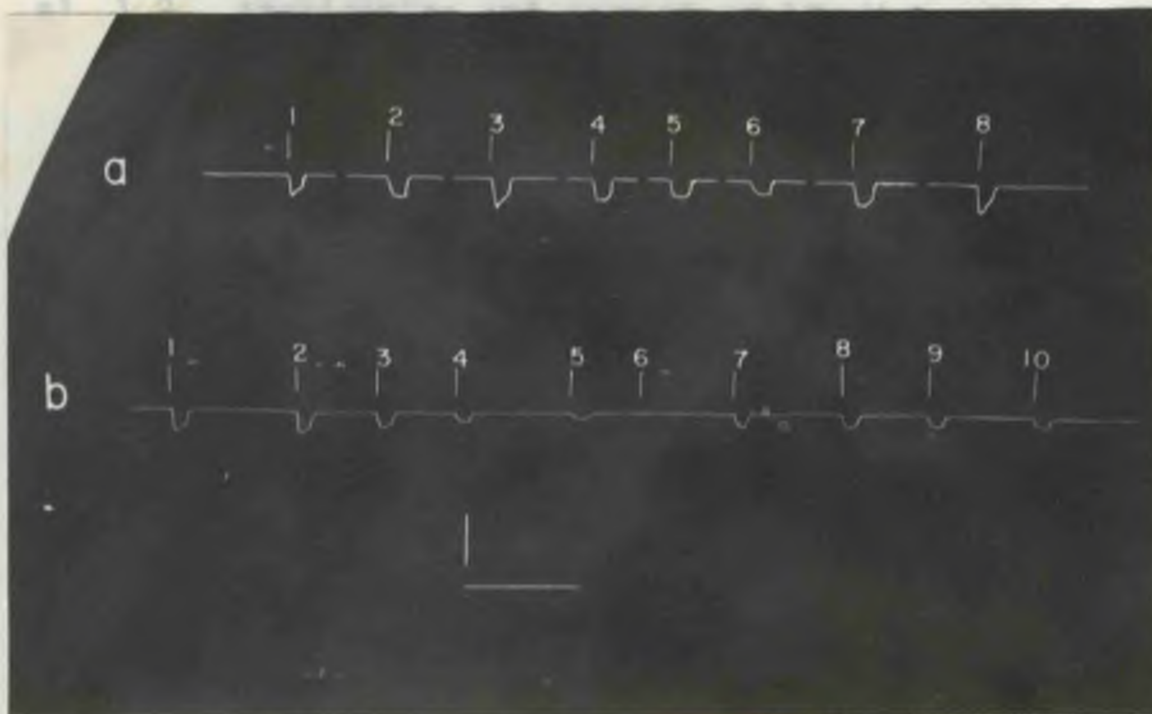


Fig. 74. Potassium contracture in Carcinus closer muscles as influenced by calcium ion concentration. Calibrations, letters and numbers as in Fig. 73. In a), breaks in the base line indicate time gaps of 2 min.

a) 1. Application and removal of 15 mM Ca, 70 mM KCl.
2. " " " " 60 mM Ca, 70 mM KCl.
3. " " " " 15 mM Ca, 70 mM KCl.
4. " " " " 20 mM Ca, 70 mM KCl.
5. " " " " 30 mM Ca, 70 mM KCl.
6. " " " " 60 mM Ca, 70 mM KCl.
7. " " " " 15 mM Ca, 70 mM KCl.
8. " " " " 5 mM Ca, 70 mM KCl.

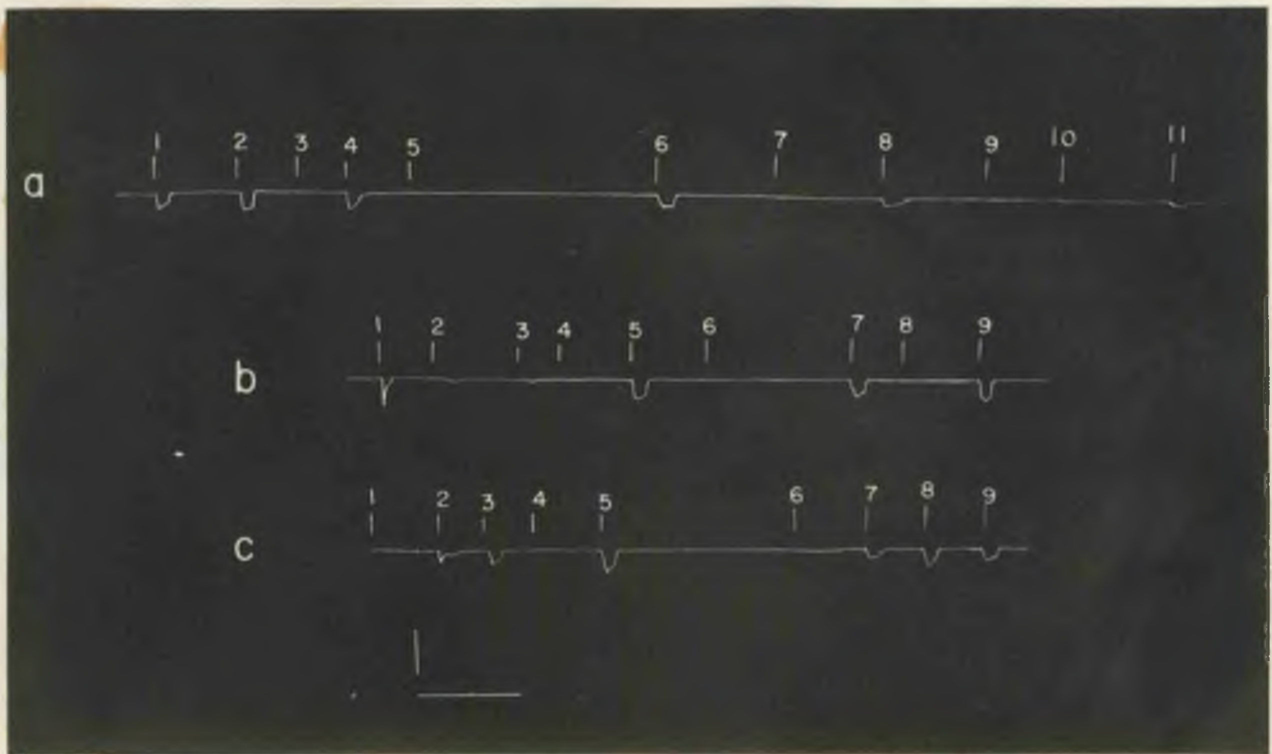
b) 1. Application and removal of 15 mM Ca, 80 mM KCl.
2 to 5. " " " " 8 mM Ca, 80 mM KCl.
6. Application of 60 mM Ca, 10 mM KCl.
7. Application of 10 mM Ca, 80 mM KCl; removal with normal saline.
8 to 10. Application and removal of 15 mM Ca, 80 mM KCl.

Fig. 75. Experiments dealing with effects of divalent cations on the potassium contracture of *Carcinus* closer muscles. Calibrations, letters, and numbers as in Fig. 73.

a) 1,2. Application and removal of 15 mM Ca, 70 mM KCl.
3. Application of 0 Ca, 17 mM Sr, 10 mM KCl.
4. Application of 0 Ca, 17 mM Sr, 70 mM KCl, and removal with 0 Ca, 17 mM Sr, 10 mM KCl.
5. Return to normal saline.
6. As in 1.
7. As in 3.
8. As in 4.
9. Application of 0 Ca, 10 mM KCl.
10. Application of 0 Ca, 70 mM KCl, and removal with normal saline after ½ minute.
11. As in 1.

b) 1. Application and removal of 15 mM Ca, 70 mM KCl.
2. Application of 0 Ca, 17 mM Ba, 10 mM KCl.
3. Application of 0 Ca, 17 mM Ba, 70 mM KCl, and removal after ½ min. with 0 Ca, 17 mM Ba, 10 mM KCl.
4. Return to normal saline.
5. Application and removal of 15 mM Ca, 70 mM KCl.
6. Application of 5 mM Ca, 17 mM Ba, 10 mM KCl.
7. Application and removal of 5 mM Ca, 17 mM Ba, 70 mM KCl.
8. Return to normal saline.
9. Application and removal of 15 mM Ca, 70 mM KCl.

c) 1. Application of 0 Ca, 17 mM Ba, 10 mM KCl.
2. Application of 2 mM Ca, 17 mM Ba, 70 mM KCl, and removal with 0 Ca, 17 mM Ba, 10 mM KCl.
3. Application of 5mM Ca, 17 mM Ba, 70 mM KCl, and removal as in 2.
4. Application of normal saline.
5. Application and removal of 15 mM Ca, 70 mM KCl.
6. Application of 5 mM Ca, 17 mM Ba, 10 mM KCl.
7. Application of 5 mM Ca, 17 mM Ba, 70 mM KCl, and removal with normal saline.
8,9. Application and removal of 15 mM Ca, 70 mM KCl.



a
1 2 3 4 5 6 7 8 9 10 11
b
1 2 3 4 5 6 7 8 9
c
1 2 3 4 5 6 7 8 9

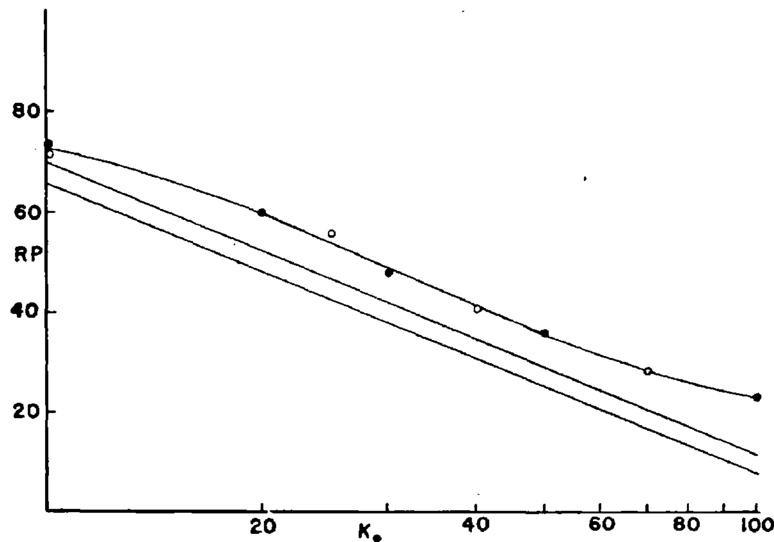


Fig. 76. Membrane potential (RP, millivolts inside negative) and external potassium ion concentration (Ko, mM, log scale) in muscle fibres of two Carcinus closer muscles. Open circles, Muscle 3 of Table 6; filled circles, Muscle 2 of Table 6. The straight lines show the theoretical behaviour of a muscle containing 140 mM K (lower line) and 160 mM K (upper line), as predicted by the Nernst equation.

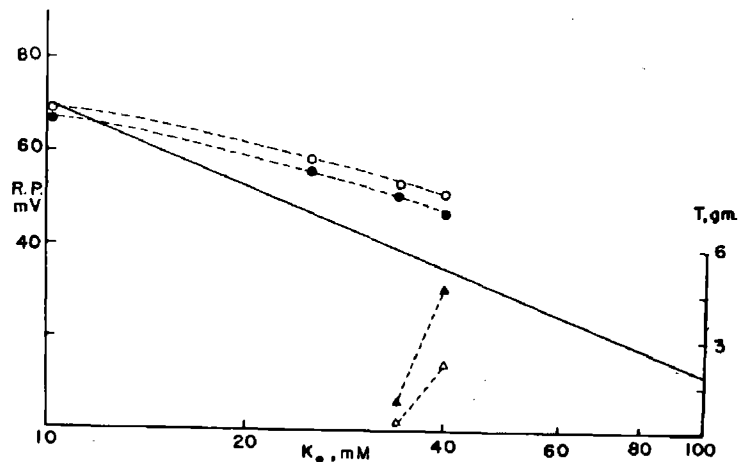


Fig. 77. "Immediate" membrane potential and tension responses of two different Carcinus closer muscles during perfusion with solutions of increasing KCl concentrations. Open circles, average membrane potentials of 8 fibres from one muscle; open triangles, corresponding tension of the whole muscle. Filled symbols are corresponding responses of a muscle from a different animal (circles, average membrane potentials of 7 fibres). Solid line indicates the theoretical membrane potential of a muscle containing 160 mM KCl. (R.P. and Ko as in Fig. 76; T, tension of the whole muscle in grams, measured at the tip of the dactyl).

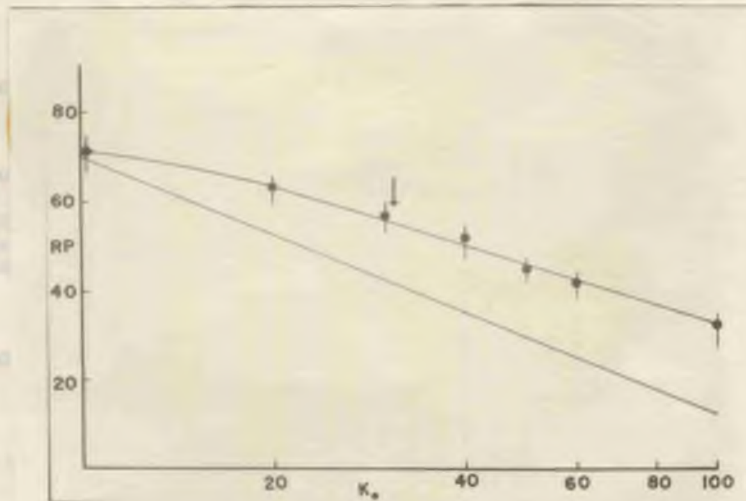


Fig. 78. Average "immediate" membrane potentials of thirty fibres from three muscles of one animal (Carcinus). Vertical lines show the variation encountered. Lower line shows the theoretical membrane potential of a muscle containing 160 mM KCl. The arrows show the average membrane potential at which tension was developed by the muscle. (RP and Ko as in Fig. 76).

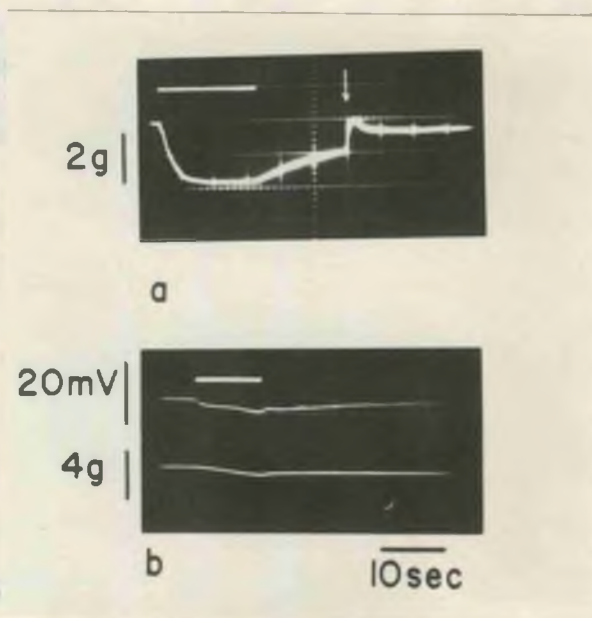


Fig. 79. Effects on contracture tension of stimulation of the inhibitor axon (Carcinus closer muscle). (a) Stimulation of the inhibitor axon at 60 per sec. (indicated by white bar above oscilloscope trace) caused complete relaxation of contracture in 40 mM KCl. At the arrow, rapid perfusion of KCl was given. (b) Stimulation of the inhibitor axon at 30 per sec. caused membrane hyperpolarization (top trace) and partial relaxation of contracture in 40 mM KCl (bottom trace). Membrane potential of the muscle fibre at the start of the experiment was 48 mV.

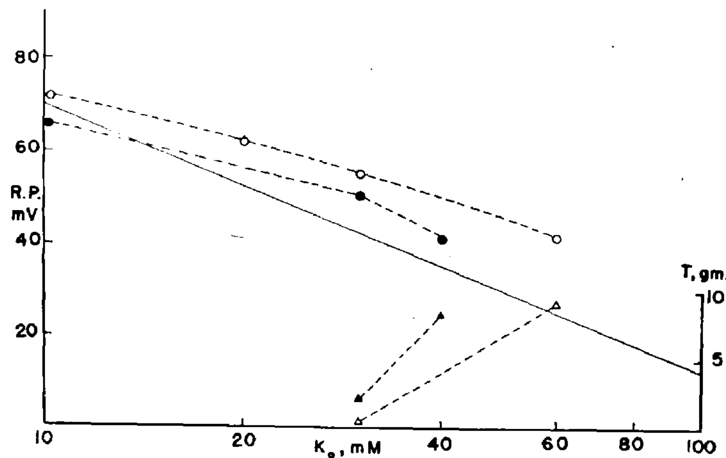


Fig. 80. "Immediate" membrane potential and tension responses from two closer muscles from the same animal (Carcinus) before (open symbols) and three days after (filled symbols) exposure of the animal to a lowered environmental temperature. Circles represent membrane potentials (average values from ten muscle fibres in each case); triangles represent tension of the whole muscle. (R.P., Ko, T, and solid line as in Fig. 77.)

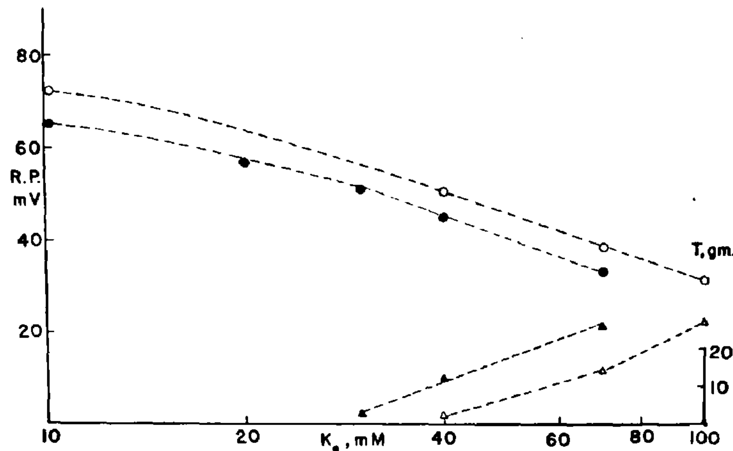


Fig. 81. Comparison of "immediate" membrane potential and tension responses of muscles from specimens of Carcinus acclimated to 16° C. for two weeks (open symbols) and to 50° C. for two weeks (filled symbols). In each case two muscles from each of three animals were used, and membrane potentials from seven fibres in each muscle were measured at each KCl concentration. All animals were the same size. (R.P., Ko, and T as in Fig. 77).

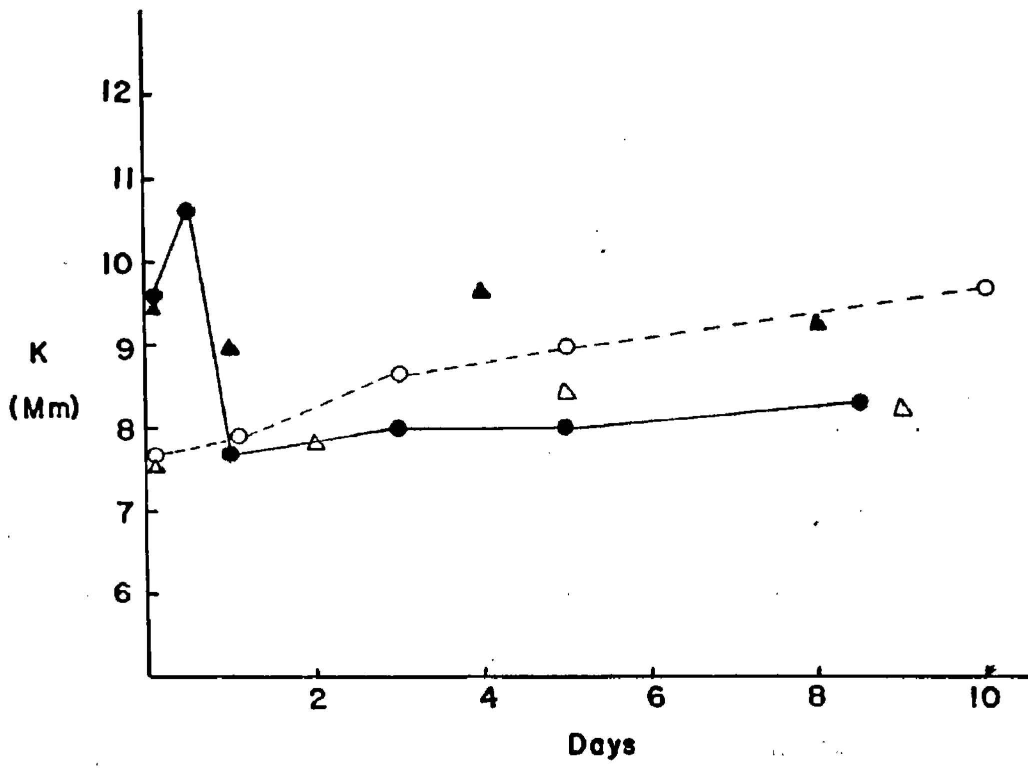


Fig. 82. Blood potassium levels as a function of time and environmental temperature in *Carcinus*. Mean values from Table 9 are plotted against time. Filled triangles, Group I; filled circles, Group II; open triangles, Group III, open circles, Group IV.

Table 6. Membrane potentials of fibres of muscles soaked in normal saline and in salines of different potassium concentrations.

Muscle 1*			Muscle 2*			Muscle 3*		
Time (min)	Ko (mM)	M.P.[1] (MV)	Time (min)	Ko (mM)	M.P.[2] (mV)	Time (min)	Ko (mM)	M.P.[3] (mV)
0	10		0	10		0	10	
40	10	71.5	35	10	73	40	10	71
47	10		45	20		48	25	
100	10	69.5	75	20	60	80	25	57
110	10		80	30		90	40	
150	10	68	130	30	48.5	130	40	41
158	10		138	50		137	70	
190	10	66.5	180	50	36.5	155	70	29
			188	100				
			220	100	24			

Ko, external potassium ion concentration; M.P., average membrane potential; time taken from initial perfusion of the muscle.

*All muscles were from the same animal.

1. Average values for 8 muscle fibres.
2. Average values for 10 muscle fibres.
3. Average values for 10 muscle fibres.

Table 7. Membrane potentials of fibres from a muscle perfused alternately for 5 min. periods with high and low KCl solutions*.

	Potassium Chloride Concentration (mM)	Membrane Potential (mV)
1.	8	73.5
2.	35	58.5
3.	8	70
4.	35	52
5.	8	63
6.	35	50.5

* average values for 10 muscle fibres.

Table 8. Potassium concentrations of *Carcinus* claw extensor muscles from animals kept at 15° C. and at 5° C.

Animal	Muscle		Potassium (mM per Kg. water)
15° C. group			
1.	ext. r. claw		154
2.	ext. r. claw		151
3.	ext. r. claw		142
4.	ext. r. claw		153
		Mean	150 ± 2.7 (S.E.)
5° C. Group			
1.	ext. r. claw		132
1.	ext. l. claw		132
2.	ext. r. claw		148
2.	ext. l. claw		146
3.	ext. r. claw		149
3.	ext. l. claw		151
5.	ext. r. claw		129
5.	ext. l. claw		124
		Mean	139 ± 3.7 (S.E.)

Table 9. Blood potassium concentrations of specimens of <u>Carcinus</u> exposed to different temperature conditions.

	Time (days)								
	0	½	1	2	3	5	8	9	10
Group I									
1.	10.3		9.8			10.0	9.4		
2.	9.9		8.9			9.8	9.4		
3.	8.9		9.0			9.4	8.9		
4.	8.5		7.8			9.2	8.8		
mean	9.4		8.9			9.6	9.2		
Group II									
1.	9.6	10.5	8.2		8.6			8.7	
2.	9.0		6.8		7.4	8.1		7.5	
3.	9.5	10.7	7.1		7.8	8.1		9.0	
4.	9.8	10.8	8.0		8.1	8.2		8.4	
5.	10.0	10.4	8.2			7.6		8.0	
mean	9.6	10.6	7.7		8.0	8.0		8.3	
Group III									
1.	7.8			8.0		8.5		8.2	
2.	7.5			7.5		8.6		8.5	
3.	7.1			(dead)					
4.	7.9			8.0		8.1		8.0	
mean	7.5			7.8		8.4		8.2	
Group IV									
1.	8.0		8.1		8.6	8.8			10.0
2.	7.8		8.0		8.8	9.1			9.5
3.	7.3		8.6			10.0			9.6
4.	7.5		7.2			9.1			9.6
5.	8.0		7.7			8.1			9.9
mean	7.7		7.9		8.7	9.0			9.7

ii) <u>Astacus: Walking Leg Opener</u>

The very small walking leg opener muscle of *Astacus* has many advantages as a preparation for the study of membrane depolarization by chemical agents, and the relation of this depolarization to tension developed by the muscle. These advantages include: (1) the small size of the muscle. A single drop of saline is sufficient to bathe it. It was estimated that the muscle contained from 30 to 50 muscle fibres in a flat sheet about 3 fibres thick. (2) The relative uniformity of its constituent fibres apparent from the study of indirectly produced electrical responses.

In the preparations used in these experiments, tension was recorded from the tip of the dactyl. A fine tube attached to a syringe was used to apply solutions to the exposed surface of the muscle. Membrane potentials from representative muscle fibres were recorded during application of solutions. Artefacts resulting from displacement of the electrode from the muscle fibre were frequent, but a large number of experiments were performed, and many successful recordings were obtained.

When solutions containing excess KCl were added to an opener muscle preparation, fibres examined in all parts of the muscle showed prompt depolarization which could be reversed by returning the muscle to normal (5 mM KCl) saline. Provided the period of KCl depolarization was

kept short, and adequate resting time (5 minutes) was allowed between successive depolarizations, KCl solutions could be applied many times to the same preparation with similar results (Fig. 83, a, b,). When fibres in different parts of the muscle were recorded from, depolarization was found to be similar in all of them (Fig. 83 c, d, e). It was sometimes seen to be several millivolts less in fibres deep in the muscle, but the total variation was not great. In six fibres of one muscle, responses to application of KCl averaged 14 $\pm$ 0.7 mV (S.E.), with extreme values of 17 mV and 12 mV. In five different preparations, in each of which three fibres were recorded from, the average depolarization response to application of 13 mM KCl was 15 mV, with a range of average values in different muscles of 13 mV to 16.5 mV. All animals were males from the same shipment, of about the same size, and kept under the same conditions. The recordings were made in December.

It was observed that KCl contracture occurred only when the membrane potentials of the fibres recorded from were lowered past an average "threshold" value of 60 mV (range, 62 to 57 mV in 10 preparations). In Fig. 83, none of the fibres recorded from showed depolarizations of sufficient magnitude to lower the membrane potential past 60 mV, and in all cases the muscle developed no tension. When KCl concentrations of 15 mM to 18 mM or greater were applied, membrane potentials were lowered past 60 mV, and

a reversible contracture was produced (Figs. 84, 85, 86). The contracture increased in size as more concentrated solutions of KCl were added (Fig. 86). When KCl concentrations of more than 40 mM were used, the contracture showed a rapid decline after reaching a peak value (Fig. 86, d, f). After such treatment, successive contractures were smaller in magnitude.

Fig. 87 summarizes results from six comparable preparations. Tension starts at an average membrane potential of 60 mV, i.e., when depolarization is about 15 to 22 mV in magnitude in fibres with resting potentials of 75 to 82 mV. Orkand (1962) found a "threshold" membrane potential of 60 mV in a different crayfish muscle when he depolarized single fibres with a microelectrode.

When Fig. 87 is compared with Fig. 52, it can be seen that tension is produced by motor nerve stimulation at much lower levels of depolarization than in the case of KCl application. The significance of this result will be considered in the Discussion.

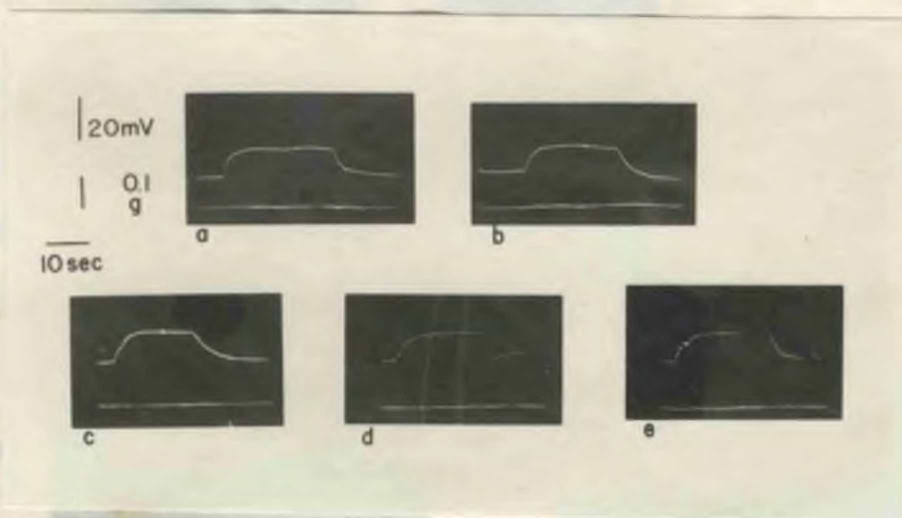


Fig. 83. Potassium chloride depolarization of muscle fibres in the opener muscle of the Astacus walking legs (a,b) Records made from the same muscle fibre during successive applications of 13 mM KCl and removal with 5 mM KCl (c,d,e) Records made from three different muscle fibres in the same muscle during successive applications of 13 mM KCl and removal with 5 mM KCl. Initial resting potentials were: (a), 78 mV; (b), 75 mV; (c) 80 mV; (d) 76 mV; (e), 76 mV. Artefacts appear in (d) and (e). Lower traces, tension of the whole muscle.

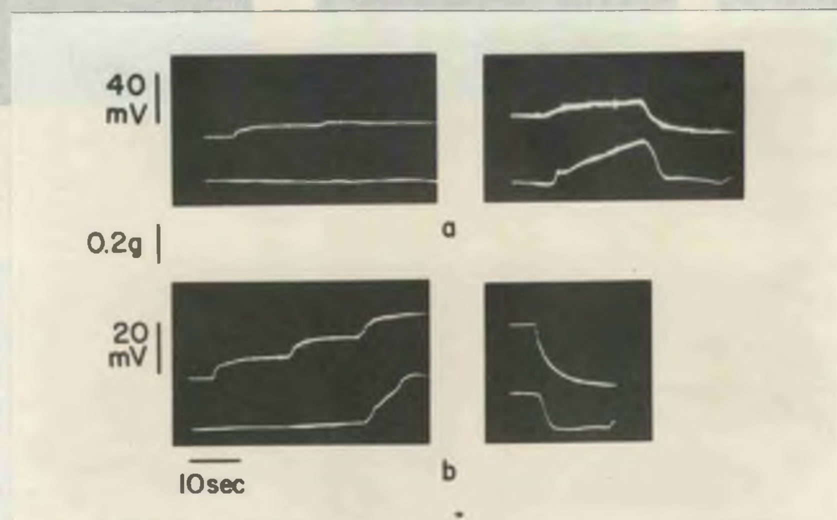


Fig. 84. Records of potassium chloride depolarization and tension development in two different preparations (a and b). In (a), solutions containing 10 mM, 13 mM, and 22 mM KCl were added successively; tension (lower traces) appeared when the latter solution was added, and disappeared when 5 mM KCl was re-added. In (b), solutions containing 10 mM, 15 mM, and 20 mM KCl were added; tension appeared when the latter solution was added, and disappeared with re-addition of 5 mM KCl. Initial resting potentials were: (a), 78 mV; (b), 78 mV.

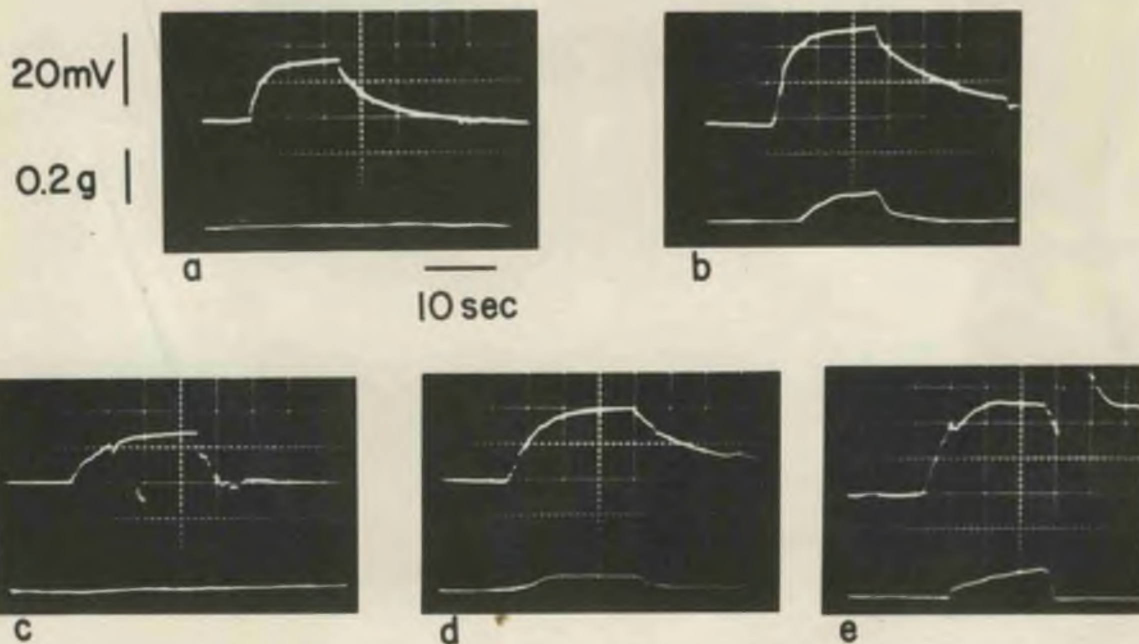


Fig. 85. Records of potassium chloride depolarization and tension development in two opener muscles of Astacus walking legs. (a) Addition of 10 mM KCl and removal with 5 mM KCl. (b) Addition and removal of 22 mM KCl in the same preparation. Initial resting potential in (a), 80 mV. (c,d,e) Records from another preparation during addition and removal of 10 mM KCl (c), 18 mM KCl (d), and 23 mM KCl (e). Initial resting potential in (c), 77 mV. Serious artefacts appear in (c) and in (e) in the membrane potential recordings. Lower traces, tension of the whole muscle.

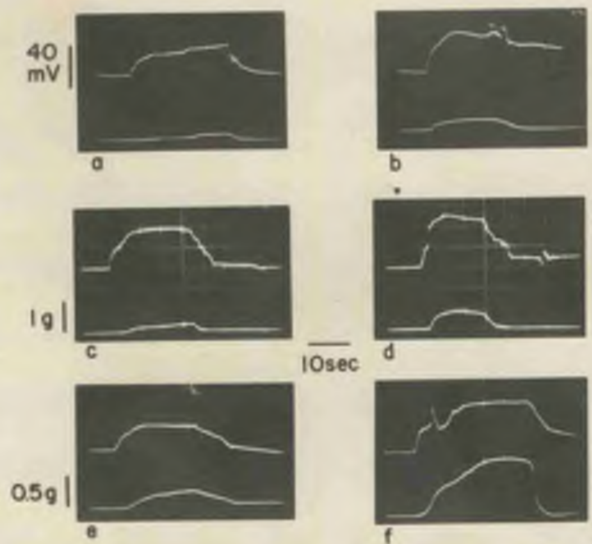


Fig. 86. Records of depolarization and tension from three preparations during application and removal of high KCl solutions. (a,b) Records from a preparation during application and removal of 23 mM KCl (s) and 35 mM KCl (b). Initial resting potential in (a), 82 mV. (c,d) Records from another preparation during application and removal of 35 mM KCl (c) and 50 mM KCl (d). Initial resting potential in (c), 80 mV. (e,f) Records from another preparation during application and removal of 25 mM KCl (e) and 50 mM KCl (f). Initial resting potential in (e), 78 mV. Artefacts occur in all of these figures in the membrane potential recordings (upper traces).

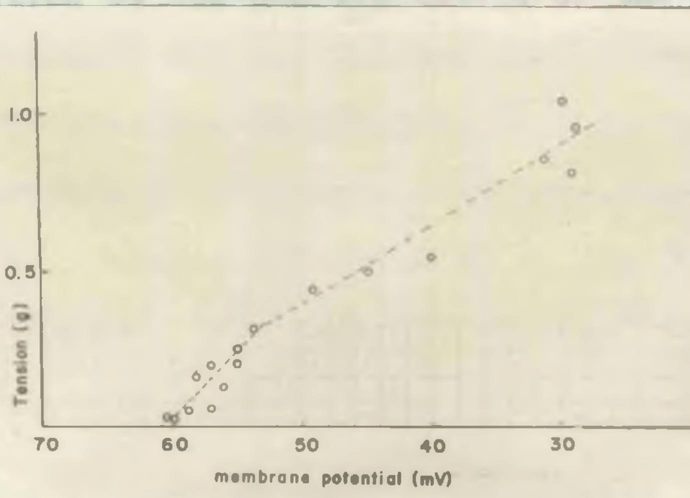


Fig. 87. Membrane potential and tension in opener muscles of Astacus walking legs. Points were obtained from six preparations in which the muscles were of the same size and in which the average initial resting potentials were similar. In each preparation measurements from only one fibre were used for membrane potential determinations.

iii) <u>Nephrops: Closer Muscle</u>

An estimate of the membrane potential level at which tension is produced in the *Nephrops* closer muscle was obtained by perfusing solutions containing excess potassium ions into the muscle, and measuring membrane potentials of muscle fibres at threshold tension. The walking leg closer muscles are small, and it was observed that muscle fibres in all parts of the muscle, even several layers down, were rapidly depolarized by solutions containing excess potassium ions.

The average value of the membrane potential threshold for 5 muscles was found to be 58 $\pm$ 1.1 mV(S.E.)--close to, but slightly higher than that estimated for *Carcinus* muscle, and slightly less than that determined for Astacus muscle. This determination by KCl depolarization of the whole muscle must be regarded as a first approximation and not exact. Nevertheless, it provides an initial basis for consideration of the tension-producing mechanisms in this muscle (see Discussion).

e). <u>Activation of single muscle fibres</u>

It is clear from the difficulties encountered in attempts to interpret the results of experiments on potassium contracture of whole muscles that more exact knowledge about the relationship between depolarization and contraction in crustacean muscles is dependent on the study of these processes in single muscle fibre preparations.

The study of Orkand (1962) on activation of single crayfish muscle fibres by means of an internal current-passing microelectrode represents the first successful attempt to record simultaneously the electrical and mechanical activity of single crustacean muscle fibres. Previously, the only attempts to observe the mechanical activity of individual crustacean muscle fibres had employed visual observation (van Harreveld, 1939)--a far from satisfactory method.

Orkand's technique of stimulating individual muscle fibres with internal microelectrodes was employed in the present study to determine the "threshold" membrane potential for contraction of single muscle fibres in crab muscles. In addition, use was made of a new technique for isolating single muscle fibres developed by Hoyle (see Methods, and Hoyle and Smyth, 1963). By means of this technique, studies on the contraction of single isolated muscle fibres responding to stimulation of the motor nerve and to potassium depolarization were made in the stretcher muscle of <u>Cancer</u>.

The techniques used in isolating single muscle fibres for tension measurements have been described previously (see Methods). Further comment is necessary to explain the means used to determine the "threshold" depolarization with current-passing internal microelectrodes.

In an isolated muscle fibre preparation, a transducer is connected to one end to record tension (Fig. 89, F); the other end of the fibre is attached to the shell. The current-passing microelectrode (Fig. 89, S) is inserted into the muscle fibre at one end. The recording electrode (R) is inserted close to the stimulating electrode.

When depolarizing current is passed through the stimulating electrode, the fibre membrane is depolarized to the greatest extent in the region of the stimulating electrode. Depolarization decreases with distance from the stimulating electrode at a rate dependent on the length constant of the muscle fibre and on its length. In the fibres studied by Orkand, the lengths were short compared with the length constants, and depolarization varied little over the entire fibre. In the crab fibres studied here, the fibres were longer, and depolarization usually declined considerably along the length of the fibre. But provided the "depolarization profile" was determined in the fibre being studied, and provided the distance between R and S was measured, it was possible to determine the depolarization at S by a measurement at R.

Typically the length constant of a fibre determined by depolarizing pulses was less than that determined by hyperpolarizing pulses (Fig. 90). Therefore it was best to use depolarizing pulses to determine the spread of potential along a fibre.

In the fibres examined in these experiments, it was found that tension could be observed as the depolarization was increased above a "threshold" value. The exact value of the "threshold" could not be determined exactly, because it was not known how great a length of the muscle fibre had to be depolarized past the "threshold" in the region of

the stimulating electrode, to cause development of measurable tension. However, by finding the depolarization at the stimulating electrode at which contraction could just be detected, and subtracting a small amount (about 0.5 mV) from this value, an estimate of the "threshold" could be obtained. (Fig. 89).

As the depolarization is increased above threshold, the strength of the contraction in the region of the stimulating electrode can be assumed to increase (cf. Huxley and Taylor, 1958), and at the same time the length of fibre activated becomes larger (Fig. 89). Comparison of tensions at different depolarization levels should take these two factors into account.

i) Carcinus: Closer Muscle

The method used to record tension from single Carcinus muscle fibres activated by depolarizing current delivered through an intracellular electrode has been described (see Methods). It was possible to examine Type C muscle fibres only; Type A and Type B muscle fibres were too inaccessible.

Measurements from some Type C fibres are shown in Figures 91, 92. In Fig. 91 (a, b) a muscle fibre with a resting potential of 66 mV was studied. The length constant of this muscle fibre was determined to be 1.6 mm, and potential decay along the fibre was exponential; the recording electrode was inserted 0.815 mm from the stimulating electrode. In response to a depolarizing pulse of 200 msec. duration and 10 mV magnitude at the recording electrode, a slight amount of tension was developed (Fig. 91a). A subsequent pulse of 7.5 mV (at the recording electrode)

produced almost no tension (Fig. 91b). Depolarizations of less than 7 mV (at the recording electrode) produced no tension. The threshold depolarization for tension development was taken to be 7.0 mV at the recording electrode which, with a length constant of 1.6 mm, corresponds to a depolarization of 12.0 mV at the stimulating electrode. Therefore the membrane potential of this muscle fibre had to be lowered to 54.0 mV at the stimulating electrode before tension was developed.

Fifteen determinations of the "threshold" for tension development in Type C muscle fibres gave values ranging from 57 to 42 mV (average, 50 $\pm$ 1.1 mV). The apparently higher threshold for single-fibre measurements compared with whole muscle measurements using potassium depolarization may reflect a failure to measure very low tension in single muscle fibres. In some cases contraction of the muscle fibre could be observed visually when no tension was recorded. It is also possible that fibres not examined (particularly Type B muscle fibres) have lower thresholds than the Type C fibres examined. Another factor to be considered is that a minimum length of muscle fibre near the simulating electrode probably has to be depolarized past the threshold before measurable tension can be developed.

As an approximation, therefore, the value of 55 mV found by potassium depolarization can be assumed to be a reasonable estimate for the threshold value for Type C muscle fibres. However, there may be considerable "scatter" about this mean.

When the depolarization is increased above threshold level the tension developed by the muscle fibre increases greatly (Fig. 91, c, d, e) as it does in frog (Hodgkin and Horowicz, 1960b) and crayfish

(Orkand, 1962) muscle fibres. In common with crayfish muscle fibres, the tension developed by the crab muscle fibres is dependent on the length of time the depolarization is maintained above the threshold level. Total tension is therefore related to the product of time and depolarization above threshold.

In some muscle fibres the rates of development and decay of tension were slower than in others having similar length and time constants, even in response to the same type of depolarization. An example is given in Fig. 91 (f,g). This raises the possibility that in the different types of muscle fibre already described there may be a wide range of contraction speeds.

When a large depolarization of the muscle fibre membrane was evoked and repeated at a frequency of about 1 per sec. or greater, it was observed that the mechanical responses of many muscle fibres became larger with successive stimuli, even though the membrane potential and depolarization did not change (Fig. 92. a,b,c). This observation may indicate a rather long-lasting "active state" in these muscle fibres.

In some muscle fibres, repeated stimulation caused a marked lowering of the resting potential. The tension developed by a given amount of depolarization then became greater than it had been before the lowering of the membrane potential. The responses shown in Fig. 92 (d,e,f) were obtained from the same fibre as in Fig. 91 (c,d,e), but after the membrane potential had fallen from 65 mV to 56 mV. It can be seen that more tension is developed in response to a given magnitude of depolarization after the fall in membrane potential. In addition, the rate of decline in tension is slower in the latter case. This phenomenon is evidence that

the absolute level of the membrane potential determines the amount of depolarization that is necessary to cause contraction; or, in other words, that the "threshold" remains fixed (cf. Orkand, 1962).

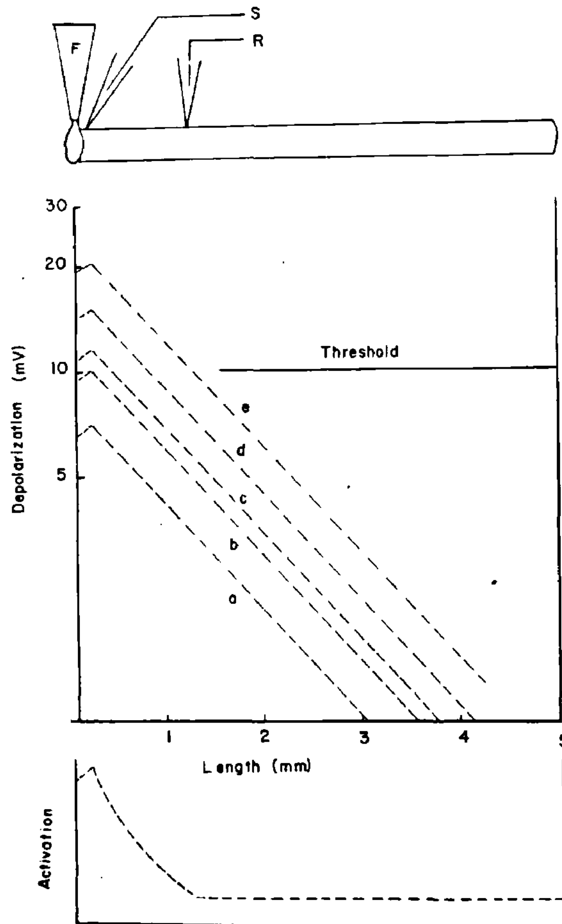


Fig. 89. Diagram to illustrate activation of a single *Carcinus* muscle fibre with intracellular electrodes. Current is passed through the stimulating electrode (S); the resulting depolarization is recorded by a second electrode (R). Tension is recorded by a transducer (F) at one end of the fibre.

In this example the fibre has a length constant of 1.47 mm and a length of 5 mm. The resting potential of the fibre was 67 mV. Depolarization along the surface of the muscle fibre during successive increasing current pulses is shown by broken lines (a to e). No contraction was measured in response to the first two pulses (a and b). A very small contraction resulted from the third pulse (c), and stronger contractions resulted from stronger pulses (d,e). The "threshold" was between (b) and (c). The depolarizations measured at the recording electrode were: 5.8 mV (c) and 5.0 mV (b). The calculated values for depolarization at the stimulating electrode were: 11.5 mV (c) and 10.0 mV (b). The threshold depolarization was estimated to be 10.5 mV, giving a "threshold" membrane potential of 56.5 mV.

Hypothetical "activation" in response to the depolarization of (e) is depicted in the lowest figure. Activation is assumed to occur only when depolarization exceeds the "threshold" and to be related to the degree of depolarization at each point on the fibre.

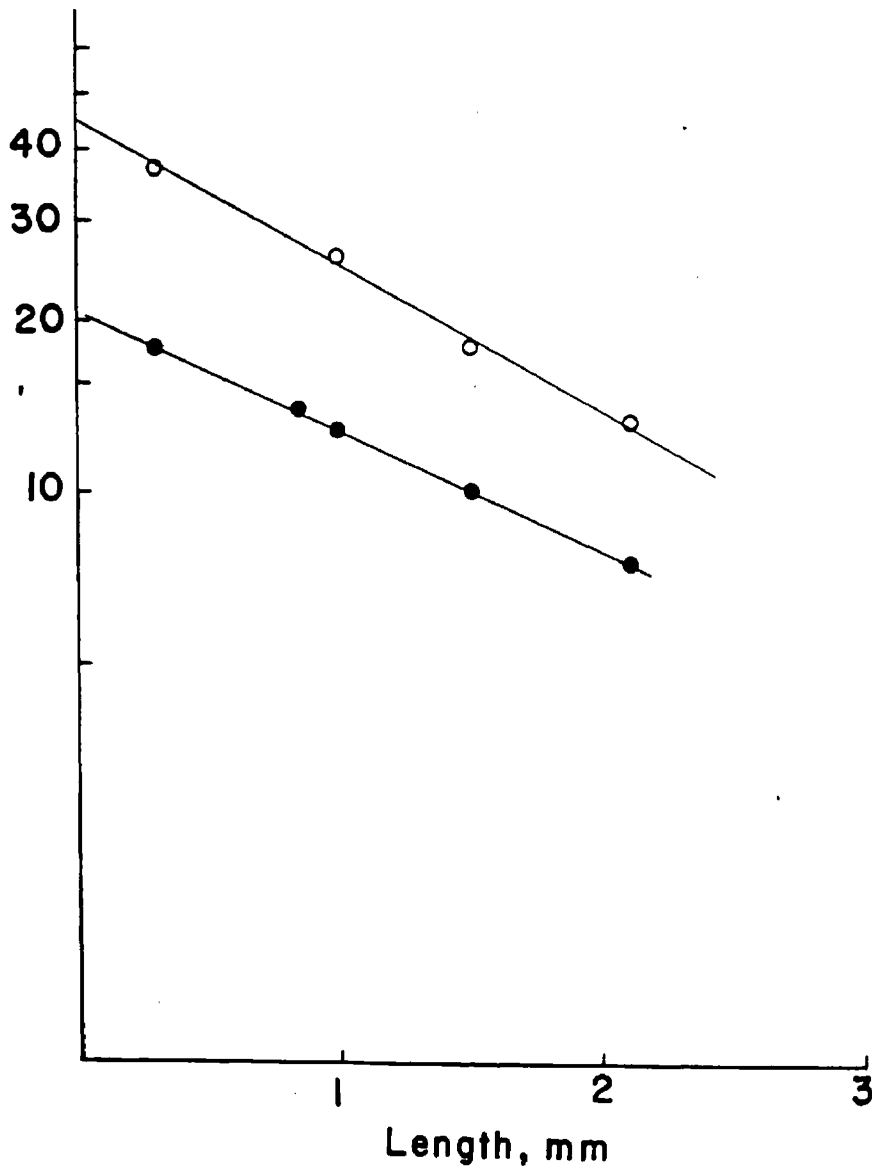


Fig. 90. Decay of hyperpolarizing pulses (filled circles) and depolarizing pulses (open circles) along a *Carcinus* muscle fibre. Length constants were 1.66 mm for depolarizing pulses and 1.95 mm for hyperpolarizing pulses.

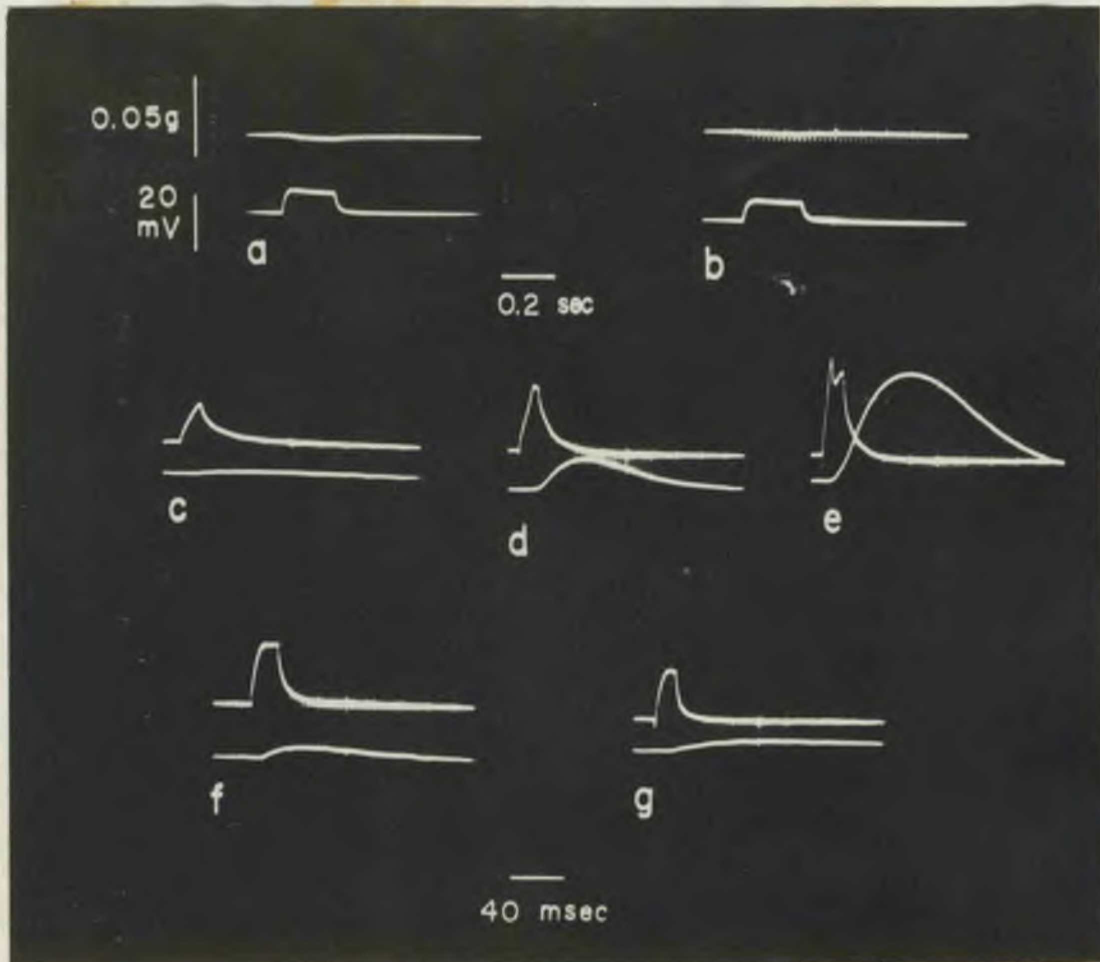


Fig. 91. Tension and electrical recordings from single Type C muscle fibres of *Carcinus* in response to direct electrical depolarization (a,b). Responses of a fibre having a length constant of 1.6 mm to pulses of 200 msec. duration. Resting potential, 66 mV; electrode separation, 0.815 mm. (Tension, upper traces).

(c,d,e) Responses of another muscle fibre to 16 msec. pulses of increasing intensity (tension, lower traces). Resting potential, 65 mV; electrode separation, 0.3 mm. (f,g) Differences in contraction speed in two Type C muscle fibres. In (f) length constant was 1.5 mm, electrode separation 0.9 mm., resting potential 70 mV. In (g) length constant was 1.3 mm., electrode separation 1.2 mm., resting potential 68 mV.

Fig. 92. (a,b,c) Responses of a Carcinus Type C muscle fibre to three stimuli in a train delivered at 1 per sec. Responses to the first, seventh, and twelfth stimuli in the train are shown. Electrode separation, 0.05 mm. (d,e,f) Responses of the fibre shown in Fig. 91 (c,d,e) after lowering of the membrane potential by repeated stimulation. Tension, lower traces.

ii) Cancer: Stretcher Muscle

The method for recording tension in isolated fibres of the stretcher muscle has been described (see Methods). The tension measurements made using this method undoubtedly yield more accurate "threshold" values, since the damping effect of other muscle fibres is minimal.

Only readily accessible fibres could be examined by this method, i.e. fibres in the "central" part of the muscle (see p. 72). As in Carcinus, other fibres not examined may have different characteristics.

Ten determinations of the "threshold" for tension development gave an average value of 56.5 ± 0.8 mV (S.E.) at the stimulating electrode. Values ranged from 59 mV to 54.5 mV. The behaviour of fibres in this part of the muscle appeared to be fairly uniform with respect to the "threshold" level.

As in Carcinus fibres, the tension increased with increasing depolarization (Figs. 93, 94, 96). In some fibres there was a marked increase in tension with time during a maintained depolarization (Fig. 93). In other fibres the tension reached a maximum value rapidly (Fig. 96).

In a few fibres rapid "spikes" occurred during depolarization, and the tension showed a rapid rise and fall (Fig. 94). In these fibres, tension could be produced without "spikes" as well.

As in Carcinus fibres, repeated stimulation sometimes brought about a fall in membrane potential, and when this occurred, the amount of tension produced by a given degree of depolarization increased (Fig. 95).

A depiction of the relation between depolarization and tension is shown in Fig 97. A measure of the activating effect of a depolarizing pulse was obtained by computing the amount of depolarization above

threshold level at each point on the muscle fibre surface. The measure of activation, in units of mV.mm, was plotted against tension for four fibres (Fig. 97). The differences in the slopes of the lines for different fibres can perhaps be attributed to small differences in cross-sectional area, rate of tension development, physiological condition, etc.

In several fibres potassium contracture was induced after measurements of responses to direct electrical depolarization had been made, in an effort to find out whether the two methods of producing depolarization were equivalent. Solutions containing high potassium were perfused rapidly into a "chamber" built around the muscle fibre with soft wax. By this means a fairly rapid depolarization of the muscle fibre could be produced. It could be reversed by addition of normal saline (Fig. 96).

If it is assumed that the depolarization produced by high potassium is distributed over the entire surface of the muscle fibre, and if it is also assumed that the potassium depolarization is equivalent to the directly produced electrical depolarization, then a given magnitude of potassium depolarization should produce a greater degree of tension than the same magnitude of electrical depolarization. The reason for this is that activation by current passed through a microelectrode is confined to a much smaller part of the muscle fibre (see Fig. 89). The inactive parts of the muscle fibre are stretched by the shortening of the active parts. Since the inactive muscle has a lower elastic modulus than the active muscle, the tension developed by shortening of the active muscle cannot all be measured by the transducer. The fraction that is measured will depend, among other things, on the relative lengths of active and

inactive muscle fibre and on the values of the elastic moduli of active and inactive muscle.

In a uniformly depolarized muscle fibre, the degree of activation, amount of shortening, and elastic modulus are the same along the length of the fibre. Therefore the tension developed by a given amount of depolarization can be more completely measured by the transducer.

The results from five muscle fibres* showed that the tension developed by potassium depolarization was slightly greater than that developed by the same amount of electrical depolarization (Fig. 98). It also appeared that a similar threshold existed for potassium depolarization and for direct electrical depolarization, provided that the threshold for potassium contracture was estimated from an initial application of high potassium solution. With subsequent applications of high KCl, the "threshold" appeared to increase (Fig. 96).

Examples taken from these results are given in Figs. 96 and 98. In Fig. 96, results of two successive applications of a high potassium solution are shown (c; d,e). Tension is not developed until a "threshold" is passed. In this case, the "threshold" for potassium contracture was about 58 mV during the first application of potassium. When a second application of potassium solution was made, the "threshold" was apparently increased to about 53 mV. The threshold for electrical depolarization was previously estimated to be 56 mV in this fibre (Fig. 96, a).

In Fig. 98, a plot of depolarization against tension is given for another fibre activated by direct electrical depolarization and by

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*All of the fibres used developed maximum tension within 0.8 sec. in response to direct stimulation.

potassium depolarization. The potassium depolarization produced more tension for a given degree of depolarization than did electrical depolarization measured at the stimulating electrode. However, the difference was not very great, and it is difficult to predict the degree of difference in magnitude of contraction for the two types of depolarization (i.e., local depolarization and uniformly distributed depolarization) on a priori grounds. Furthermore, it could not be ascertained whether or not the potassium depolarization was uniform along the length of the muscle fibre. Therefore, all of the results on potassium contracture in single muscle fibres must be regarded with extreme reservation; more and better experiments are obviously needed.

The provisional conclusions which can be suggested are: (1) The thresholds for direct electrical depolarization and for potassium depolarization are initially about the same; (2) Repeated potassium depolarization may raise the threshold for subsequent potassium depolarizations in these muscle fibres.

Tension measurements were made from five single muscle fibres which were prepared with motor innervation still functional. Examples of these measurements are shown in Figs. 99, 100.

It was found that these fibres showed no contraction unless total depolarizations of 10 to 20 mV were built up by stimulation of the motor nerve. In the "centre" fibres studied, stimulation at a rate of 30 per second or greater was necessary to produce this amount of depolarization and a resulting contraction (Fig. 99).

In one fibre a series of twitches occurred when the motor nerve was stimulated at a high frequency (Fig. 100). These twitch-like

mechanical responses were accompanied by rapid excursions in the membrane potential of 5 to 10 mV. It is possible that these excursions were larger in another part of the muscle fibre, although in the muscle fibres studied the electrical events due to nerve stimulation were observed to be fairly uniform in all parts of the muscle fibre.

The results of the studies on these single-fibre preparations indicate that a depolarization "threshold" exists for indirectly produced contraction in Cancer stretcher muscle fibres. This "threshold" appeared to be about the same as those for potassium contracture and contraction produced by direct electrical depolarization. Some uncertainty arose in determining the exact threshold, because the electrical response of the membrane during nervous excitation is not a steady depolarization but a rapidly fluctuating one. However, the threshold was estimated by comparing records of responses to different frequencies of stimulation of the motor nerve and finding the total depolarization just sufficient to produce a contraction. The threshold depolarization was estimated to lie between the contraction-producing total depolarization and the largest total depolarization which did not produce a contraction. In five fibres, estimates of the membrane potential "threshold" lay between 61.5 mV and 54 mV.

It would have been of interest to obtain records of all three types of contraction and depolarization in the samemuscle fibres, but this will be attempted in future studies.

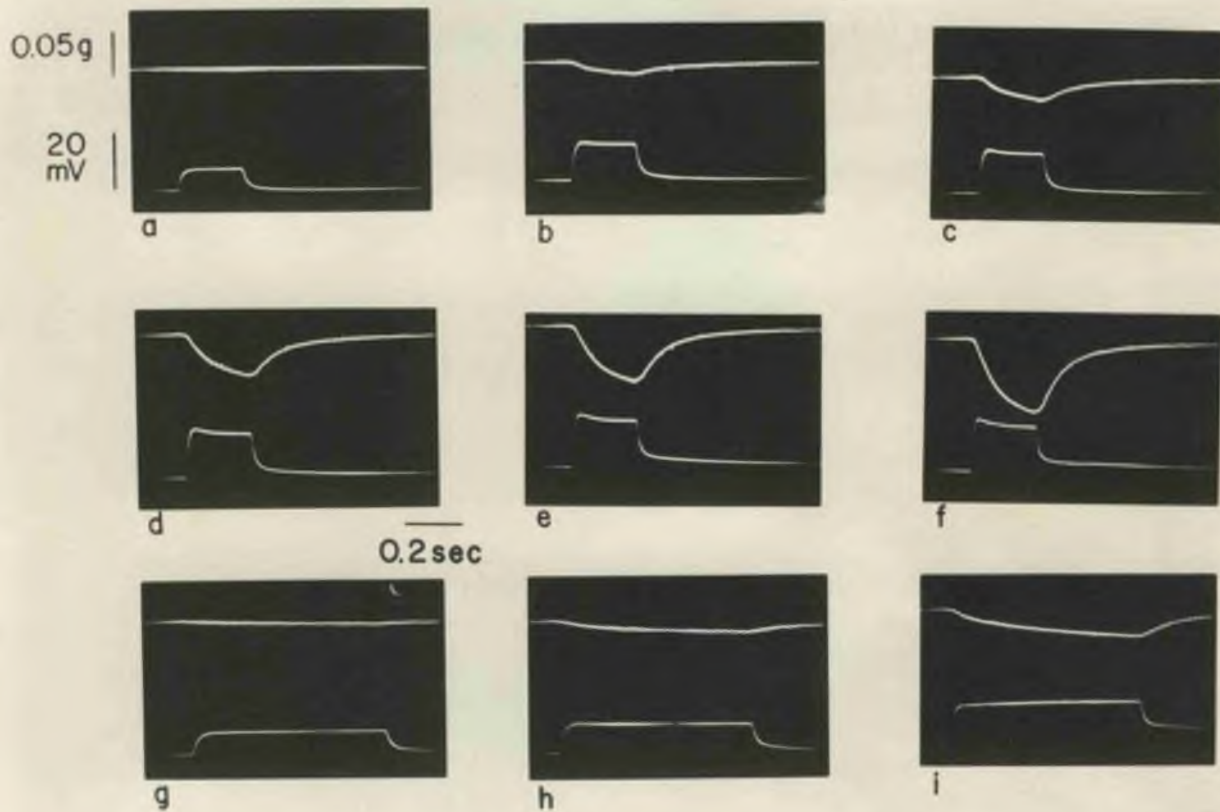


Fig. 93. Responses of a single Cancer muscle fibre to direct electrical depolarization (tension, upper traces). Electrode separation, 0.7 mm; length constant, 1.9 mm; resting potential, 67 mV. This fibre showed a slow rate of tension development.

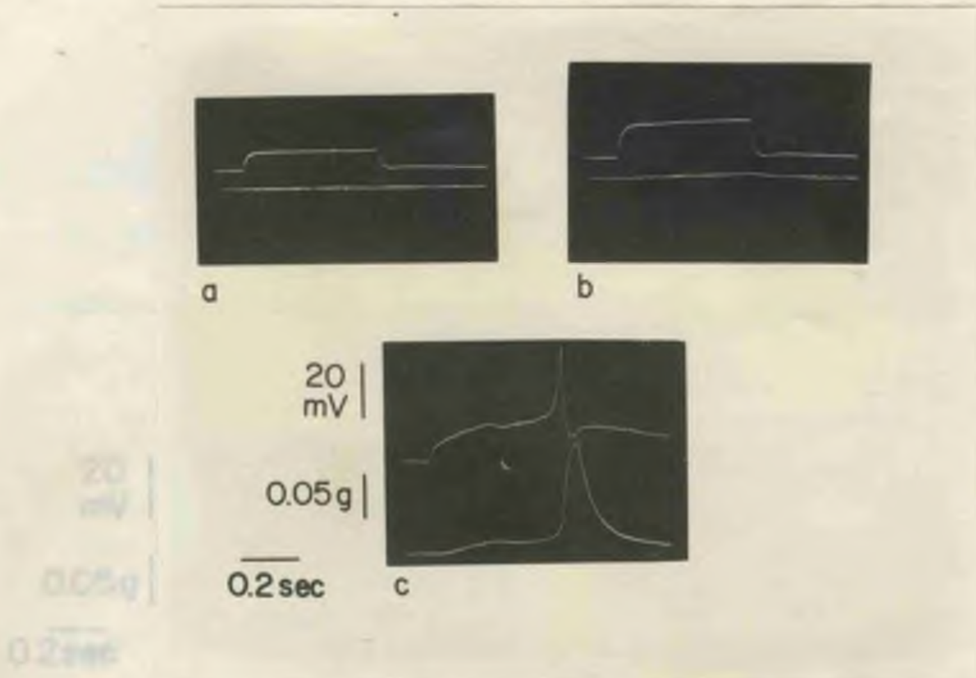


Fig. 94. Responses of a Cancer muscle fibre to direct electrical deploarization. Electrode separation, 0.5 mm; length constant, 2.1 mm; resting potential, 66 mV (a,b); 63.5 mV (c). Tension, lower traces.

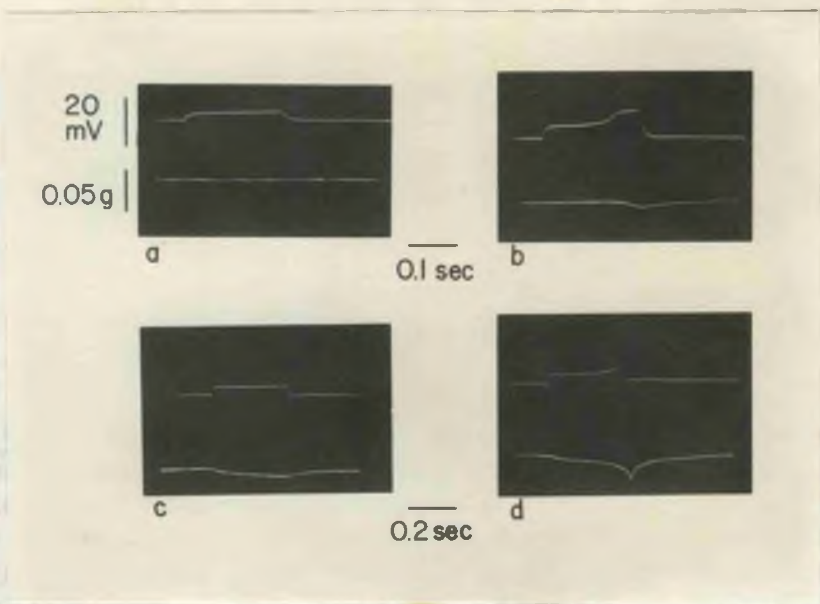


Fig. 95. Responses of a Cancer muscle fibre before (a,b) and after (c,d) lowering of the membrane potential by repeated stimulation. Electrode separation, 0.35 mm; resting potential, 65 mV (a,b), 57 mV (c,d). Tension, lower traces.

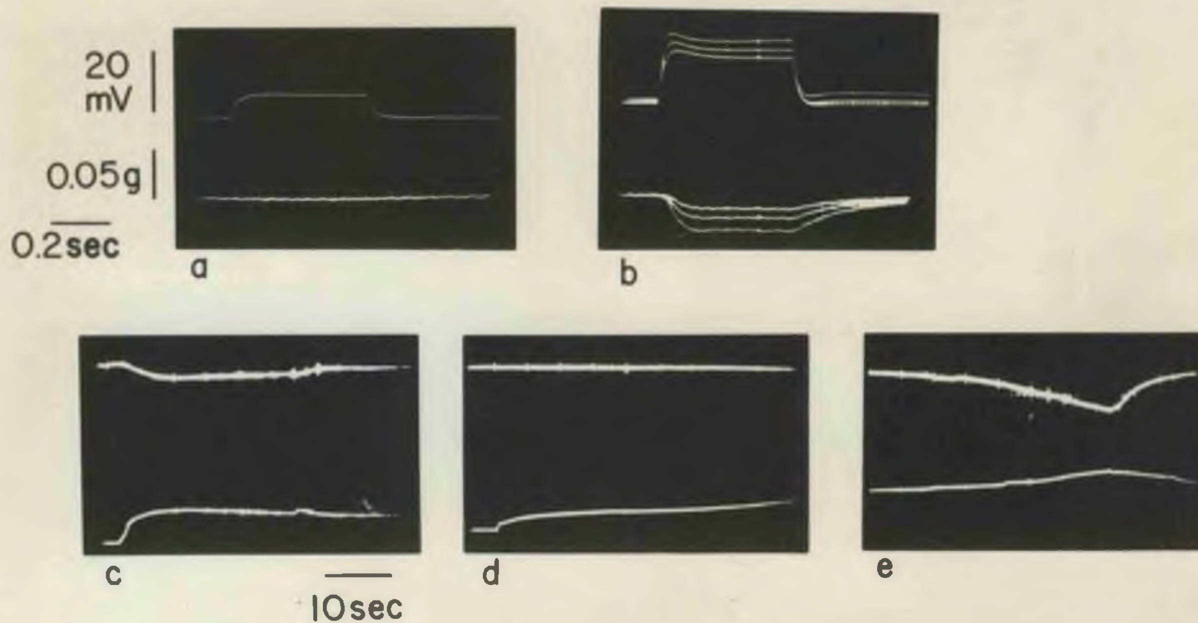


Fig. 96. Activation of a single *Cancer* muscle fibre by direct electrical depolarization (a,b) and by application of a high potassium solution (c,d,e). In (a,b), electrode separation was 0.62 mm, length constant 1.85 mm, resting potential 67 mV (in a). In (c), excess KCl was applied, then removed with normal saline. The initial resting potential was 65 mV. In (d) a fresh application was made, and in (e) the excess KCl was removed with normal saline (end of trace). The initial resting potential in (d) was 63 mV. Tension, lower traces in (a,b); upper traces in (c,d,e); increasing tension is indicated as a downward deflection.

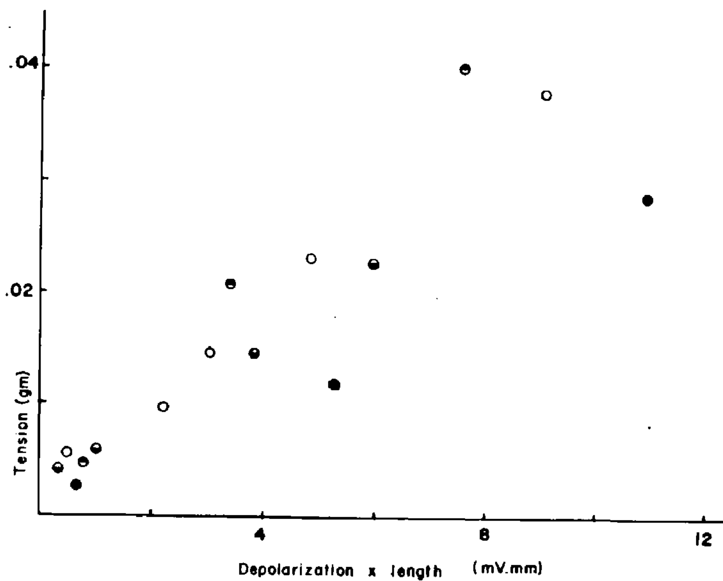


Fig. 97. Activation (depolarization above threshold integrated with respect to length) and resulting tension in four single *Cancer* muscle fibres. In all cases tension was measured at the end of a 0.5 sec. pulse of stimulation.

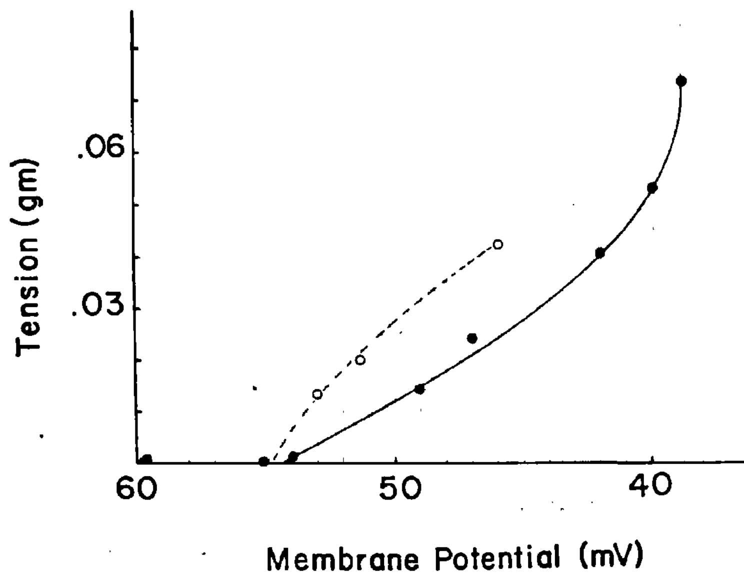


Fig. 98. Comparison of activation of a single *Cancer* muscle fibre by direct electrical depolarization (filled circles) and by high KCl solutions (open circles). The data were obtained from a fibre which responded to direct electrical stimulation in a manner similar to that shown in Fig.96. Points for potassium activation were obtained from a single application of KC .

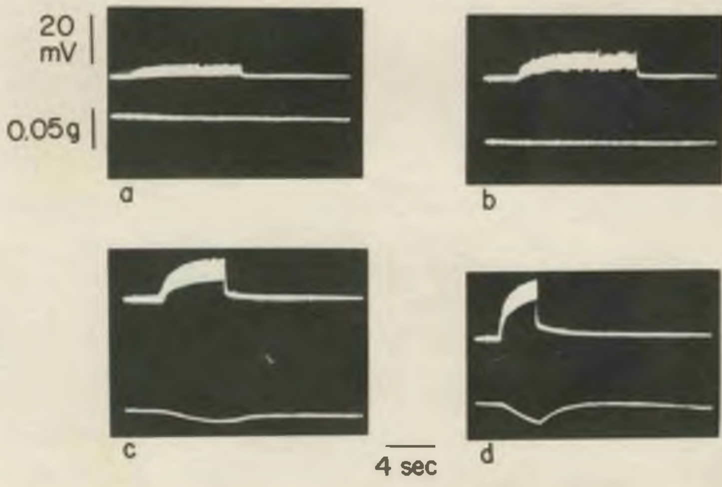


Fig. 99. Responses of a single *Cancer* muscle fibre to stimulation of the motor nerve at 20 per sec. (a), 30 per sec. (b), 40 per sec. (c), and 50 per sec. (d). Tension, lower traces.

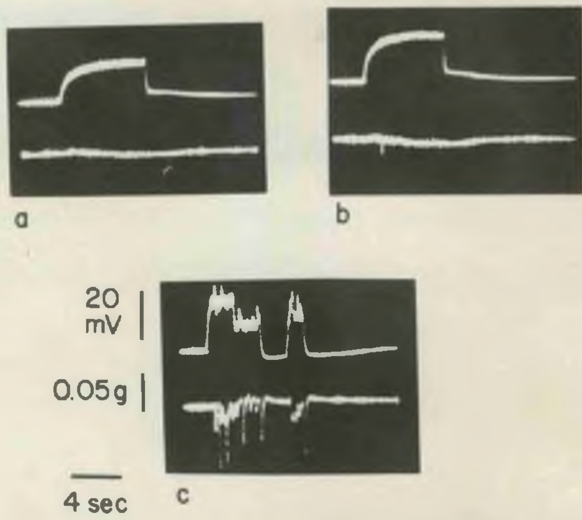


Fig. 100. Responses of a single *Cancer* muscle fibre to stimulation of the motor nerve at 50 per sec. (a), 60 per sec. (b), and 70 per sec. (c). Tension, lower traces.

iii) Pachygrapsus: Closer Muscle

The muscle fibres in the Pachygrapsus closer muscle were too small for effective single-fibre isolation. However, a few observations were made on the "threshold" for contraction in response to direct electrical depolarization. For this purpose, recording and stimulating electrodes were placed close together in the muscle fibre, and the contraction of the muscle fibre was observed by eye. The amount of depolarization necessary to produce a visible contraction of the muscle fibre was determined.

Two examples of these determinations are shown in Fig. 101. Both muscle fibres were of the non-spiking type giving large p.s.ps. in response to single "fast" nerve impulses. It is evident from the figure that depolarizations of 15 to 20 mV are necessary to produce contractions in these fibres. Resting potentials in the fibres studied ranged from 80 to 69 mV, and "threshold" membrane potentials from 59 mV to 52 mV (five fibres).

Unfortunately it was not possible to find "slow" fibres or spiking fibres sufficiently exposed to permit comparative observations on threshold levels.

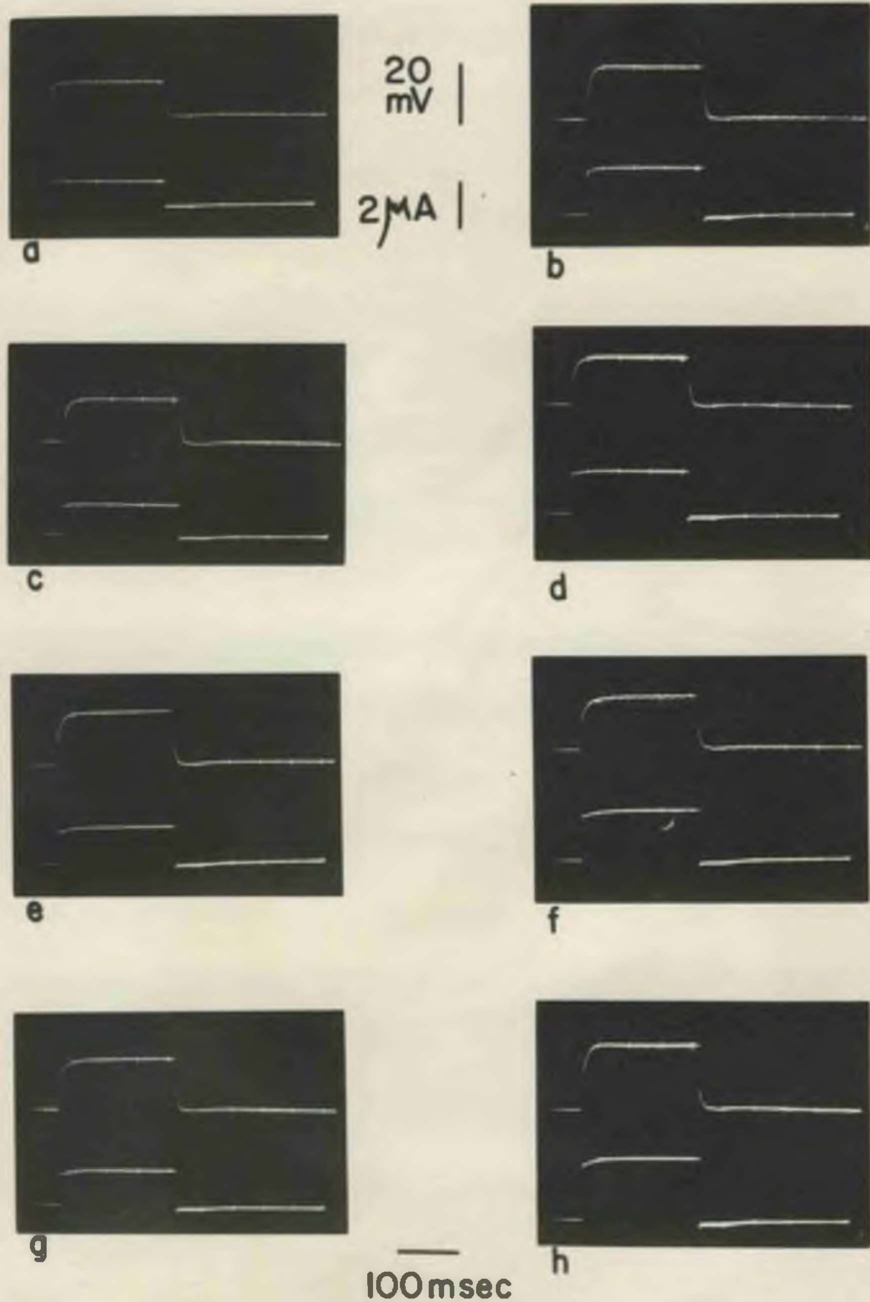


Fig. 101. Estimation of the threshold for contraction of *Pachygrapsus* muscle fibres by direct electrical depolarization. Records (a, c, e, g) were from one fibre, (b, d, f, h) from another. Contraction was visible in (e, f, g, h) and not in (a, b, c, d). Electrodes were placed close together. Resting potentials were: (a) 69 mV. and (b) 71 mV.

f) Effects of potassium ions on neuromuscular transmission in crustacean muscles.

Investigations of the mechanisms involved in the initiation of muscular contraction by nerve impulses have often employed as a tool the alteration of the external ionic environment of the muscle. This method has often yielded valuable results. The experiments described in this section deal with the effects of altering the potassium ion concentration of the perfusion saline on indirectly produced electrical and mechanical responses of several crustacean muscles.

The experiments were suggested by a paper by Waterman (1941), in which it was found that raising the potassium ion concentration of the perfusion solution increased the "fast" mechanical response of the crayfish claw, and simultaneously decreased the "slow" mechanical response. Waterman concluded that this result showed differences in the neuromuscular transmission mechanisms of the "fast" and "slow" systems.

Since Waterman's paper, no further work has appeared relating to the phenomenon which he described. However, Wiersma and Zawadzki (1948) investigated the effects of ions (including potassium) on the effectiveness of peripheral inhibition and found no changes in the frequency of stimulation of the inhibitory axon required to produce a given degree of inhibition.

The aim of the present study was to explore the effects of potassium ions on "fast" and "slow" systems of crustacean muscles in an attempt to throw some light on the phenomenon described by Waterman, and to obtain more information about

the relation of indirectly produced electrical activity to muscular contraction.

i) Nephrops: Closer Muscle

The *Nephrops* closer muscle proved to be a good preparation for studies involving alteration of the ions in the perfusing fluid. The muscle was small and could be rapidly perfused with the experimental saline. Intracellular electrodes could be left in place during perfusion, and changes in resting potential and in responses to stimulation of the motor axons could be observed in the same fibre over a period of time. A disadvantage of this method was that reliable data could be obtained on one or two fibres only during an experiment. It was necessary to make observations on many preparations to build up a picture of changes in the electrical events of the muscle as a whole.

When the normal solution, containing 8 mM KCl, was replaced by a solution containing more than 20 mM KCl, contracture of the muscle resulted, often accompanied by superimposed, regularly occurring twitches. It was not determined whether these were due to spontaneous firing of one of the motor axons or to myogenic activity, but the former alternative seemed more likely because of the rhythmic nature of the contractions.

If the experimental saline contained less than the amount of KCl required for contracture, it was found that the mechanical response of the muscle to single or repetitive stimuli applied to the "fast" axon was considerably augmented (Fig.

102, a, b; Fig. 103, f, g). In older preparations in which the twitch response to a single stimulus had disappeared due to fatigue, an increase in the potassium ion concentration of the perfusion saline had the effect of restoring the twitch. These effects of increased potassium appeared very rapidly. If the high-potassium saline was perfused into the muscle for a prolonged period, the mechanical response gradually diminished in size. (Of course, even in normal saline the mechanical response of this muscle becomes smaller with time and with repeated stimulation; see p. 54).

The electrical responses showed marked changes during perfusion with solutions containing raised KCl concentrations. In most fibres the size of the p.s.p. became progressively smaller as the resting potential was lowered (Fig. 102, a to d). However, in some fibres an electrically excitable response was produced in raised KCl solution (Fig. 102, e, f). The total electrical response to a single stimulus was larger in high-potassium solution in such fibres.

In many fibres in which the response to a single stimulus was smaller in raised KCl solution, it was found that repetitive stimulation gave rise to larger electrically excitable responses than were seen in the same fibres before treatment with high-KCl saline (Fig. 102, c, d; Fig. 103). The total amount of depolarization required to produce active membrane responses was often very small indeed in raised KCl solutions (Fig. 102, d).

Unlike Waterman's crayfish muscle, the closer muscle

of *Nephrops* developed more tension in response to "slow" axon stimulation when perfused with solutions containing raised KCl concentrations than when perfused with normal saline (Fig. 104). The electrical activity of muscle fibres giving large "slow" responses was often increased in raised KCl. Spikes were more frequently observed, and these spikes arose at levels of depolarization which did not produce spikes in normal saline. Total depolarization due to nervous activity was therefore greater in raised KCl.

This situation was not permanent. After a prolonged (20 to 40 min.) stay in high KCl, "slow" electrical and mechanical activity declined. The mechanical activity declined relatively more rapidly.

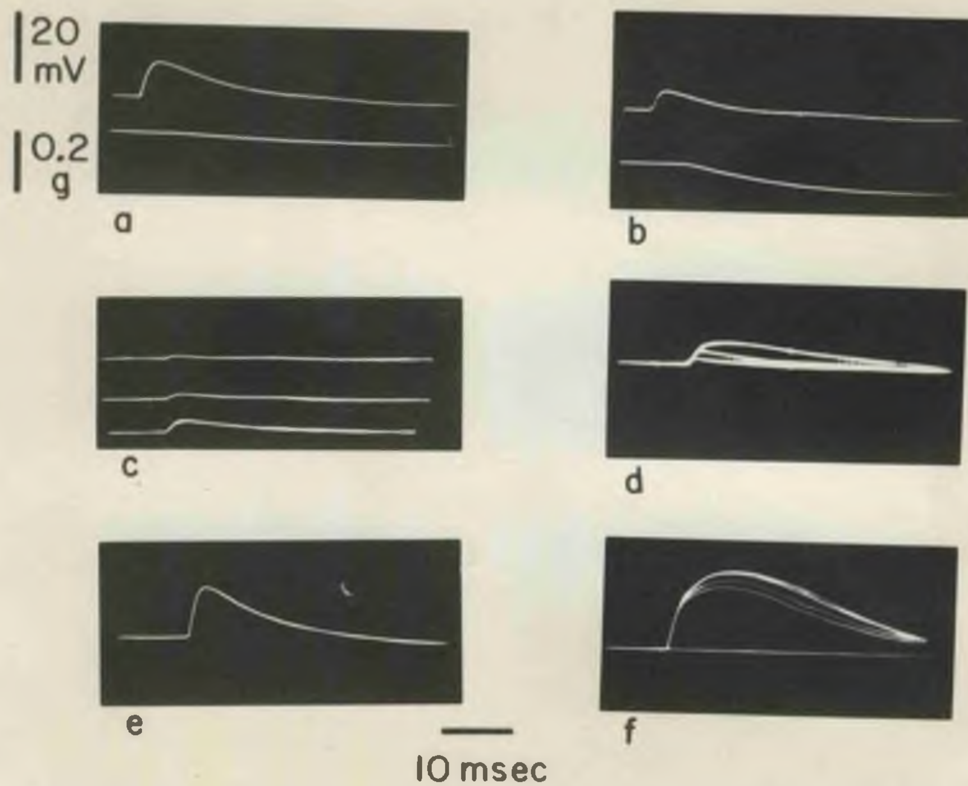


Fig. 102. Effects of potassium ions on "fast" responses in *Nephrops*. (a) Responses to a single shock in normal saline (a; resting potential, 72mV) and after perfusion with 17 mM KCl (b; resting potential, 60 mV). The electrical response is smaller in (b), but the mechanical response (lower trace) is larger. (c) Responses to a single shock in another preparation during perfusion with 20 mM KCl; initial resting potential, 75 mV. (d) Responses of the same fibre to stimulation of the "fast" axon at 8 per sec., after perfusion with 20 mM KCl. (e,f) Response of another preparation to stimulation of the "fast" axon at 2 per sec. in normal saline (e; resting potential, 75 mV) and after perfusion with 20 mM KCl (f; resting potential, 60 mV).

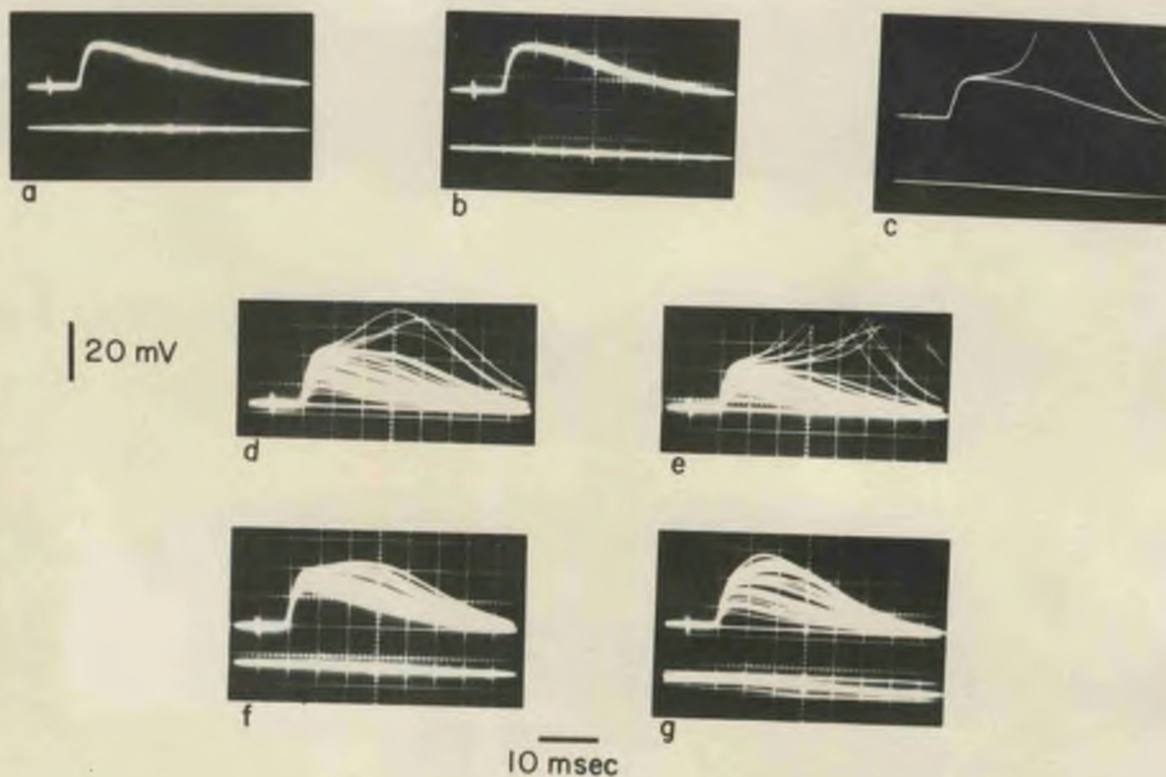


Fig. 103. Effects of potassium ion on "fast" responses in Nephrops. (a, b, c) Responses to stimulation of the "fast" axon at 2 per sec. in normal saline (a: resting potential, 72 mV) and during perfusion with 17 mM KCl (b: resting potential 65 mV, and (c: resting potential, 61 mV). (d,e) Responses of another preparation to stimulation at 10 per sec. in normal saline (d: resting potential, 71 mV) and during perfusion with 15 mM KCl (e: resting potential, 65 mV). Stimulation was given for 2½ secs. in each case. (f, g) Responses to the same stimulation in another preparation during perfusion with normal saline (f: resting potential, 71 mV) and with 15 mM KCl (g: resting potential, 66 mV). Note increased mechanical response (lower trace) in (g).

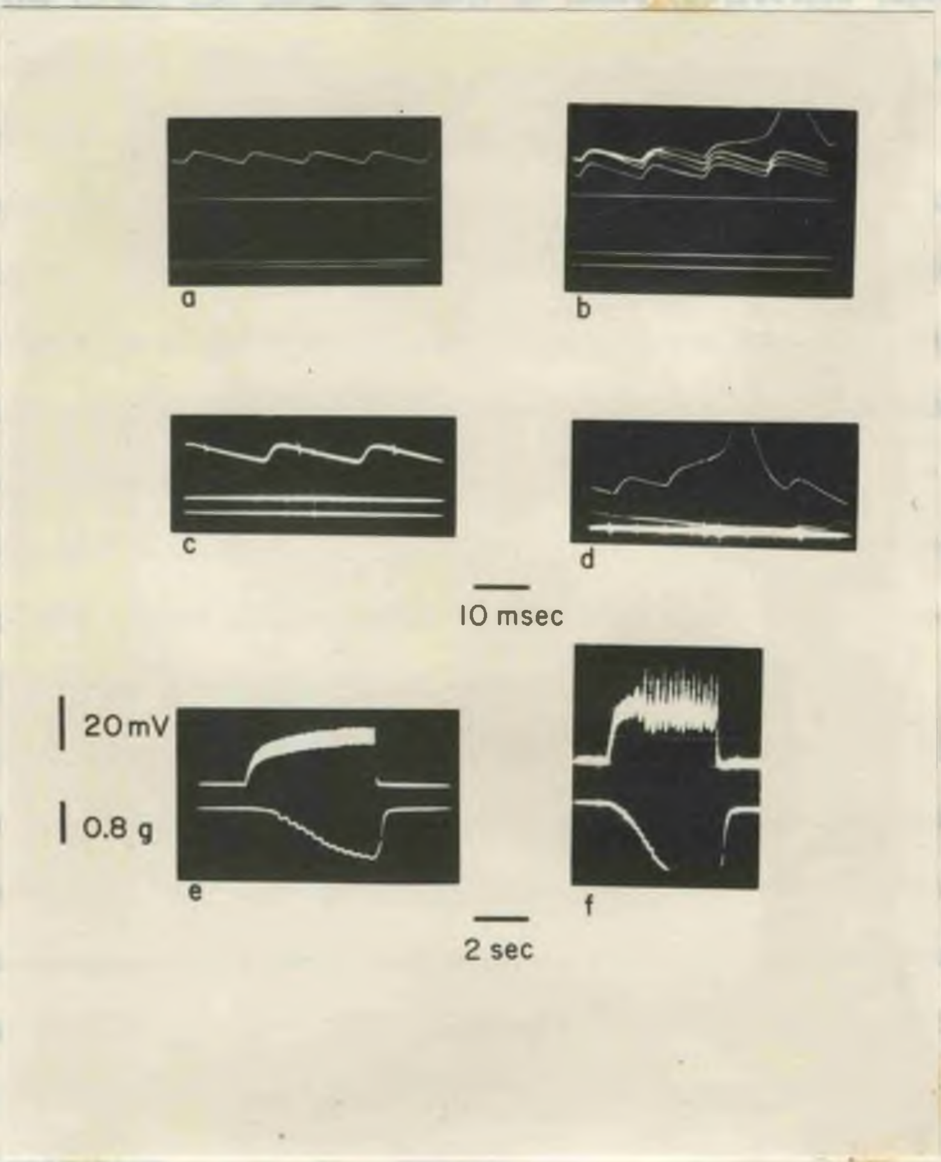


Fig. 104. Effects of potassium ion on "slow" responses in *Nephrops*. (a, b) Responses during stimulation at 90 per sec. in normal saline (a: resting potential, 70 mV) and in 15 mM KCl (b: resting potential, 64 mV). Mechanical response (lower trace) is larger in (b). (c, d) Responses of another preparation to stimulation at 45 per sec. in normal saline (c: resting potential, 70 mV) and in 15 mM KCl (d: resting potential, 65 mV). (e,f) Responses of another preparation to stimulation at 60 per sec. in normal saline (e: resting potential, 72 mV) and in 15 mM KCl (f: resting potential, 64 mV).

ii) Crayfish muscles

The various muscles of the crayfish studied previously were subjected to treatment with solutions containing KCl concentrations intermediate between that of normal saline (5 mM) and that necessary to cause contracture (about 18 to 21 mM). The electrical and mechanical responses to indirect stimulation were observed before and during treatment.

The "fast" responses of the walking leg closer muscle were studied in a number of preparations. In most cases the twitch response to a single stimulus was considerably augmented in raised KCl (Fig. 105, a to d); in a few cases the effect was rather slight (Fig. 105, e, f). After about 10 to 20 minutes the mechanical response gradually declined.

In those fibres giving simple p.s.ps. the size of the electrical response was reduced in high KCl as the resting potential was lowered (Fig. 105, a to d).

In spiking fibres the total electrical response was reduced as the resting potential was lowered (Fig. 105, e, f; Fig. 106). Initially the p.s.p. remained about the same size and the spike typically reached the same final level, near zero membrane potential or slightly overshooting. However, as time went on the p.s.p. and spike both became greatly reduced, and eventually the spike disappeared (Fig. 106). This latter behaviour is reminiscent of that described by Hoyle (1953) for spiking fibres of an insect muscle treated with high potassium.

In the closer muscle of the crayfish claw, the mechanical response to a single stimulus applied to the "fast" axon was always greatly augmented by adding solutions containing raised KCl concentrations (Fig. 107). Both the strength and the duration of the response were increased.

No definitive intracellular records of the potassium

effect were obtained for this muscle. However, interesting results were obtained with external electrodes. The electrical activity of the muscle appeared to be more prolonged after treatment with high potassium, and to show humps and other irregularities not present in normal saline. Two shocks applied closely together (2.0 to 3.0 msec.) elicited greatly increased electrical and mechanical activity. It appeared probable that treatment with high potassium facilitated the appearance of electrically excitable responses to a single nerve impulse, as in _Nephrops_.

In a number of muscles the "slow" mechanical response was considerably augmented by raised potassium (Fig. 107). However, in one other case the reverse situation was found: raising the potassium concentration depressed the "slow" response slightly. The magnitude and direction of the potassium effect appeared to be a function of the particular animal selected for the experiment. Definitive intracellular records of the potassium effect on the "slow" system were not obtained for this muscle.

When the opener muscle of the walking leg was treated with raised potassium, the mechanical response of the muscle was usually slightly increased at first (Fig. 108, a to d). The relaxation time of the contraction was often increased (Fig. 108 d). After a prolonged stay in high KCl solution, the response became much smaller than in normal saline (Fig. 108 f).

Electrical recordings from individual muscle fibres showed that as the resting potential was lowered in raised

potassium solutions, the size of the electrical response was at first only slightly smaller than in normal saline (Fig. 108, a to d).

It is of interest to note in this connection that Boistel and Fatt (1958) observed that in a crayfish claw opener muscle the excitatory p.s.ps. begin to decrease in size only when the membrane potential is lowered about 10 mV below the resting potential. When the membrane potential is increased above the resting potential level, the p.s.ps. also decline in size, due to a fall in membrane resistance with hyperpolarization. The crayfish muscle is apparently unique in this respect and does not behave in the same way as crab muscle (Fatt and Katz, 1953c). Crayfish muscle has been found to have a comparatively low potassium conductance (Girardier et al., 1961).

After a prolonged stay in KCl concentrations slightly less than that required for contracture, the electrical responses became much reduced (Fig. 108, e, f), and in some fibres no responses could be recorded after about 20 min. in KCl concentrations slightly less than that required to produce contracture.

A number of preparations of the opener muscle of the claw were studied during application of raised KCl concentrations. In most cases it was found that the mechanical response was less than in normal saline when the frequency of stimulation was below 20 per sec., but that the mechanical response was considerably augmented when the frequency of stimulation was greater than 20 per sec. (Fig. 109). Unfortunately no

good electrical recordings were obtained for this muscle, but those which were obtained provided an indication that the effect of raised KCl on the size of the electrical response was related to its effect on the size of the mechanical response.
At lower frequencies of stimulation the electrical responses appeared to be considerably smaller in high KCl, but as the frequency of stimulation was increased the electrical responses became similar in size to those in normal saline (Fig. 109).

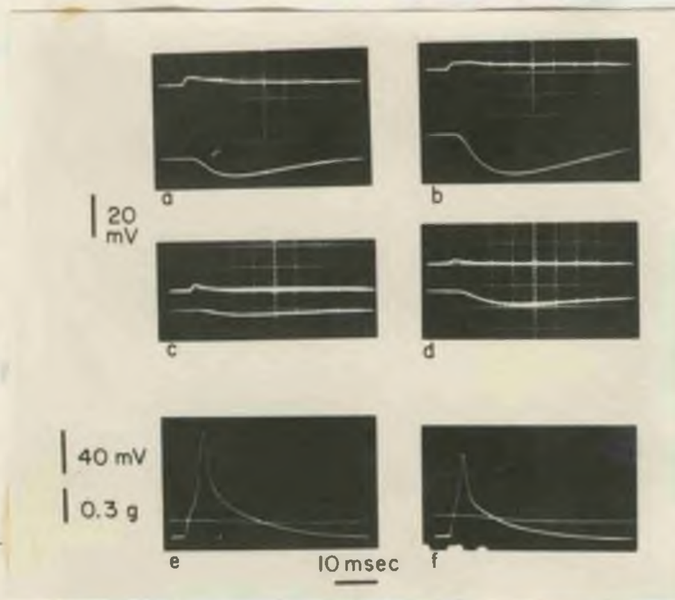


Fig. 105. Effects of potassium ion on "fast" responses of the Astacus walking leg. (a, b) Responses to a single shock during perfusion with normal saline (a: r.p. 75 mV) and with 12 mM KCl (b: r.p. 62 mV). (c, d) Responses of another preparation to the same stimulation during perfusion with normal saline (c: r.p., 76 mV) and with 15 mM KCl (d: r.p., 60 mV; slight contracture observed). (e, f) Responses of another preparation to the same stimulation during perfusion with normal saline (e: r.p., 78 mV) and with 12 mM K (f: r.p., 65 mV).

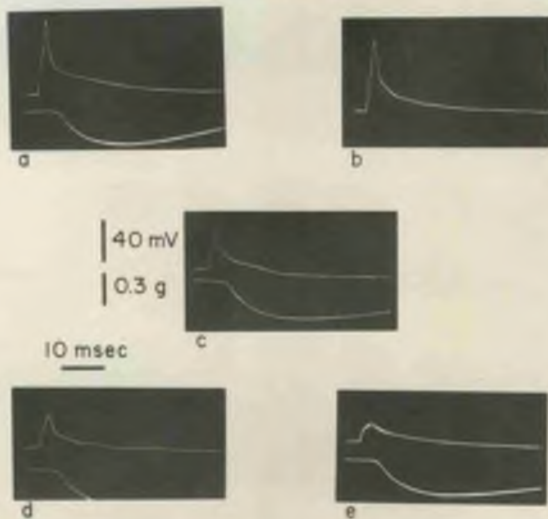


Fig. 106. Progressive effects of potassium ion on the "fast" electrical response of a spiking fibre in a preparation of the Astacus walking leg. Stimulation was given at 1/sec. In (a) the resting potential was 80 mV. (b) to (e) show successive stages during perfusion with 14 mM KCl over a 15 minute period. In (e) the resting potential was 60 mV. The mechanical response was slightly smaller in (e) than in (a).

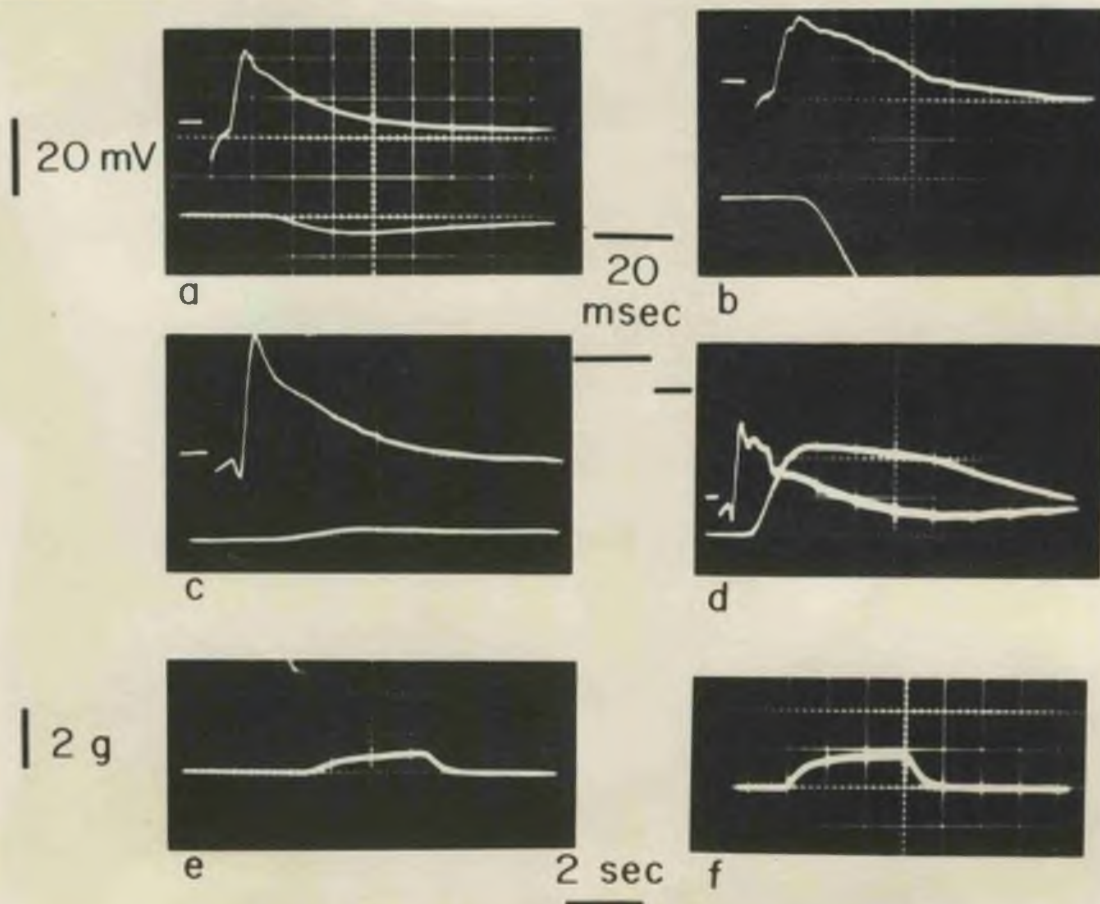


Fig. 107. Effects of potassium ion on "fast" and "slow" responses of the closer muscle of the crayfish claw. (a, b) "Fast" responses to a single shock during perfusion with normal saline (a) and with 17 mM KCl (b). A slight contracture was observed in (b). Electrical recording was done with external electrodes. (c, d) Responses of another preparation to the same stimulation during perfusion with normal saline (c) and with 15 mM KCl (d). (e, f) Mechanical response to stimulation of the "slow" axon at 12 per sec. during perfusion with normal saline (e) and with 15 mM KCl (f). Same preparation as in (c,d).

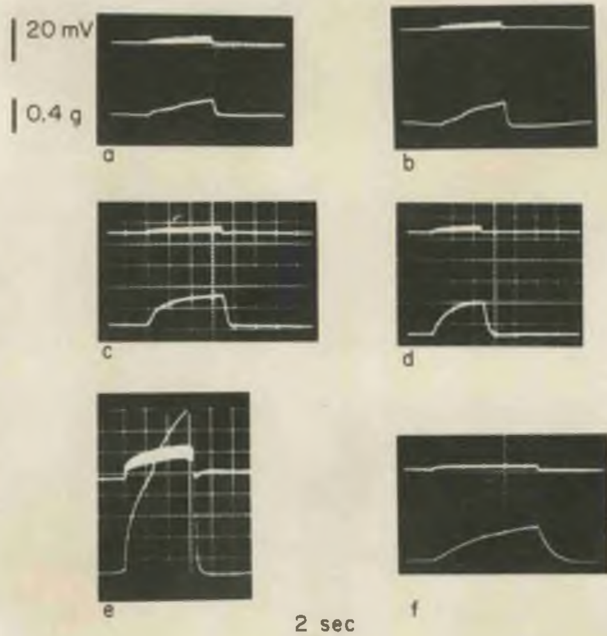


Fig. 108. Effects of potassium ion on the responses of the *Astacus* walking leg opener. (a, b) Responses to stimulation at 30 per sec. in normal saline (a: r.p., 78 mV) and in 10 mM KCl (b: r.p., 67 mV). (c, d) Responses of another preparation to the same stimulation in normal saline (c: r.p., 76 mV) and in 10 mM KCl (d: r.p., 67 mV). (e, f) Responses of another preparation to stimulation at 90 per sec. in normal saline (e: r.p., 80 mV) and after 25 min. in 18 mM KCl (f: r.p., 59 mV).

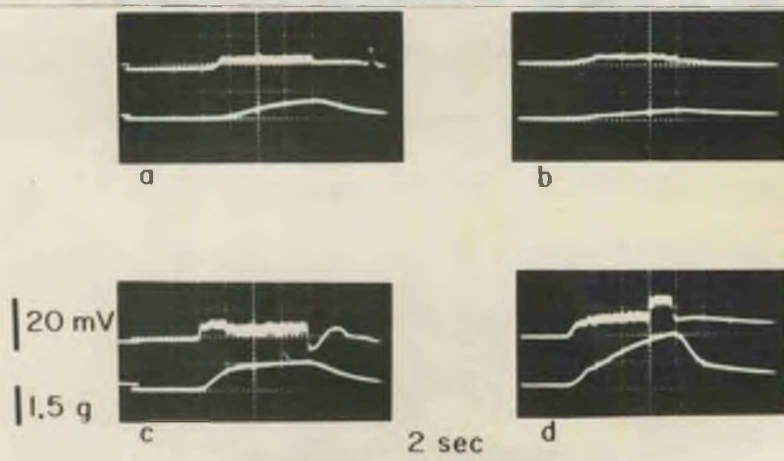


Fig. 109. Effects of potassium ion on the responses of a preparation of the opener of the claw of *Astacus*. (a, c) Reponses in normal saline during stimulation at 15 per sec. (a) and 30 per sec. (c: r.p., 70 mV). (b, d) Responses in 13 mM KCl during stimulation at 15 per sec. (b) and 30 per sec. (d: r.p., 63 mV). The electrical records (upper traces) show considerable distortion but provide an indication of the size of the p.s.ps.

iii) <u>Pachygrapsus: Closer Muscle</u>

A number of *Pachygrapsus* closer muscle preparations were studied during application of sub-contractural KCl concentrations.

The fast mechanical response to a single stimulus was always markedly increased in raised KCl (Fig. 110). The increased response declined rather slowly with time and remained larger than in normal saline for at least 30 minutes.

Electrical recordings from spiking fibres showed results similar to those obtained from spiking fibres in the crayfish walking leg closer (Fig. 110, d to g). As the membrane potential was lowered, the total size of the electrical response was reduced, although the spike reached the same absolute level (close to zero membrane potential). However, the spike declined slowly in size with a prolonged stay in high KCl, and eventually the absolute level reached by the spike was less than in normal saline.

When non-spiking fibres showing large "fast" responses to single stimuli were recorded from during application of raised KCl, it was frequently found that the electrical response increased in absolute size as the membrane potential was lowered (Fig. 110, a to c). This may have been an indication of an electrically excitable response, but other factors, such as an increased release of transmitter substance, cannot be entirely ruled out.

The effect of raised KCl on the slow mechanical response was rather small (Fig. 111). The electrical responses of the specialized "slow" fibres were changed considerably as their

membrane potentials were lowered during application of raised KCl. Typically, the size of the p.s.p. became slightly less, and the maintained depolarization at higher frequencies of stimulation was very markedly reduced (Fig. 111, a to d). These changes were partly reversible provided the stay in high KCl was not prolonged (Fig. 111, e, f). In spite of the smaller maintained depolarization in high KCl, the absolute membrane potential level reached during depolarization was approximately the same as in normal saline at higher frequencies of stimulation. In Fig. 111, for example, the absolute membrane potential levels reached during indirectly produced depolarization in (b), (d), and (f), were 33 mV, 31 mV, and 33.5 mV, respectively. At lower frequencies of stimulation the absolute membrane potential level reached in high KCl (c) was slightly lower (39 mV) than in normal saline (a: 44mV, and e: 45 mV).

The smaller maintained depolarization in high KCl appeared to be largely attributable to a decrease in the time constant of decay of the p.s.p. In Fig. 111, the time constant of deday was estimated to be 53 msec. in normal saline and 46 msec. in high KCl.

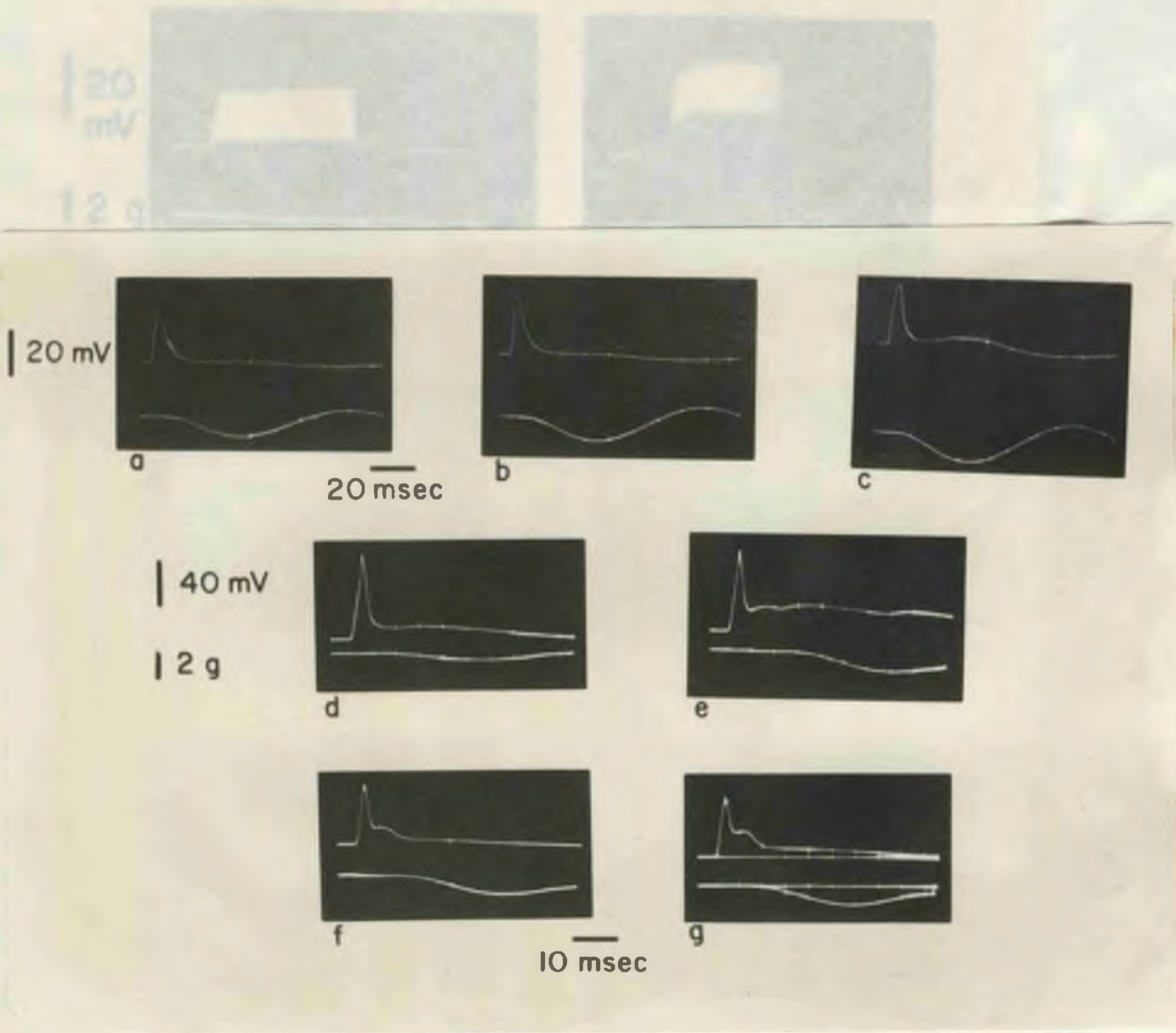


Fig. 110. Effects of potassium ion on "fast" responses of the *Pachygrapsus* closer muscle. (a, b, c) Responses of a preparation to single shocks during perfusion with normal saline (a: r.p., 71 mV) and with 20 mM KCl (b: r.p., 65 mV, and c: r.p., 59 mV). Electrical and mechanical responses were both larger in (c). (d, e, f, g) Responses of another preparation in normal saline (d: r.p., 78 mV) and during perfusion with 20 mM KCl for 10 minutes (e to g). In (g) the resting potential was 60 mV.

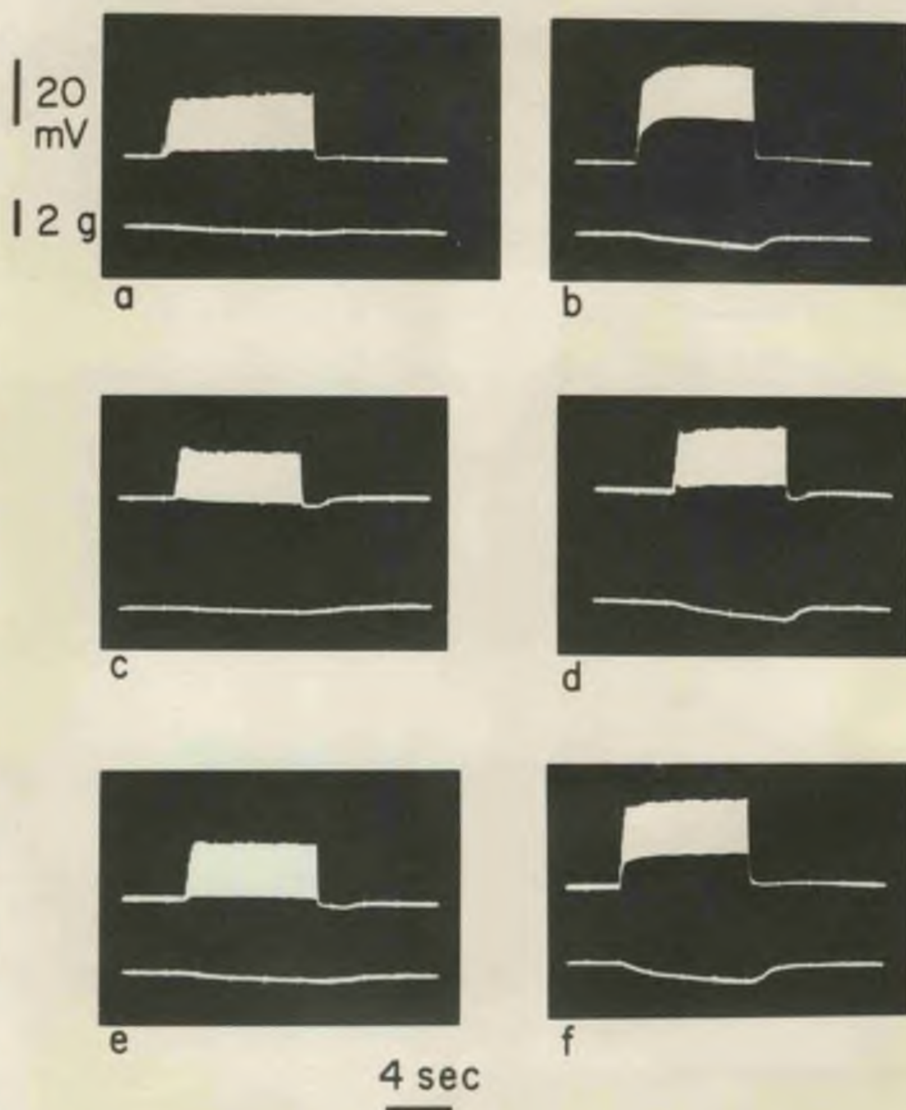


Fig. 111. Effects of potassium ion on "slow" responses of the Pachygrapsus closer muscle. Stimulation was given at 10 per sec. (a, c, e) and at 20 per sec. (b, d, f). (a, b) Responses in normal saline; r.p., 65 mV. (c,d) Responses in 20 mM KCl; r.p., 55 mV. (e.f) Responses after return to normal saline; r.p., 60 mV.

iv) Carcinus: Closer Muscle

Relatively few preparations of this muscle were studied with respect to the potassium effect. However, a number of points of interest emerged from the observations which were made.

The fast mechanical response was almost always augmented slightly by raising the KCl concentration of the perfusion saline (Fig. 112, a, c; Fig. 113, a). When the preparation was returned to normal saline after a short stay in high KCl, the fast mechanical response was usually reduced from its initial magnitude in normal saline. This reduction in magnitude was greater than could be accounted for by normal fatigue.

The "slow" mechanical response was reduced by high KCl in some preparations. An example is given in Fig. 112 (b,d). In this example the total tension response was reduced by high KCl, but the superimposed "twitch" responses were slightly augmented. When normal saline was restored, the tension response was very drastically reduced, and the "twitches" could no longer be seen (Fig. 112, f).

In the majority of muscles studied, the "slow" mechanical response was either unchanged in raised KCl, or slightly augmented (Fig. 113). The type of response which was obtained depended primarily on the experimental animal selected. All muscles from a given animal gave the same type of response in raised KCl, but the differences between muscles from different animals were sometimes considerable.

A feature which was observed in most cases was reduction

of size of the mechanical response when normal saline was restored to the muscle after a brief period of perfusion with high KCl. An extreme example is shown in Fig. 113 (c). In this case a slight contracture resulted from application of high KCl.

Electrical measurements showed that the sizes and time constants of decay of both "fast" and "slow" p.s.ps. were reduced. Data for Type A and Type B fibres were not complete, but in the case of Type C fibres this reduction was always seen (Fig. 112). The reduction in size of the p.s.ps. with lowered membrane potential appeared to be greater in raised KCl than when the membrane potential was reduced by means of a current-passing microelectrode (Fig. 114). However, part of the difference may have arisen from the uniform nature of KCl depolarization compared with the non-uniform nature of microelectrode depolarization (cf. Burke and Ginsborg, 1956 b).

Membrane resistances were considerably reduced in raised KCl. In one muscle, five Type C fibres of about the same size had an average length constant of 1.76 mm in normal saline; of 1.52 mm in 17 mM KCl saline; and of 1.25 mm in 25 mM KCl saline. Since membrane resistance is proportional to the square of the length constant, the ratios of the membrane resistances in the respective salines can be estimated as 3.1 : 2.3 : 1.6. In other words, membrane resistance is about half normal in 25 mM KCl.

It is well known that membrane resistance falls in raised KCl in other excitable cells (e.g. Hodgkin, 1947 ; Keynes, 1954). This fall in membrane resistance is very likely the main

factor responsible for the reduction in size of p.s.ps. in raised KCl and for the associated decrease in time constant of decay, and may account for the fact that in Type C fibres the size of the p.s.p. appears to be reduced to a greater extent than during direct electrical depolarization (Fig. 114).

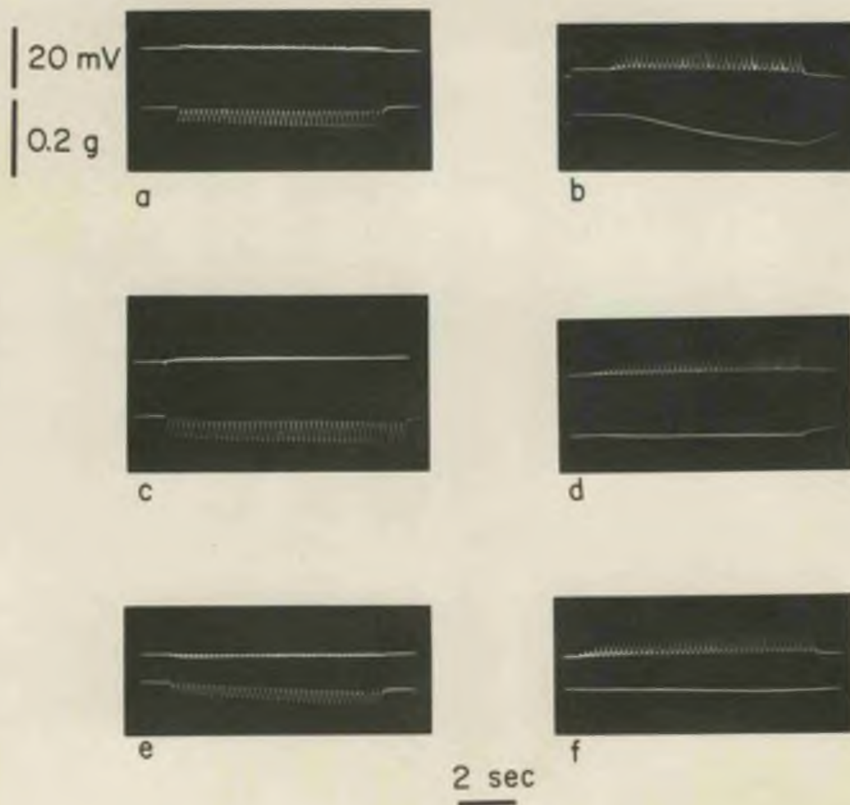
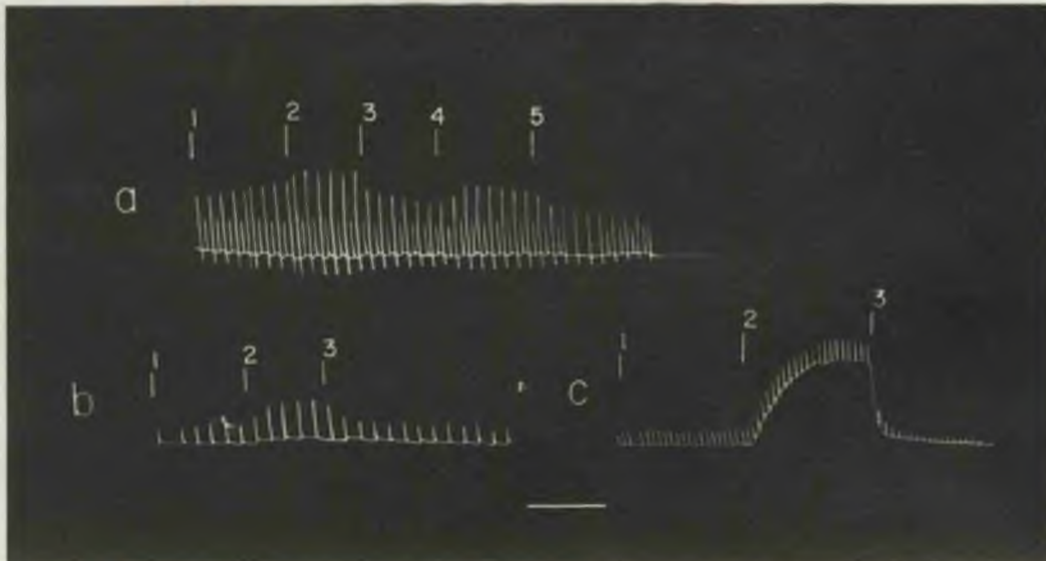


Fig. 112. Effects of potassium ions on Carcinus neuromuscular transmission. All recordings were made from the same preparation. Electrical recording was from a Type C muscle fibre. (a, b) Responses to "fast" (a) and "slow" (b) axon stimulation at 6 per sec.; normal saline; resting potential, 70 mV. (c, d) Responses to "fast" (c) and "slow" (d) axon stimulation at 6 per sec.; saline with 22 mM KCl; resting potential, 59 mV. (e, f) Responses to "fast" (e) and "slow" (f) axon stimulation 2 minutes after return to normal saline; resting potential, 67 mV. Tension, lower traces.

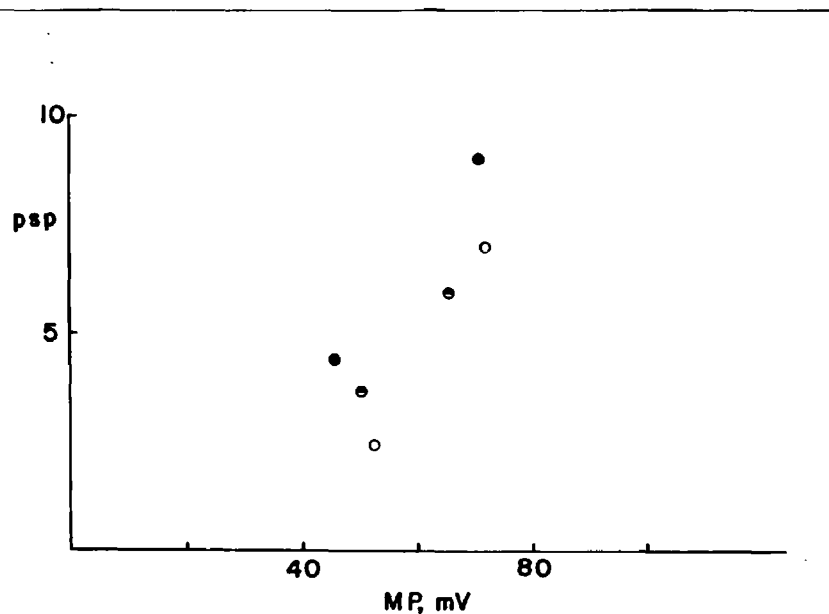


<u>Fig. 113.</u> Effects of potassium on the mechanical responses of three <u>Carcinus</u> closer muscles (isotonic recording). The time calibration is 2 minutes.

a) 1. Perfusion with normal saline. The "fast" axon (large deflections) and the "slow" axon (small deflections) were stimulated alternately with bursts of 40 per second lasting $\frac{1}{4}$ second.
2. Perfusion with 20 mM KCl.
3. Return to normal saline.
4. Perfusion with 20 mM KCl.
5. Return to normal saline.

b) 1. Perfusion with normal saline. The "slow" axon was stimulated with bursts of 40 per sec. lasting $\frac{1}{4}$ second.
2. Perfusion with 30 mM KCl.
3. Return to normal saline.

c) 1. Perfusion with normal saline. Stimulation of the "slow" axon as in (b, 1).
2. Perfusion with 30 mM KCl (Note contracture).
3. Return to normal saline.



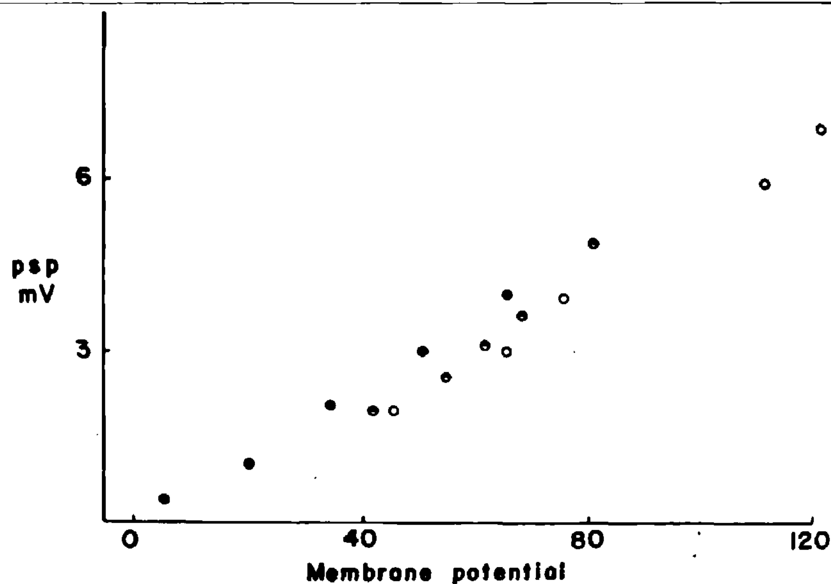


Fig. 114. Depolarization and "slow" p.s.p. size in *Carcinus*. In the top figure "slow" p.s.p. size before and after depolarization with 30 mM KCl is shown for three fibres. In the bottom figure the effect on p.s.p. size of changing the membrane potential with a current-passing microelectrode is shown for three fibres of the same muscle. P.s.p. responses were recorded from Type C fibres during stimulation of the "slow" axon at 8 per sec. No "reversal potential" (Burke and Ginsborg, 1956b) was observed during direct depolarization.

v) General Remarks

Muscles of different crustacean species, and even muscles from different animals of the same species, show a variety of responses to alteration of the potassium concentration of the perfusion fluid. However, certain rough generalizations can be made.

In almost all cases, the "fast" mechanical responses of the preparations studied were enhanced initially by increased potassium concentrations. This was also true of the majority of the "slow" systems studied, although in some cases depression of the "slow" response resulted from an increase in potassium. Prolonged treatment with high potassium led to a gradual decline of both fast and slow mechanical responses.

A similar pattern of initial enhancement and eventual decline in high potassium solutions has been found for other muscles (e.g. Sandow and Kahn, 1952, frog muscle; Hajdu et al., 1950, rat diaphragm preparation).

Possible explanations for the effects found in crustacean muscles will be considered later (see Discussion).

g.) Effects of barium ion on *Nephrops* and *Carcinus* muscles

The effects of barium ion on crayfish and lobster muscles have been thoroughly analyzed by Fatt and Ginsborg (1958) and by Werman and Grundfest (1961). In these muscles barium converts the normally graded membrane responses to direct electrical stimulation into prolonged, all-or-nothing spikes, apparently by delaying and reducing the conductance of the membrane to potassium ions.

In lobster muscle barium greatly reduces the depolarization required to evoke electrically excitable activity. The "threshold" for spike production is shifted towards the resting potential.

No information has been given in the above studies about the effects of barium on the mechanical activity of the muscles concerned. It is reasonable to expect that the increased electrical activity would bring about increased mechanical activity, if the membrane depolarization theory of initiation of contraction is valid. On the other hand, barium has been shown to inhibit potassium contracture in frog muscle (Frank 1962). Therefore it is not possible to predict on *a priori* grounds the effect of barium on the mechanical response of crustacean muscle. The study reported here was made with the intention of discovering the effect of barium on electrical and mechanical activities of the closer muscles of *Nephrops* (an animal fairly closely related to the American lobster studied by Werman and Grundfest) and of *Carcinus*.

i) *Nephrops*: Closer muscle

The effects of barium on the responses of this muscle to indirect stimulation were studied by perfusing the muscle with solutions containing 0 to 80 mM $BaCl_2$ (substituted for equivalent NaCl) and recording the electrical responses of proximal fibres during stimulation of the "fast" axon (usually with single shocks).

For a few minutes after the start of perfusion of a muscle with a solution containing 20 to 80 mM Ba, there was little change in the size of the indirectly produced electrical response and of the mechanical response of the muscle (Fig. 115, d, e). However, changes became evident as the perfusion was continued. The electrical responses of many fibres in the proximal part of the muscle changed from simple p.s.ps. to large and prolonged spikes in a very short space of time (Fig. 115, a to f). The higher the concentration of barium, the sooner these changes occurred. There was no marked change in the resting potentials of the barium-treated fibres, although often a hyperpolarization, and occasionally a depolarization, of a few millivolts was apparent. There was little doubt that the effect of barium on the indirectly produced electrical responses of this muscle was similar to the effect on the directly produced responses of lobster American lobster muscle (Werman and Grundfest, 1961). The threshold for production of electrically excitable responses was apparently lowered to such an extent that these responses could be triggered even by relatively small p.s.ps. The sizes of the p.s.ps. themselves showed little change right up to the moment of spike production.

Associated with the increased electrical activity, and appearing at the same time was a large increase in the mechanical activity of the muscle. As the barium treatment continued, however, the mechanical activity became progressively less, and eventually disappeared (Fig. 115, j). Very large electrical responses were still recorded when the mechanical activity had been reduced to zero (Fig. 115, j). These responses were, however, considerably smaller than responses recorded from the same fibres earlier (Fig. 115, g).

When stimuli were given at a low frequency (about one per second), the first electrical response of a barium-treated fibre was usually somewhat larger than subsequent responses (Fig. 115, h). The mechanical response to the first stimulus was invariably a great deal larger than subsequent responses in muscles treated with barium for longer than 10 mins. In many muscles the mechanical response declined almost to zero after about ten stimuli had been given (at one per sec.). However, this phenomenon was observed only in muscles left in barium for at least 10 mins. Tests conducted sooner after the start of the perfusion showed that the mechanical response did not decline rapidly then.

If, after repeated stimulation and decline of the mechanical response, the muscle was given a rest period of about 30 secs., the first response thereafter showed considerable recovery.

The reason for the decline of the mechanical response to zero after a prolonged stay in high barium solution, in spite

of the continued presence of large electrical responses, is not immediately clear. It is possible that barium exerts an inhibitory action on the contractile mechanism or on the excitation-contraction coupling process, perhaps by competitive inhibition of a calcium-utilizing step. It is also possible that the large electrical responses cause rapid exhaustion of a step in the excitation-contraction coupling process which cannot easily be "reprimed" in the presence of barium ions.

The reason for the decline of the electrical response (more gradually than the mechanical response) is also not immediately apparent.

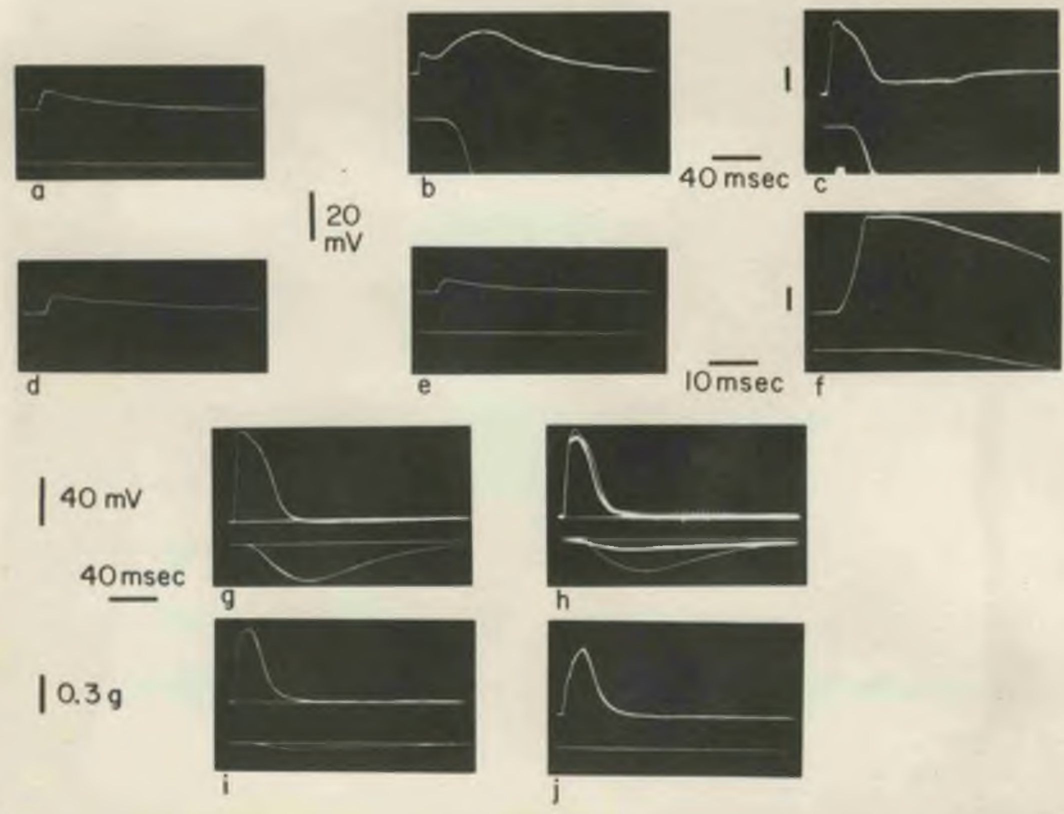


Fig. 115. Effects of barium ion on the "fast" responses of the *Nephrops* closer muscle. (a, b, c) Responses of a preparation to single shocks to the "fast" axon during perfusion with normal saline (a); 5 min. after perfusion with 40 mM Ba saline (b), and 5½ min. after perfusion with this solution (c). Note reduced time scale in (b) and (c). (d, e, f) Responses of another preparation to the same stimulation during perfusion with normal saline (d), 5 min. after perfusion with 40 mM Ba (e), and 6 min. after perfusion with this solution (f). (g to j) Responses to stimulation of the "fast" axon at 1 per sec. 8 min. after perfusion with 60 mM Ba saline (g), 14 min. after start of perfusion (h), 17 min. after start of perfusion (i), and 21 min. after start of perfusion (j).

ii) Carcinus: Closer Muscle

Electrical and mechanical responses to indirect stimulation were recorded from preparations of the Carcinus closer muscle during perfusion with solutions containing 0 to 100 mM Ba.

With barium concentrations of 15 to 30 mM, the "slow" and "fast" mechanical and electrical responses showed slight but definite increases in size. An example is shown in Fig. 116 (a to d), in which the electrical responses of a Type B muscle fibre were recorded during "slow" axon stimulation. Marked potentiation of the electrical response of this fibre occurred after treatment with Ba, and the mechanical response of the muscle was also definitely larger.

After treatment with these same concentrations of barium, it was found that stimulation with double shocks (about 3 msec. separation) produced a marked change in the "fast" mechanical response, but little change in the "slow" mechanical response (Fig. 116, e to h). In the same muscles, the "fast" and "slow" mechanical responses to stimulation with single shocks were only slightly potentiated after barium treatment.

When higher concentrations of barium were used (40 to 100 mM), it was found that a single shock to either the "slow" or the "fast" axon gave a powerful and prolonged contraction of the muscle. Recordings from muscle fibres indicated that several p.s.ps. were produced by a single shock. It appeared that the nerve was firing repetitively in such cases (Fig. 116, i). Repetitive (and spontaneous) firing of the nerve has also been described for Romalea following barium treatment (Werman et al., 1961). Electrical responses of Type C fibres were

often small, but in Type A and Type B fibres much larger responses would be generated by this repetitive firing. No successful recordings were made for these latter two fibre types.

The marked decline of mechanical activity observed in Nephrops was not found in Carcinus. However, following a powerful contraction in high barium, another contraction could be obtained only after a rest period of several seconds.

The responses of Carcinus fibres to direct stimulation following treatment with barium were somewhat different than those which have been described for lobster muscles (Werman and Grundfest, 1961). Initially, application of barium caused a slight degree of hyperpolarization in most fibres, usually only a few millivolts. After the muscle had been soaked in barium, depolarizing pulses failed to evoke all-or-nothing spikes at membrane potential levels of 30 to 40 mV below the resting potential level (Fig. 117, a,b). In fact, the responses in barium were quite similar to those in normal saline except for a steepening of the voltage-current relationship, indicating a definite, but not drastic, increase in membrane resistance (Fig. 118). The increase in membrane resistance was reflected also in an increase in length constant. Four Type C fibres of similar characteristics had average length constants of 1.7 mm in normal saline and 2.2 mm in 60 mM barium, indicating an increase in membrane resistance by a factor of 1.7 in barium solution. No lowering of the threshold for electrically excitable responses was evident (Fig. 118).

The increase in membrane resistance in barium accounts

for the increase in size of "fast" and "slow" p.s.ps. described previously.

In several cases repeated stimulation of a fibre with depolarizing pulses led to marked lowering of the resting potential. When this occurred, spike responses could be obtained by direct stimulation. These responses were of the prolonged type typical of lobster muscle (Fig. 117, c to f).

From these observations it is evident that the effects of barium are rather different in crab and lobster muscles. Barium spikes can be produced in crab fibres only after considerable depolarization. The threshold for spike production is not lowered in the manner characteristic of _Nephrops_ and American lobster muscles.

During repetitive firing of the nerve in high barium solutions, it is probable that many of the crab muscle fibres (particularly Type A and Type B muscle fibres) are depolarized to the point at which barium spikes are produced, and that such prolonged spikes generate in turn the powerful mechanical response of the muscle. Similarly, at lower concentrations of barium, double shocks to the "fast" axon probably cause much greater depolarization in Type A fibres than similar stimulation of the "slow" axon causes in Type B and Type C fibres. Some of the Type A fibres may produce barium spikes in response to double shocks to the nerve, but not to single shocks; this would give rise to the observed effects on the mechanical responses.

The variety of mechanisms found in different crustacean muscles and even within the same muscle is further emphasized by the observations on barium effects.

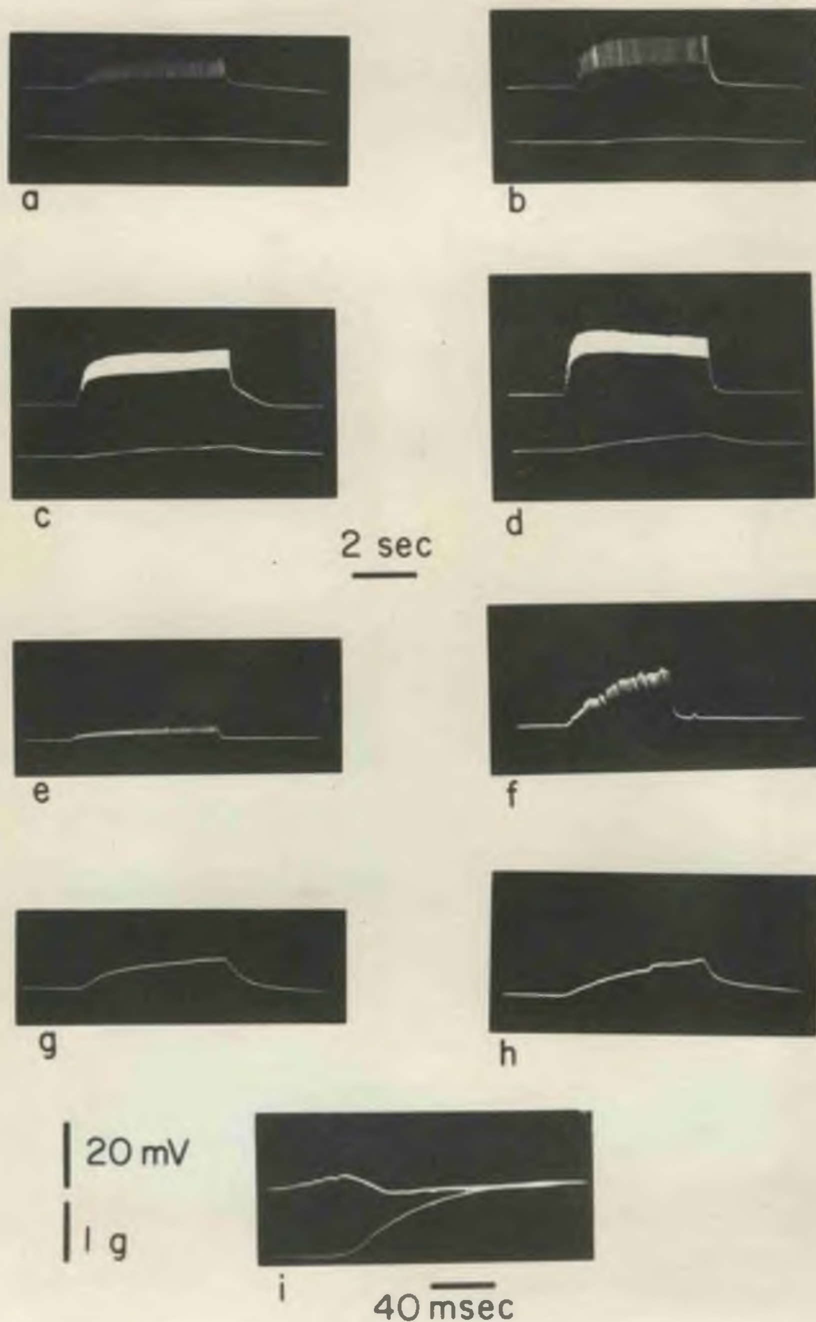


Fig. 116. Effects of barium ion on indirectly produced responses of Carcinus muscle. (a, c) Responses of a Type B fibre to stimulation of the "slow" axon at 10 per sec. (a) and 20 per sec. (c). (b, d) Responses of the same fibre after perfusion with 20 mM Ba saline; note that the mechanical response is slightly increased. (e) Mechanical response to stimulation of the "fast" axon at 10 per sec. with double pulses (2.5 msec. separation). (f) Response to the same stimulation after perfusion with 20 mM Ba. (g, h) "Slow" mechanical response to the same stimulation in normal saline (g) and after perfusion with 20 mM Ba (h). (i) Responses to a single stimulus applied to the "fast" axon after perfusion of the muscle with 60 mM Ba for 5 min. Electrical record (top) was from a Type C muscle fibre. Note multiple p.s.p.s. and the powerful mechanical response of the muscle.

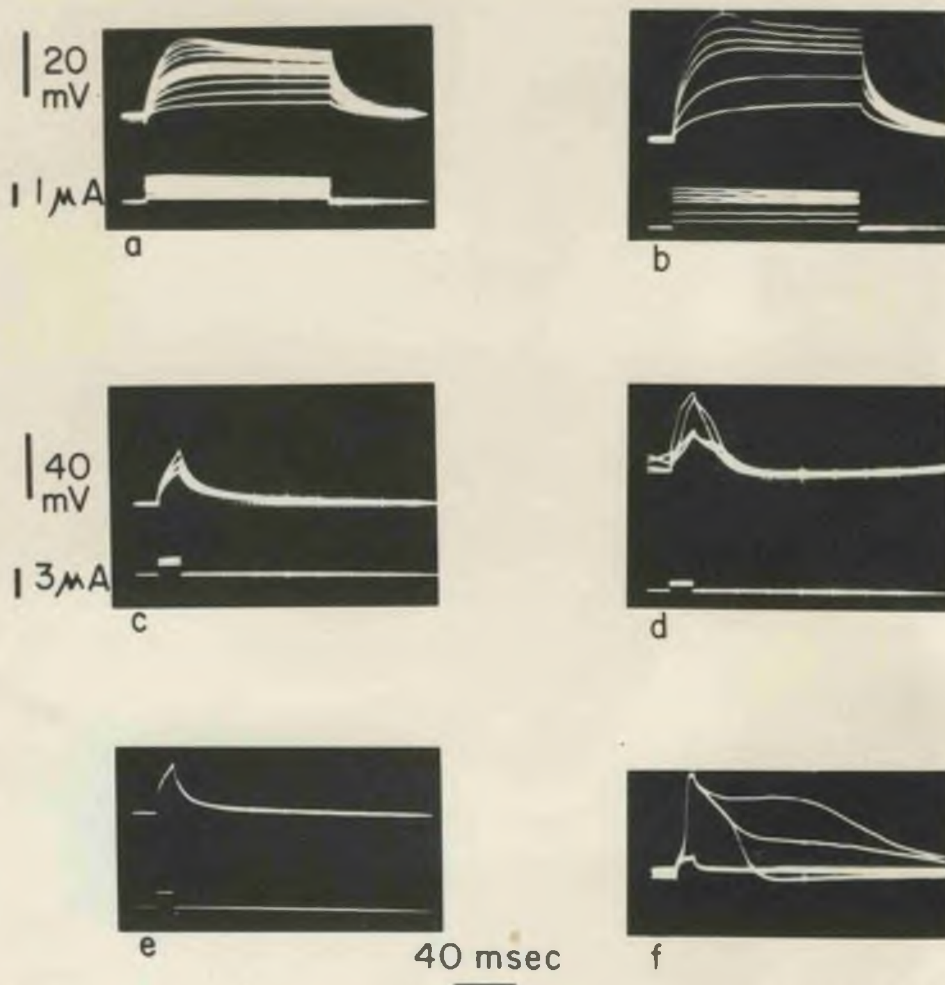


Fig. 117. Responses to direct stimulation in Type C muscle fibres of Carcinus. (a) Responses to depolarizing pulses in normal saline; resting potential, 70 mV. (b) Responses to depolarizing pulses in 80 mM Ba; r.p., 75 mV. (c) Responses to depolarizing pulses of another Type C muscle fibre in 80 mM Ba; r.p., 71 mV. (d) Responses of the same fibre after depolarization following repeated stimulation; r.p., 50 mV. (e) Responses of another fibre in 80 mM Ba; r.p., 65 mV. (f) Responses of the same fibre after depolarization following repeated stimulation; r.p., 52 mV.

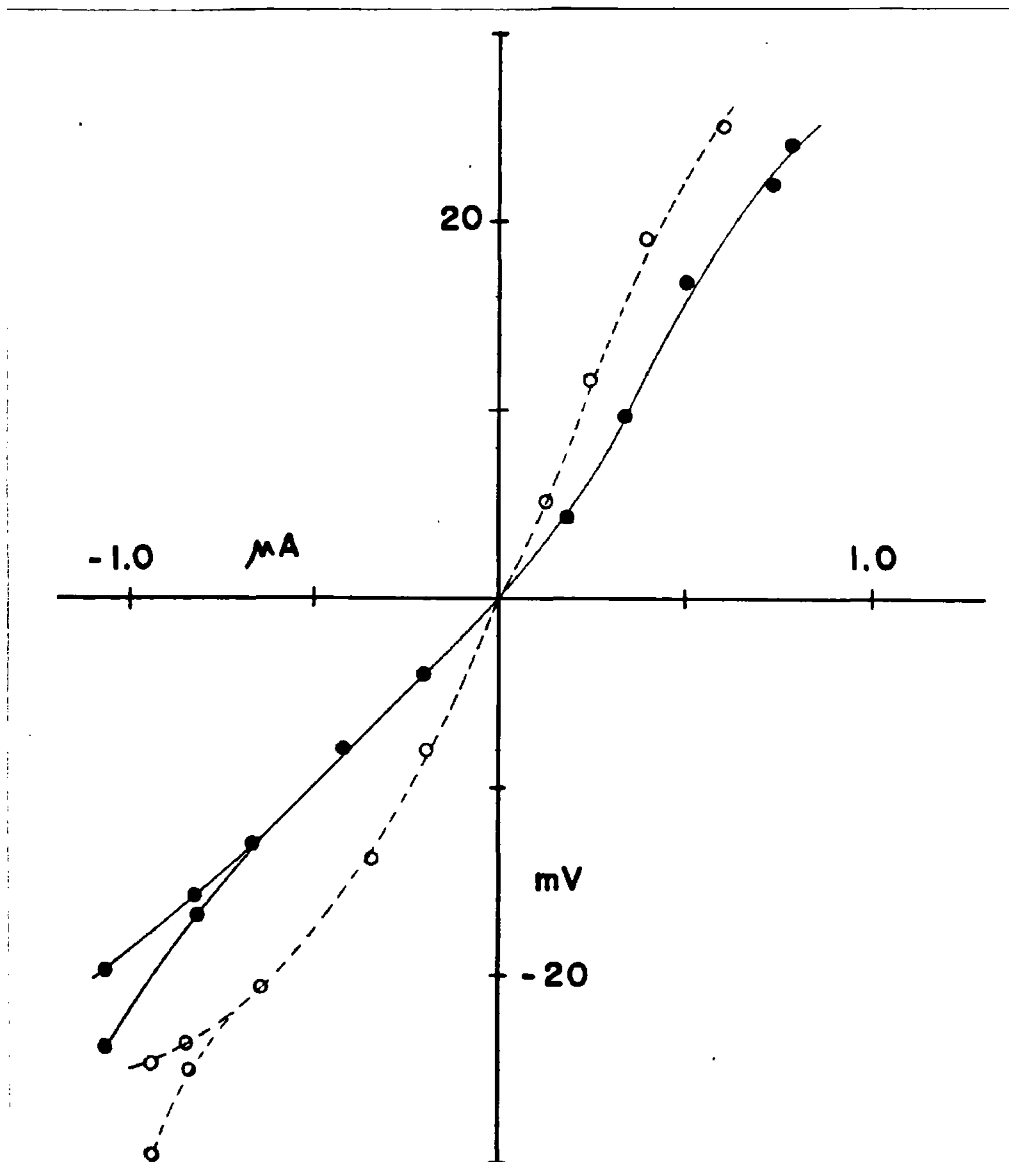


Fig. 118. Voltage-current plots for a Type C fibre in normal saline (filled circles; r.p., 71 mV) and in 60 mM Ba (open circles; r.p., 76 mV). Hyperpolarization is taken as positive. The divided line in the lower left-hand quadrant represents initial and final membrane potential responses to depolarization (See Fig. 66).

h) <u>Effects of substituted anions on electrical and mechanical responses of crustacean muscles</u>.

Since the description of the potentiation of the mechanical response of frog muscle by substitution of bromide, nitrate, or iodide for the chloride of normal Ringer's solution (Kahn and Sandow, 1950), a number of studies have been carried out to determine the mechanisms of this effect (e.g. Hill and Macpherson, 1954; Ritchie, 1954; Hutter and Padsha, 1959; Hodgkin and Horowicz, 1960 c; and others). These studies have shown that in frog muscle the substituted anions increase the duration of the active state of the muscle, giving rise to an increased twitch height. In addition, and associated with this change, the spike and after-potentail are prolonged (Hutter and Noble, 1960); the membrane resistance is increased; and the membrane potential "threshold" at which contraction starts is shifted towards the resting potential.

Other muscles besides the usual frog preparations have been subjected to treatment with anions of this series. In smooth muscle, spike frequency and tension are increased by nitrate (Axelsson, 1961). In heart muscle, mechanical performance is reduced by nitrate as are the rate of rise and magnitude of the action potential (Peterson and Feigen, 1962). Certain insect muscles show potentiation of the twitch responses similar to that observed in frog muscles (Hoyle, personal communication).

Many other studies of the effects of anions on muscle have been made, but this brief survey indicates that the usual effect on striated muscle is potentiation of the mechanical

responses. Usually it has been found that there is an associated prolongation of the electrical response. In heart muscle, however, both electrical and mechanical responses are reduced by foreign anions.

No information exists on the effects of substituted anions on crustacean muscles, apart from a brief note by Reuben (1959). Since such a study seemed likely to be of importance in shedding light on possible mechnaisms of excit- excitation-contraction coupling in crustacean muscle, experiments were performed to determine the effects on crustacean muscles of substituting bromide, nitrate, iodide, and thiscyanate for the chloride of the normal perfusion solution.

Most of the experiments were done with the Carcinus closer muscle. However, a number of additional experiments (with nitrate) were performed with muscles of other animals, and these showed that the effects of nitrate were not the same in all crustacean muscles.

i) Carcinus: Closer Muscle

The methods used for recording electrical and mechanical responses and for perfusing altered solutions into the muscle were the same as those described previously. Several types of experiment were performed including: stimulation of "fast" and "slow" axons before and after substitution of foreign anions; application of high potassium solutions before and after anion substitution; determination of the effects of foreign anions on membrane resistance of single muscle fibres; and stimulation of single muscle fibres by intracellular electrodes before and after addition of foreign anions.

<u>Effects on responses to indirect stimulation</u>. The effects of foreign anions on responses to "fast" and "slow" axon stimulation depended on the particular anion being tested, its concentration, and the particular axon being stimulated.

When concentrations of nitrate ranging from 20 to 400 mM were perfused into the muscle, both "fast" and "slow" mechanical responses were depressed (Figs. 120 to 122). The "slow" response was affected to a greater extent than the "fast" (Fig. 121). The extent of the effect depended on the concentration of nitrate employed; at low nitrate concentration (below 50 mM) the effect was slight, but when higher nitrate concentrations were employed, the depression became increasingly pronounced. When nitrate solution was replaced by the normal saline, the mechanical response recovered very slowly.

The effects of bromide substitution on the muscle's mechanical responses were similar to the effects of nitrate but much less pronounced. At bromide concentrations below 150 mM the depression was very slight, and even at higher concentrations it was not comparable in magnitude to that produced by nitrate.

Observations were made on the electrical responses of Type A, Type B, and Type C fibres before and after treatment with nitrate. In all cases it was found that the electrical responses were augmented considerably by nitrate treatment. In Type C (Fig. 120) and Type B (Fig. 121) fibres, the sizes of both "fast" and "slow" p.s.ps. were increased, as were the

maintained depolarization "plateaus". In the case of Type A fibres (Fig. 122) it was observed that the sizes of "fast" p.s.ps. were increased, and that in some instances spikes were fired in nitrate at frequencies of stimulation which did not produce this result in normal (chloride) perfusion saline. In spite of this very evident increase in electrical activity in all types of muscle fibre, the associated mechanical responses were markedly reduced.

After a prolonged stay in nitrate solution, it was observed that electrical responses of many impaled Type A fibres showed a gradual decline in magnitude (Fig. 122, g, h). However, it was not entirely clear whether or not this result was due to membrane damage caused by prolonged impalement of the same fibre with the recording microelectrode.

The effect of nitrate on the resting potentials of impaled muscle fibres appeared to be slight when these fibres had resting potentials of about 70 mV. Fibres with lower resting potentials showed in some cases hyperpolarization by 2 to 4 mV in nitrate solutions.

The effects on the mechanical response of substitution of iodide and thiocyanate for chloride were somewhat different from those described for nitrate and bromide. When concentrations of iodide and thiocyanate of 5 to about 100 mM were used, the "fast" mechanical response was increased in size, whereas the "slow" mechanical response was depressed (Fig. 123, a to d; Fig. 124, c, d). These effects were slowly reversible after return to normal saline. When higher concentrations of these anions were used, both "fast" and "slow"

mechanical responses were depressed, often irreversibly. High concentrations of these anions appeared to have a deleterious effect on this muscle.

The electrical responses to both "fast" and "slow" axon stimulation were markedly increased in iodide and thiocyanate solutions (Figs. 123 to 126). The increase in size was very pronounced for Type C (Figs. 123, 124) and for Type A (Fig. 126) muscle fibres. In some Type C fibres, electrically excitable responses were seen during stimulation of the "fast" axon at a high frequency (Fig. 124, e, f). In Type A fibres, the electrical response to a single stimulus applied to the "fast" axon was changed from a graded p.s.p. to a much larger p.s.p. which commonly evoked a spike (Fig. 126 a,b). In thiocyanate, a smaller increase in size was observed for many Type B muscle fibres (Fig. 125, a to d). After a prolonged stay in thiocyanate, responses of both Type A and Type B fibres became reduced in size (Figs. 125, 126), sometimes below their levels in normal saline. In Fig. 125 (f), it can be seen that the time constant of decay is reduced after some time in SCN.

It was commonly found that application of iodide and thiocyanate resulted in hyperpolarization of the muscle fibre membranes. This hyperpolarization was of the order of 2 to 8 mV in many cases.

Effects of anion substitution on potassium contracture. It has been shown by Kahn and Sandow (1955) and by Hodgkin and Horowicz (1960c) that substitution of anions such as nitrate for chloride produced a marked increase in the tension developed by frog muscle during application of a given

concentration of potassium. Hodgkin and Horowicz found that the "threshold" for tension development was shifted towards the resting potential, and that less potassium was required to produce contracture in solutions containing substituted anions than in normal Ringer's solution.

With the experiments of Hodgkin and Horowicz in mind, tests were made to determine the effects of anion substitution on the potassium contracture of the Carcinus closer muscle.

When bromide and nitrate were substituted for chloride, it was found that the amount of tension developed in response to application of a given potassium concentration was not increased, as in frog muscle, but very markedly reduced (Fig. 127, a, b). The amount of reduction depended on the amount of substituted anion present: The higher the concentration of nitrate or bromide, the greater the reduction of tension. When the muscle was returned to normal saline, the ability to give normal contractures was restored.

It was observed that nitrate was more effective than bromide in reducing tension developed in response to application of high potassium solutions.

When iodide was substituted for chloride, a definite reduction in tension was observed, which was more marked in higher iodide concentrations (Fig. 127, c). If normal saline was perfused into the muscle for a minute or two after treatment with iodide, and if the muscle was then tested with a contracture-producing potassium solution, the tension response was much greater than it had been before iodide treatment (Fig. 127c). Only by perfusing the muscle with normal

saline for about 20 minutes could this "after-effect" of iodide be eliminated.

When thiocyanate concentrations of 5 to about 150 mM were used, it was found that the contraction produced by a test potassium concentration was enormously increased (Fig. 127, d). When higher concentrations of SCN were employed, the contraction did not show this pronounced increase. As in iodide, a very pronounced "after-effect" was observed unless the muscle was perfused for a considerable time with normal saline. After a number of contractures in thiocyanate, the muscle often went into partial rigor, as in Fig. 127 (d).

Electrical correlates of these mechanical changes were sought, using the methods described previously (p. 110). Once more, it was necessary to distinguish between "immediate" and "delayed" effects of potassium on the membrane potential, because it was found that certain foreign anions could affect the "immediate" response, but not the "delayed" response.

This point is best illustrated by results obtained using solutions in which nitrate was substituted for chloride. The "delayed" effects of potassium in nitrate-substituted and nitrate-free solutions are illustrated in Fig. 128. No significant difference was apparent between the two curves.

When the "immediate" responses were tested, however, a different result was obtained. It was found that the substitution of nitrate for chloride resulted in less effective depolarization by potassium. An example illustrating these results is given in Fig. 129. In this case muscles of the same size from the same animal were used, and it was apparent

that the tension response was lower in nitrate, even when the average recorded depolarization was greater.

Experiments illustrating the same "immediate" effects are illustrated in Fig. 130. In these examples the same muscles were used. When one muscle was switched from chloride to nitrate in 40 mM KCl, hyperpolarization resulted. When the other muscle was switched from 50 mM KCl, 400 mM NO_3 to 40 mM KCl, 0 NO_3, marked depolarization resulted. In Fig. 130 (lower), it can be seen that less tension was developed in 70 mM KCl, 400 mM NO_3 than in 40 mM KCl, 0 NO_3, in spite of the fact that depolarization was greater in the former case.

The "threshold" membrane potential for tension development was estimated to be the same in nitrate as in chloride (about 55 mV). However, the membrane potential-tension relationship appeared to be altered in nitrate. Less tension was recorded in nitrate for the same degree of depolarization as in chloride. This was true both in the case of paired muscles treated separately, and in the case of a single muscle treated first with one anion, then with the other.

In all of the experiments performed above, care was taken to change rapidly from one solution to another to ensure responses that were as "immediate" as possible.

Results obtained using solutions with iodide or thiocyanate substituted for chloride were not very satisfactory. In iodide solutions the muscles deteriorated rapidly when excess potassium was introduced. Many fibres were observed to break up and disintegrate.

However, it was found that the "threshold" for depolarization was about the same as in chloride, although slightly higher potassium concentrations were required to attain it. Observations also indicated that the tension developed for a given depolarization was approximately the same as that in chloride (Fig. 133). However, it was found that the tension decay was much more rapid in iodide. Very shortly after the maximum "immediate" tension was attained, the response started to decline rapidly.

In thiocyanate solutions a different problem was encountered. As the potassium concentration was increased and the membrane potential was lowered past the "threshold" for contraction (which was estimated to lie between 58 mV and 55 mV), some of the fibres in the muscle typically went into violent contraction. These fibres were found to have very low resting potentials. The majority of the fibres remained at a higher resting potential close to the "threshold", and showed no evidence of strong contraction. Any increase in potassium concentration of the perfusing saline, however slight, caused more fibres to contract violently. Because of the non-uniform behaviour of thiocyanate-treated muscles, no reliable estimate could be made of the tension developed for a given degree of depolarization in these muscles. The only conclusion which could be drawn concerning the relationship between membrane potential and tension in thiocyanate-treated muscles was that the "threshold" for tension development lay between 60 and 55 mV resting potential -- close to the value found in thiocyanate-free solutions. The potassium concentrations required for

threshold contracture in thiocyanate solutions were less than those required in thiocyanate-free solutions -- about 20 to 25 mM as compared with 30 to 35 mM.

The thiocyanate "after-effect" (p. 164) appeared to be at least partly attributable to a marked lowering of the resting potentials of many muscle fibres when a test potassium solution was applied shortly after thiocyanate treatment.

Contractures in thiocyanate were typically very prolonged, and if they were allowed to last for more than about one minute, some degree of irreversible rigor usually resulted.

Effects of substituted anions on membrane resistance. It was suspected that some of the changes in the electrical responses to indirect stimulation brought about by treatment with foreign anions could be due to changes in membrane resistance similar to those reported for frog muscle (Hutter and Padsha, 1959). Accordingly, membrane resistances of many Type C muscle fibres were measured before and after treatment with a foreign anion.

In all cases, it was necessary to perfuse a muscle with an altered solution for about 5 to 10 minutes in order to observe the full effect of this solution on membrane resistance.

All four of the anions tested (bromide, nitrate, iodide, thiocyanate) increased the "input resistances" and time constants of the muscle fibres investigated. Examples of these effects are shown in Fig. 132 (nitrate) and in Fig. 133 (thiocyanate). In the same muscle fibres, the sizes of both "fast" and "slow" p.s.ps. were markedly increased.

Estimates of the relative membrane resistances of muscle

fibres before and after treatment with various concentrations of substituted anions were made by determining the input resistance of the fibre and its length constant, and multiplying these two quantities together. The product is a relative measure of the membrane resistance.

Results of representative measurements are given in Tables 10, 11, and 12. In Table 10, results of tests on fibres treated with nitrate and bromide are shown. The effect of bromide appeared to be very slight; even at high concentrations of this ion the membrane resistance was increased by a factor of only 1.2. By contrast, only 50 mM nitrate was required to produce a comparable change in many fibres. When concentrations of 200 to 400 mM nitrate were used, membrane resistance was often nearly doubled (although in some cases much smaller changes were noted, as in Fibre 6, Table 10). It appeared that the membrane resistance changes were greater for the first 100 mM of nitrate substituted than for additional increments.

When fibres were returned from nitrate solutions to the normal saline, membrane resistance was observed to fall. In some cases membrane resistance fell below its initial level in normal saline. (This could have been due to fibre damage).

Examples of membrane resistance changes in iodide are shown in Table 11. It is evident that addition of 50 mM iodide typically brings about a considerably greater increase in membrane resistance than does the same concentration of nitrate. However, as the concentration of iodide is increased to 100 mM, the membrane resistance usually fell, and

in some cases became less than in normal saline.

Thiocyanate had the most pronounced effect on membrane resistance of any of the anions tested (Table 12). Even 12.5 mM SCN raised the membrane resistance of one fibre (Fibre 3) by a factor of 1.65. In 50 mM SCN, membrane resistance was usually about twice that in normal saline. As the concentration of SCN was increased above 100 mM, the membrane resistance continued to increase, although more slowly.

In comparing the results for the four anions tested, it can be concluded that the effectiveness of these anions in increasing membrane resistance follows the order: bromide $<$ nitrate $<$ iodide $<$ thiocyanate, as in the lyotropic series. However, a curious discontinuity exists for iodide, since high concentrations of this anion actually lower membrane resistance. None of the other anions showed this effect at high concentrations.

Effects of substituted anions on tension responses of single muscle fibres. Potassium depolarization of whole muscles is, at best, a crude tool for investigating membrane potential-tension relationships. The effects of substituted anions on the membrane potential-tension relationships of single muscle fibres were therefore investigated by means of the techniques described previously (p. 125). Observations were limited to the effects of nitrate and thiocyanate.

When nitrate solutions were added to a single-fibre preparation, stimulation of the fibre with a current sufficient to give a vigorous contractile response in normal saline produced a much less vigorous response in the nitrate

solution (Fig. 134, a,b). The magnitude of the electrical response in nitrate was generally greater for the same stimulating current, but the membrane potential level reached was usually slightly less than in normal saline, because a hyperpolarization of the muscle fibre membrane of 4 to 8 mV usually occurred after addition of nitrate to the preparation. In order to cause the electrical response of the membrane to reach the same level of membrane potential in nitrate as in chloride, it was necessary to apply a higher current in most cases. In Fig. 134 (d), which shows a response in nitrate, the absolute level of membrane potential reached during the electrical response was lightly greater than in Fig. 134 (c), but the tension response was slightly less in nitrate.

In seven of ten fibres tested, the tension responses in nitrate were slightly less than those in chloride for the same absolute level of membrane potential. A graph illustrating one of these results is shown in Fig. 135. In three of ten cases, the tension responses in nitrate were slightly larger than in chloride at the same membrane potential level. However, in all cases the "threshold" for tension development was about the same in nitrate and in chloride (Fig. 135).

Previously it was shown that in nitrate the length constants of these muscle fibres are increased. This means that the length of fibre depolarized past the "threshold" level is greater in nitrate than in chloride for the same depolarization. A simple plot of membrane potential against tension, as in Fig. 135, fails to take into account the effects of

changes in length constant. In a number of additional experiments the depolarization "profile" of the muscle fibre was determined in chloride and in nitrate, and the depolarization-tension curves were found for the same fibres. The depolarization above threshold (which was taken to be 55 mV) was then integrated along the length of the fibre (see p. 131), and the result (L x D) was plotted against tension as in Fig. 136. In all five cases examined, the tension per depolarization-length unit was less in nitrate.

When thiocyanate solutions were applied to single fibre preparations, the tension developed in response to the same, or less, current and depolarization was considerably increased (Fig. 137). At the same absolute level of membrane potential, the tension in thiocyanate was always greater than in normal saline. However, when account was taken of the large increase in length constant in thiocyanate it was found that the tension per depolarization-length unit was about the same in thiocyante as in chloride. An example to illustrate this point is given in Fig. 138. In this example it was also found that the result was the same in 50 mM SCN as in 200 mM SCN.

In plotting the results for Fig. 138, the assumption was made that the "threshold" for contraction was the same in thiocyanate as in normal saline. This assumption may not have been entirely valid, because the contraction of single fibres was detectable at smaller depolarizations in thiocyanate. However, the large increase in length constant in thiocyanate, and the consequent more effective spread of

depolarization along the muscle fibre may have given the appearance of a lower threshold in thiocyanate. It is probable that the true threshold in thiocyanate was not greatly different from that in chloride, although it may have been 1 to 4 mV lower. If the threshold was lower in thiocyanate, the tension developed per depolarization-length unit would be somewhat less than that shown in Fig. 138. In addition, thiocyanate increases the membrane time constant very markedly, and this factor is also not taken into account in Fig. 138. The possibility exists, therefore, that the muscle develops tension less efficiently in thiocyanate than in normal saline. Difficulties in determination of the threshold in single fibre preparations made this point uncertain.

Throughout the experiments on direct stimulation of Type C muscle fibres, no marked changes in the electrically excitable responses were observed in either nitrate or thiocyanate solutions. On two occasions stimulating and recording electrodes were introduced into Type A muscle fibres, and it was found that in these fibres the electrically excitable responses to direct stimulation were slightly larger after addition of thiocyanate. The effects of substituted anions on the electrically excitable responses of most of the muscle fibres must be regarded as slight except possibly in the case of Type A fibres, and even there the effects were not great.

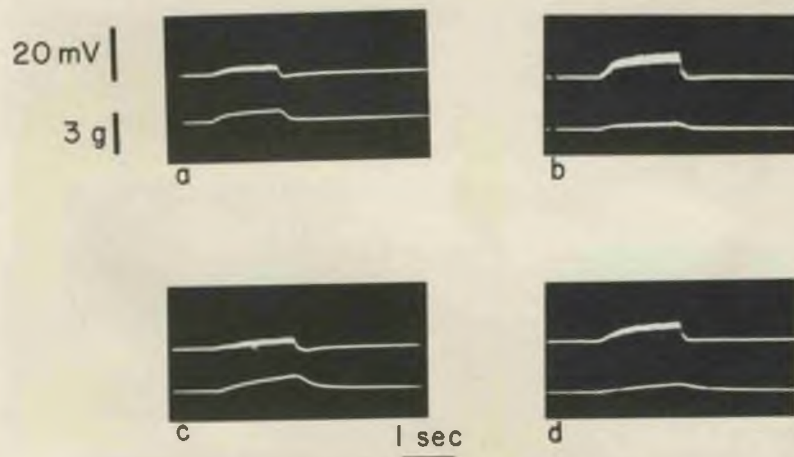


Fig. 120. Effects of nitrate ions on responses to indirect stimulation in the Carcinus closer muscle. (a, c) Responses of a Type C fibre in normal saline during stimulation of "fast" (a) and "slow" (c) axons at 30 per sec. (b, d) Responses of the same fibre in 200 mM NO_3 during stimulation of "fast" (b) and "slow" (d) axons at 30 per sec. Lower traces, muscle tension.

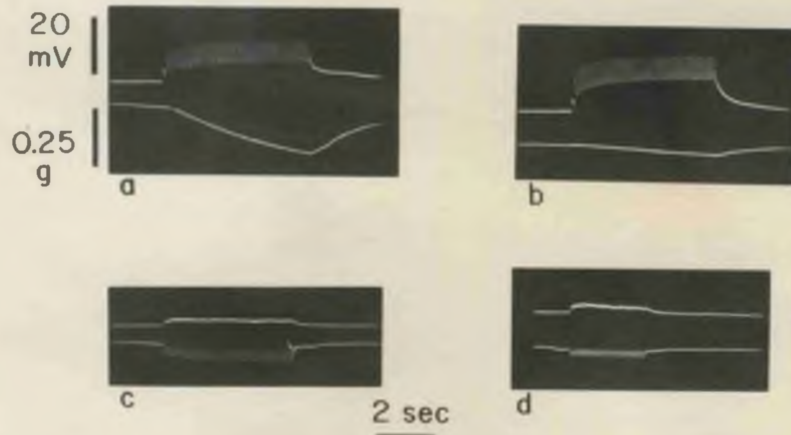


Fig. 121. Effects of nitrate on responses to indirect stimulation of a Carcinus Type B muscle fibre. (a, c) Responses in normal saline during stimulation of "slow" (a), and "fast" (c) axons at 10 per sec. (b, d) Responses in 400 mM NO_3 during stimulation of "slow" (b) and "fast" (d) axons at 10 per sec. Lower traces, muscle tension.

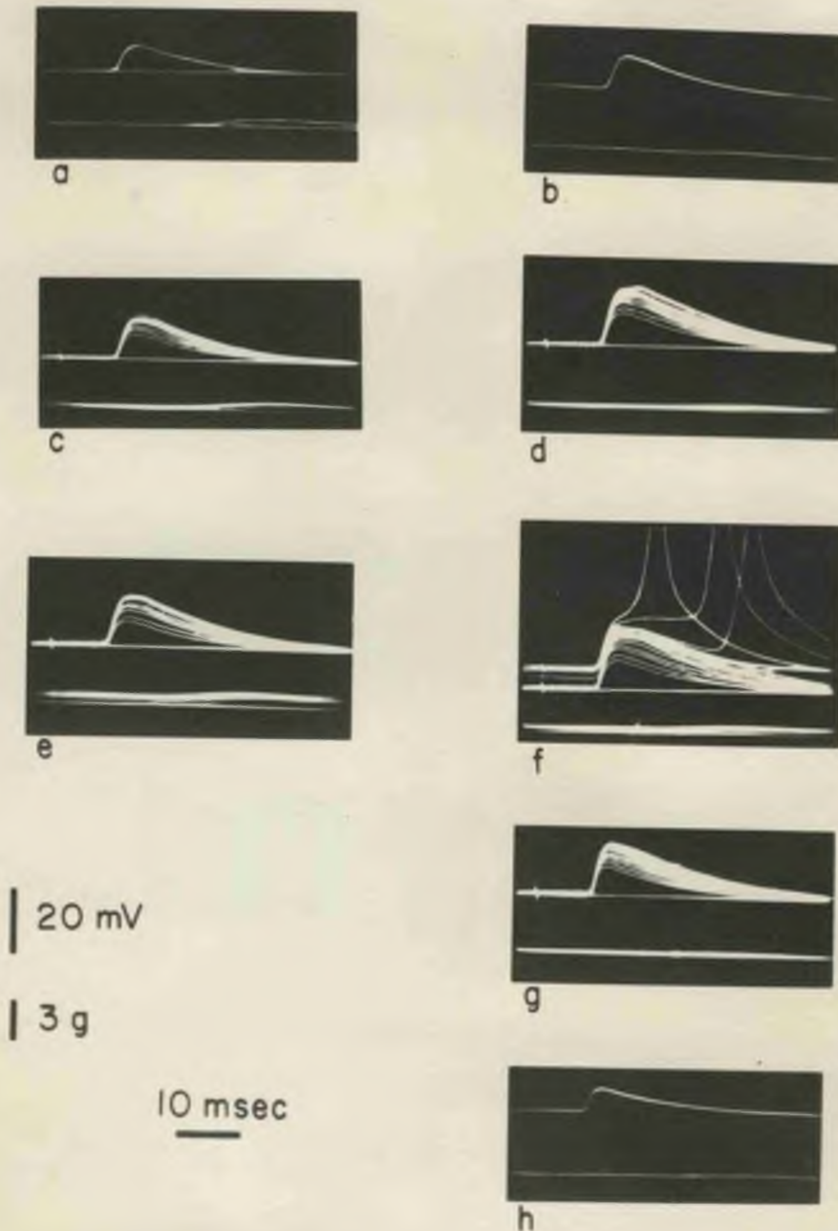


Fig. 122. Effects of nitrate on responses of a *Carcinus* Type A muscle fibre to indirect stimulation. (a, c, e) Responses in normal saline during stimulation of the "fast" axon at 1 per sec. (a), 8 per sec. (c), and 10 per sec. (e); 1 second periods of stimulation were employed in the latter two cases. (b, d, f, g, h) Responses in 350 mM NO_3 during stimulation of the "fast" axon at 1 per sec. (b), 8 per sec. (d) and 10 per sec. (f). Notice reduction of the mechanical response (lower traces) in spite of increased electrical activity. (g,h) Responses after 15 min. in NO_3; stimulation at 10 per sec. (g) and at 1 per sec. (h).

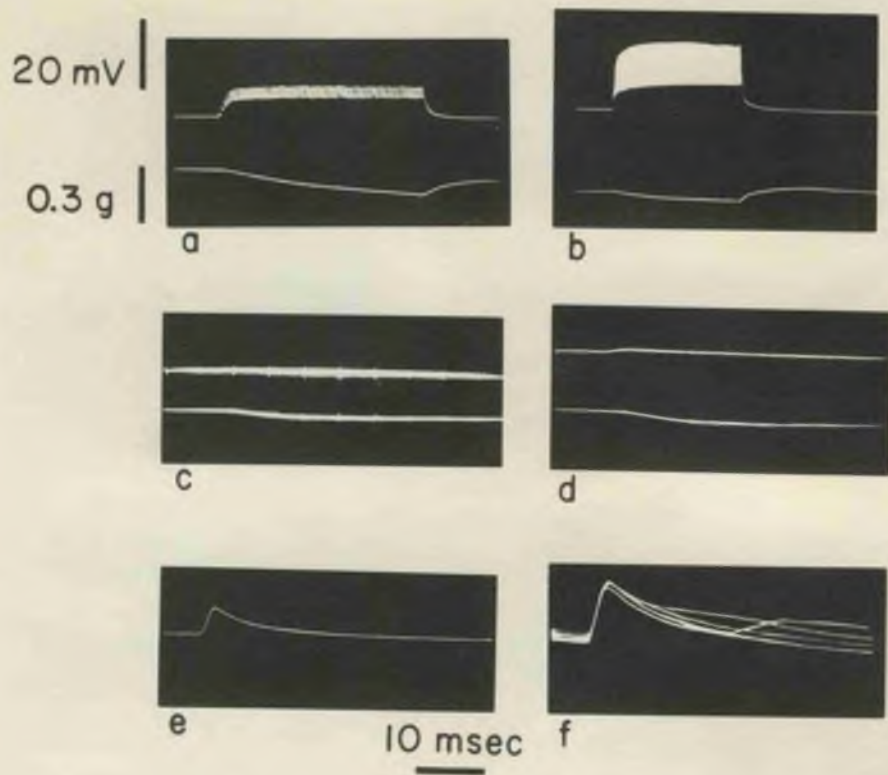


Fig. 123. Effects of iodide ions on responses to indirect stimulation in Carcinus muscles. (a, b) Responses to stimulation of the "slow" axon at 15 per sec. of a preparation in normal saline (a) and after perfusion with 70 mM I (b). (c, d) Responses of the same preparation to stimulation of the "fast" axon at 10 per sec. in normal saline (c) and in 70 mM I (d). (e, f) Electrical responses of another preparation during stimulation of the "fast" axon at 10 per sec. in normal saline (e) and in 80 mM I (f). Time calibration, 2 sec. in (a, b), 10 msec. in (c, d, e, f). Lower traces show muscle tension.

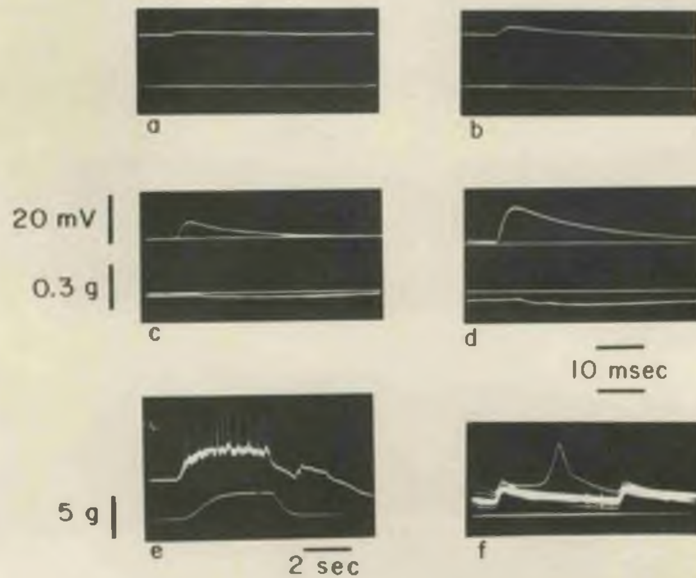


Fig. 124. Effects of thiocyanate ions on responses of Carcinus Type C fibres to indirect stimulation. (a, b) Responses during "slow" axon stimulation at 10 per sec. in normal saline (a) and after addition of 50 mM SCN (b). (c,d) Responses of the same preparation to stimulation of the "fast" axon at 10 per sec. in normal saline (c) and after addition of 50 mM SCN (d). (e, f) Responses of another preparation during stimulation of the "fast" axon at 40 per sec. in 50 mM SCN. Lower traces, muscle tension.

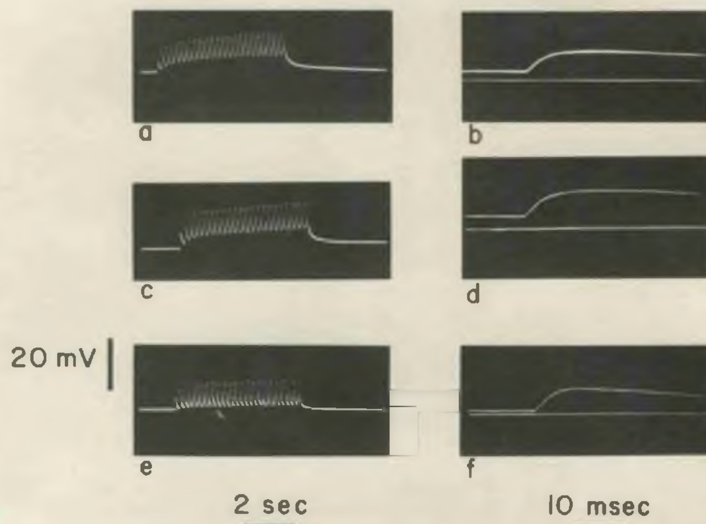


Fig. 125. Effects of Thiocyanate ions on responses of a Carcinus Type B fibre to stimulation of the "slow" axon at 6 per sec. (a, b) Responses in normal saline. (c, d) Responses 5 min. after perfusion with 50 mM SCN; (e, f) Responses 20 min. after perfusion with 50 mM SCN.

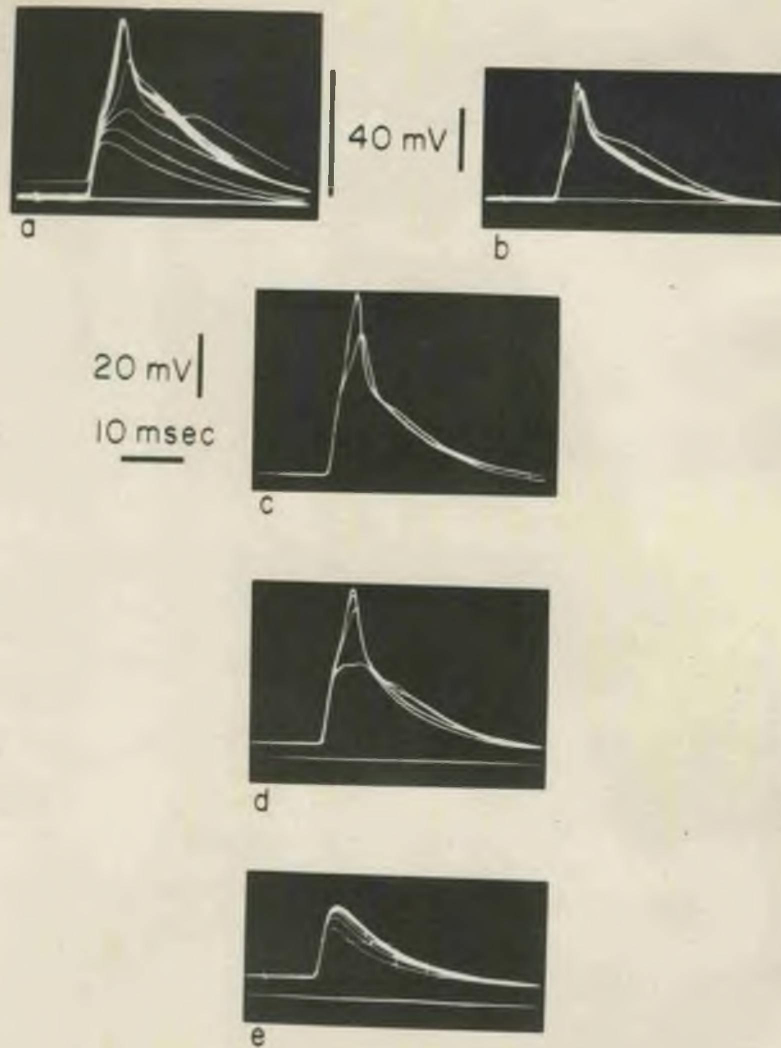


Fig. 126. Effects of thiocyanate ion on responses of a Carcinus Type A fibre to stimulation of the "fast" axon. (a) Responses in normal saline; stimulation at 8 per sec. (b) Responses to stimulation at 1 per sec. after treatment with 50 mM SCN; each stimulus evokes a spike. (c) Responses of the same fibre 15 min. after treatment with SCN. (d) Responses recorded 20 min. after treatment with SCN; stimulation at 2 per sec. (e) Responses recorded 25 min. after treatment with SCN; stimulation at 2 per sec.

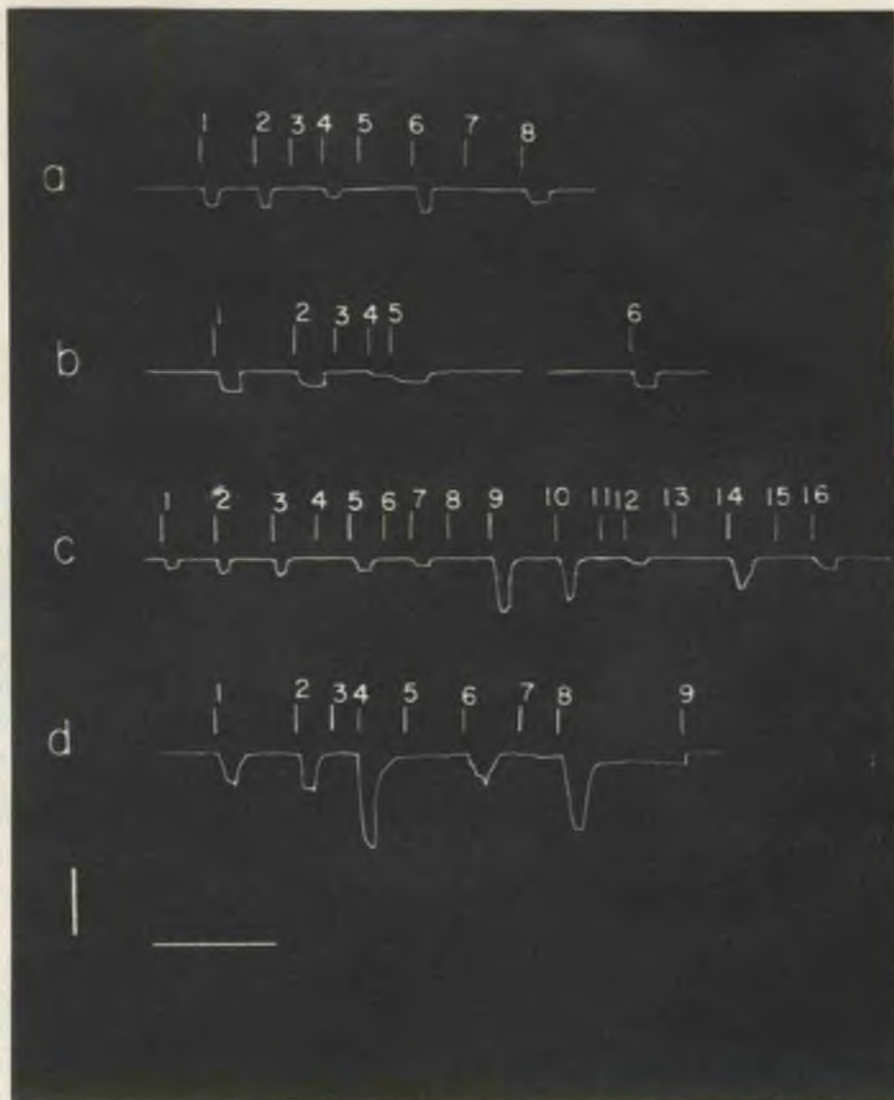
Fig. 127. Effects of anion substitution on potassium contracture of *Carcinus* muscles. Time calibration, 5 min.; Tension calibration, 30 g.

a) Effects of bromide ion.
   1. Application of 70 mM KCl, 0 Br, and removal with normal saline.
   2. As in 1.
   3. Application of saline with 500 mM Br.
   4. Application and removal of 70 mM KCl, 500 mM Br.
   5. Application of normal saline.
   6. Application and removal of 70 mM KCl, 0 Br.
   7. Application 100 mM Br.
   8. Application and removal of 70 mM KCl, 100 mM Br.

b) Effects of nitrate ion.
   1. Application and removal of 50 mM KCl, 0 NO_3.
   2. As in 1.
   3. Application of 400 mM NO_3.
   4. Application of 50 mM KCl, 400 mM NO_3.
   5. Application of 100 mM KCl, 400 mM NO_3, and removal with 10 mM KCl, 400 mM NO_3.
   6. Application and removal of 50 mM KCl. 0 NO_3, after perfusion for 10 min. with normal saline.

c) Effects of iodide ion.
   1. Application and removal of 60 mM KCl, 0I.
   2,3. As in 1.
   4. Application of 50 mM I.
   5. Application and removal of 60 mM KCl, 50 mM I.
   6. Application of 400 mM I.
   7. Application and removal of 60 mM KCl, 400 mM I.
   8. Application of normal saline.
   9. As in 1.
   10. As in 1.
   11. As in 6.
   12. As in 7.
   13. As in 8.
   14. As in 1.
   15. As in 4.
   16. As in 5.

d) Effects of thiocyanate ion.
   1. Application and removal of 70 mM KCl, 0 SCN.
   2. As in 1.
   3. Application of 70 mM SCN.
   4. Application and removal of 70 mM KCl, 70 mM SCN.
   5. Application of 400 mM SCN.
   6. Application and removal of 70 mM KCl, 400 mM SCN.
   7. Application of normal saline.
   8. Application and removal of 70 mM KCl, 0SCN.
   9. Return to base line after 5 min.



a
1 2 3 4 5 6 7 8
b
1 2 3 4 5 6
c
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
d
1 2 3 4 5 6 7 8 9

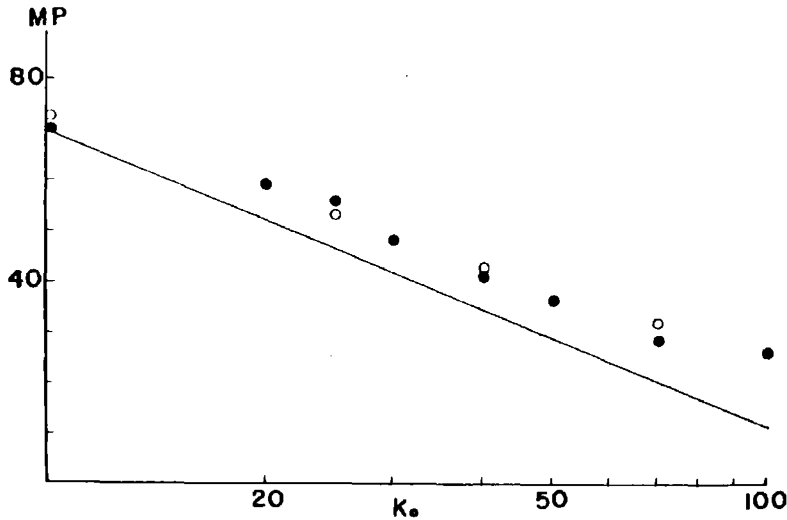


Fig. 128. "Delayed" effect of potassium on the resting potential of Carcinus muscle fibres. Filled circles represent responses in normal saline (data from Table). Open circles show responses averaged from 6 fibres of a muscle from the same animal, treated with increasing potassium in 400 mM NO_3. In both sets of results the muscles were left for about 40 min. at each potassium concentration. The straight line shows the theoretical effect of potassium on a muscle fibre containing 160 mM K. MP, resting membrane potential (mV, inside negative); Ko, external potassium concentration (mM).

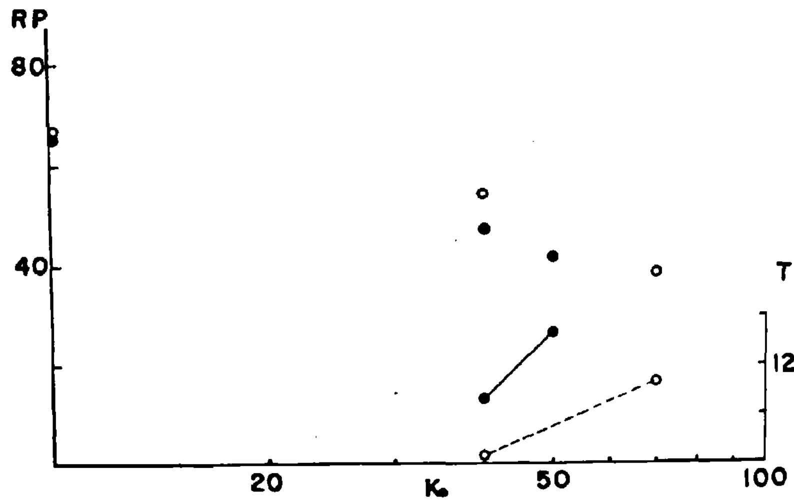
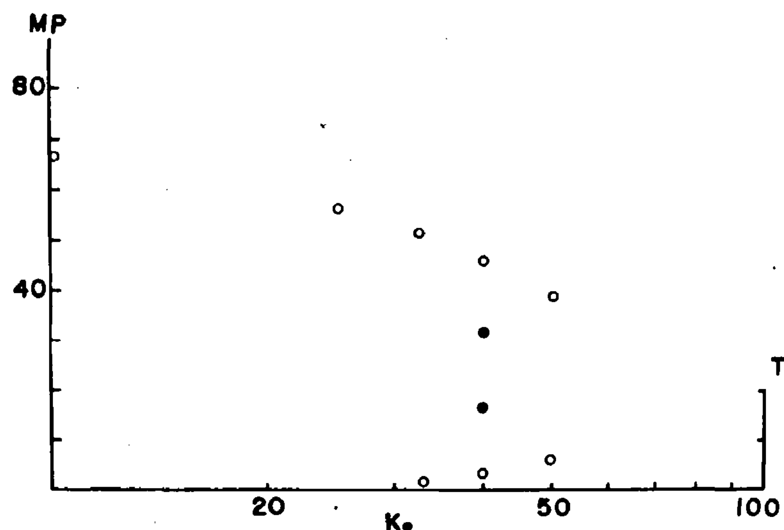


Fig. 129. "Immediate" effects of potassium (Ko, mM) on resting potential (RP, mV) and tension (T, gm) of two muscles. One muscle was subjected to increasing potassium concentrations in the absence of nitrate (filled circles). The other muscle was treated with solutions of increasing potassium concentration and containing 400 mM NO_3 (open circles). Lines connect tension measurements from each muscle. Membrane potential measurements were averaged from 6 fibres in each case.



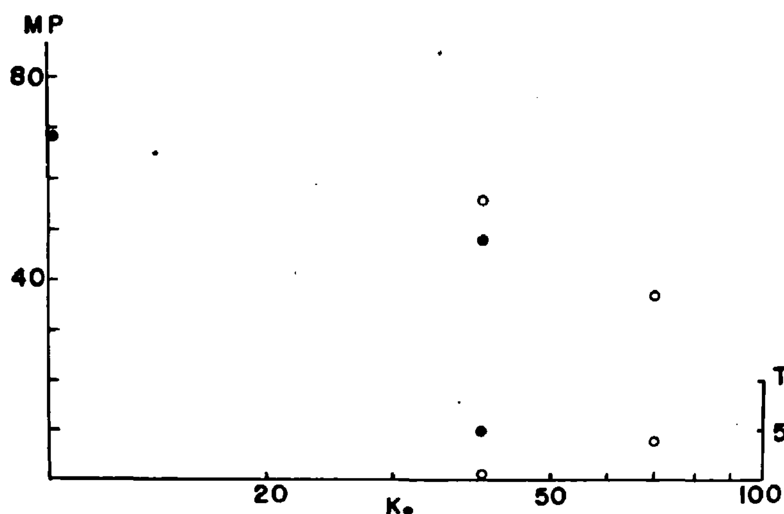


Fig. 130. "Immediate" effects of potassium (Ko, mM) on resting potential (MP, mV) and tension (T, gm) of two muscles from the same animal.

In the upper figure, a muscle was subjected to solutions of increasing KCl concentration in 400 mM NO_3 (upper open circles). As the potassium concentration was increased, tension was developed (lower open circles). At 50 mM KCl, the muscle was quickly returned to 0 NO_3, 40 mM KCl. A marked depolarization resulted (upper filled circle) and additional tension was developed by the muscle (lower filled circle).

In the lower figure, a second muscle was treated with 40 mM KCl, 0 NO_3 after soaking in 10 mM KCl. Membrane potentials are shown by the upper filled circles; tension in 40 mM KCl is shown by the lower filled circle. At 40 mM KCl the muscle was quickly perfused with 40 mM KCl, 400 mM NO_3. A marked hyperpolarization resulted, (upper open circle), and tension fell (lower open circle). As potassium was increased, tension rose.

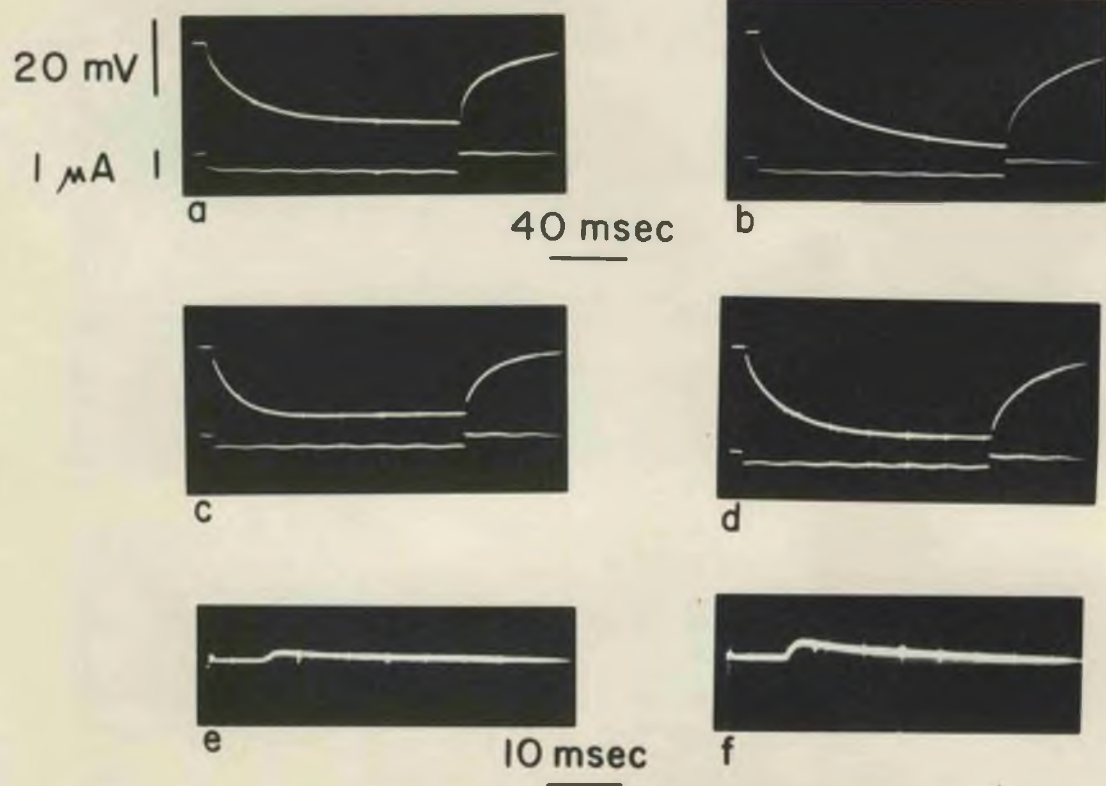


Fig. 132. Effects of nitrate on membrane resistance and indirectly produced electrical responses. (a,b) Responses to hyperpolarizing stimulation in normal saline (a), and after treatment with 350 mM NO_3 (b). (c, d) Similar results for another muscle fibre (normal saline, c; 350 NO_3, d). Note increases in time constant and input resistance in NO_3. Electrodes were less than 100 m apart in both cases. (e, f) Responses to stimulation of the "slow" axon at 5 per sec. in normal saline (e) and in 350 mM NO_3 (f); same fibre as in (c,d).

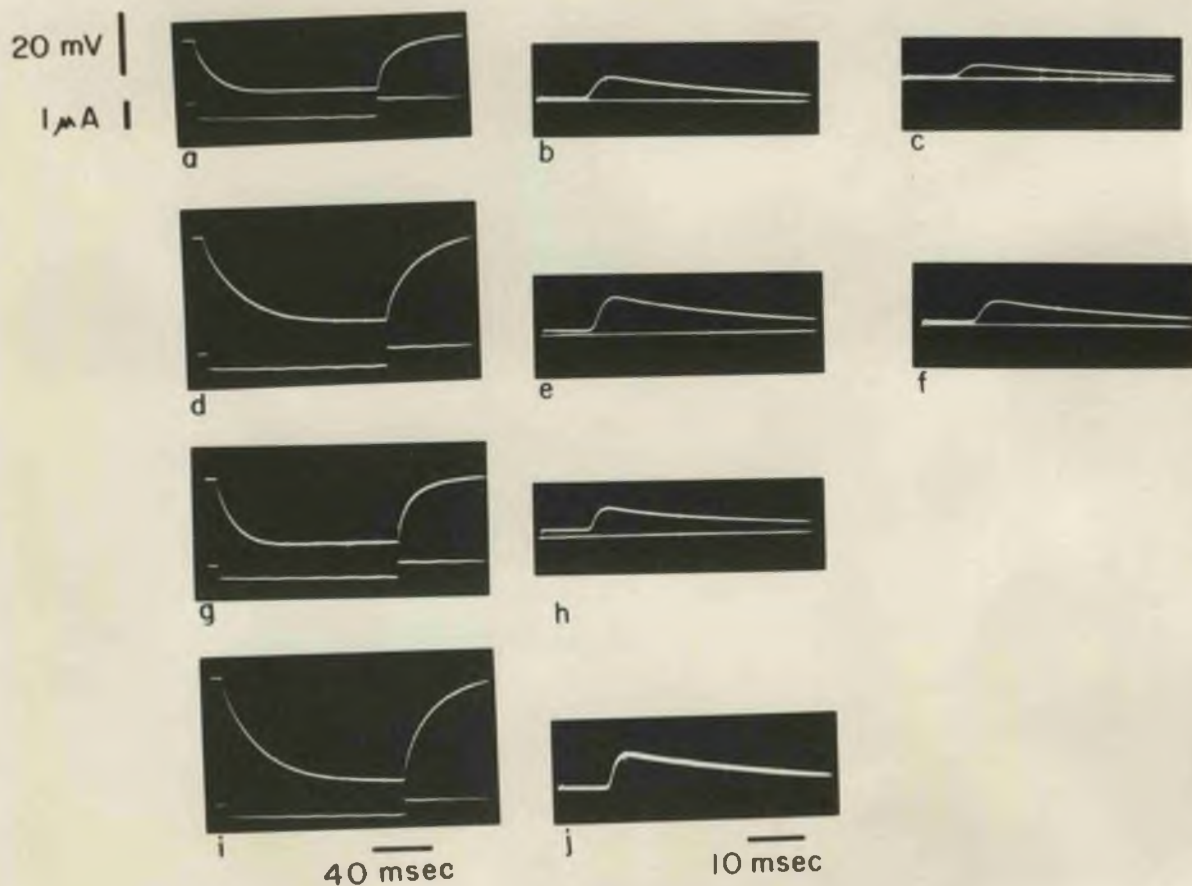


Fig. 133. Effects of thiocyanate on membrane resistance and indirectly produced electrical responses. (a) Response of a muscle fibre to a hyperpolarizing pulse. (b, c) Responses to "slow" (b) and "fast" (c) axon stimulation at 6 per sec. (d, e, f) Corresponding responses of the same muscle fibre to the same stimuli after perfusion with 50 mM SCN. (g) Response of another muscle fibre to a hyperpolarizing pulse in normal saline, and (h) response of the same muscle fibre to stimulation of the "slow" axon at 8 per sec. (i, j) Corresponding responses of the same muscle fibre to the same stimulation after perfusion of the muscle with 50 mM SCN. In (a, d, g, i) the recording and stimulating electrodes were about 100 μ apart.

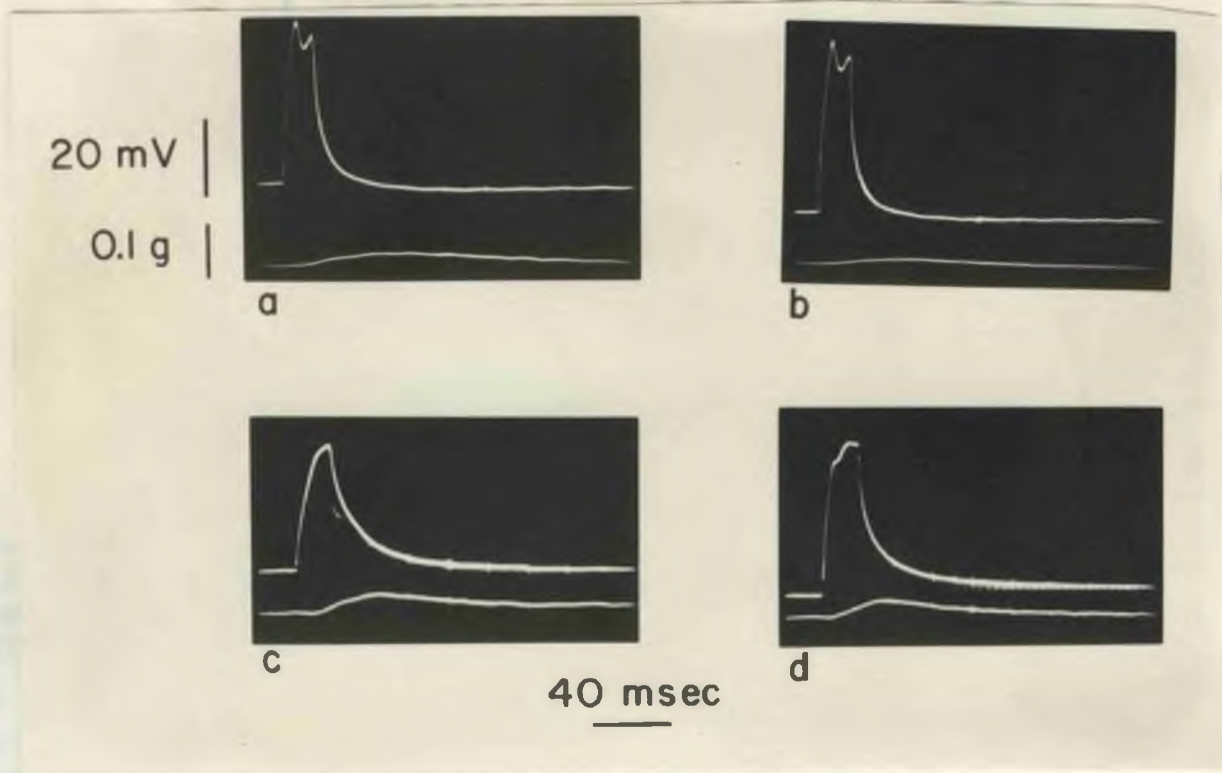


Fig. 134. Activation of single muscle fibres with depolarizing pulses delivered through intracellular microelectrodes. In all cases the stimulating and recording electrodes were 0.2 mm apart. (a, c) Responses of two muscle fibres in normal saline. (b, d) Responses of the same muscle fibres after addition of 400 mM NO_3. Resting potentials were: (a), 65 mV; (b), 69 mV; (c), 67 mV; (d) 69.5 mV. In (a) and (b) the stimulating current was the same; in (d) the stimulating current was larger than in (c). Tension of the muscle fibres is shown in the lower traces.

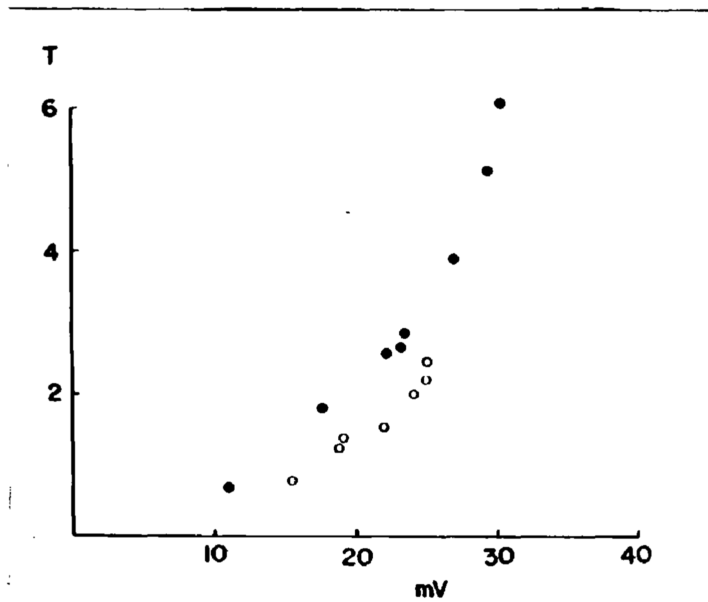


Fig. 135. Activation of a single muscle fibre by intracellular stimulation, in normal saline (filled circles), and after addition of 400 mM NO_3 (open circles). Abscissa: depolarization (mV) in excess of 55 mV membrane potential. Ordinate: tension of the muscle fibre (arbitrary units).

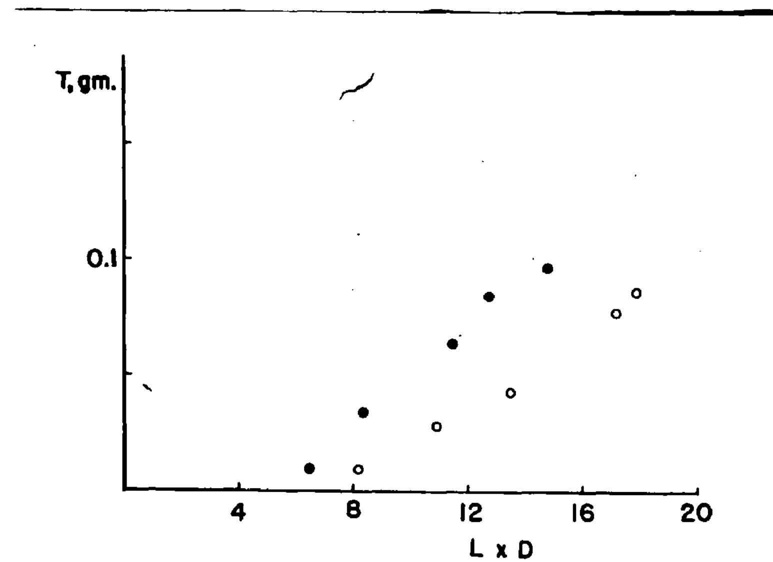


Fig. 136. Effect of nitrate on tension developed by activation of a single muscle fibre with intracellular stimulation. Abscissa: depolarization in excess of 55 mV integrated along the length of the fibre (arbitrary units; see Fig. 97). Ordinate: tension of the muscle fibre (gm). Filled circles show the relation obtained for the fibre in normal saline, and open circles show values obtained after addition of 400 mM NO_3.

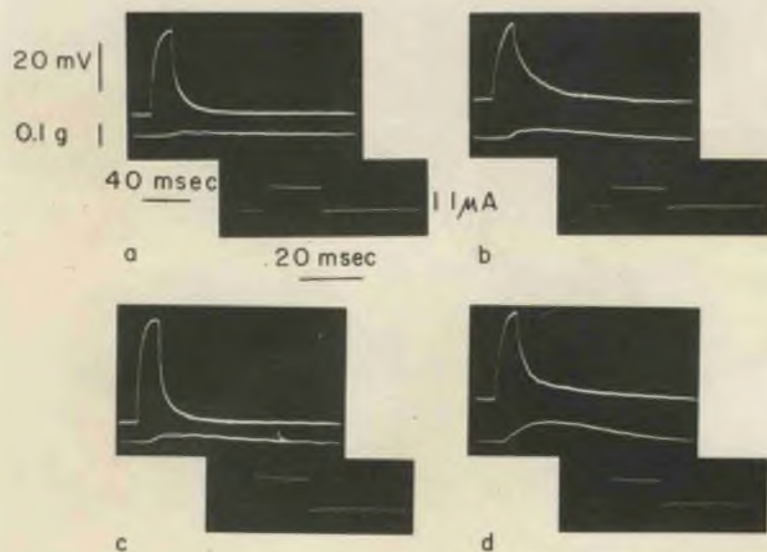
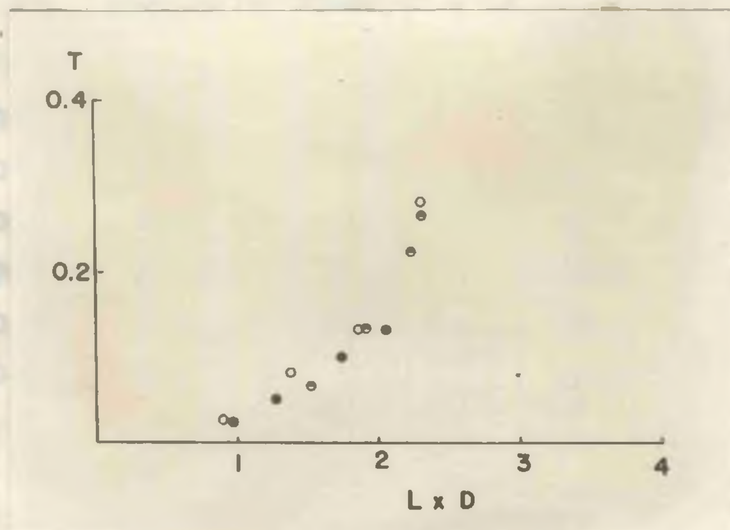


Fig. 137. Effects of thiocyanate on activation of single muscle fibres with intracellular stimulation. (a, c) Responses in normal saline; (b, d) Responses of the same fibre after addition of 50 mM SCN. Resting potentials were: (a, c) 66 mV, (b, d) 70 mV. Stimulating and recording electrodes were 0.2 mm apart. Current was monitored on a second oscilloscope.



. Fig. 138. Effects of thiocyanate on tension developed by a muscle fibre during direct electrical depolarization with an intracellular microelectrode. Ordinate and abscissa as in Fig. 136. Filled circles show the initial responses, measured in normal saline; open circles show responses in 50 mM SCN; half-filled circles show responses in 200 mM SCN.

Table 10. Effects of nitrate and bromide ions on membrane properties of <u>Carcinus</u> muscle fibres.

Fibre	Solution (mM)	Length Constant (mm)	Input Resistance ($x\ 10^3$ ohms)	Product (arbitrary units)	Relative Membrane Resistance
1.	0 NO_3	1.80	56.5	102	1.0
	50 "	2.08	57.5	120	1.2
2.	0 "	1.66	26	43	1.0
	50 "	2.23	24	54	1.24
3.	0 NO_3	1.15	21.6	25	1.0
	40 "	2.3	23.4	54	2.17
4.	0 "	1.88	31.4	59	1.0
	50 "	2.19	36.9	81	1.37
	100 "	2.26	37.5	85	1.44
5.	0 "	2.17	49	85	1.0
	200 "	2.86	50.7	145	1.72
	400 "	2.41	65.7	158	1.87
6.	0 "	1.79	31.5	56	1.o
	100"	1.89	36	68	1.2
	200"	2.03	37.5	76	1.35
	400"	1.92	36.7	71	1.25
7.	0 Br	1.28	31.2	39.5	1.0
	250 Br	1.32	31.9	42.1	1.07
	530 Br	1.50	32.3	48.5	1.23

Table 11. Effects of iodide ions on membrane properties of *Carcinus* muscle fibres.

Fibre	Solution (I)	Length Constant (mm)	Input Resistance ($x10^3$ ohms)	Product (arbitrary units)	Relative Membrane Resistance
1.	0 mM	1.95	37.5	73	1.0
	50 mM	2.14	57.5	123	1.68
2.	0 mM	1.73	19.5	34	1.0
3	50 mM	1.89	30.0	57	1.68
3.	0 mM	1.5	38	57	1.0
	50 mM	2.68	65	174	3.1
4.	0 mM	1.75	21.7	38	1.0
	50 mM	2.40	22	53	1.39
	100mM	2.70	17	46	1.21
5.	0 mM	1.62	41	66	1.0
	50 mM	1.89	48	91	1.37
	100 mM	1.42	36.4	52	0.78
	200 mM	1.44	25	36	0.54

Table 12. Effects of Thiocyanate ions on membrane properties of <u>Carcinus</u> muscle fibres.

Fibre	Solution (mM SCN)	Length Constant (mm)	Input Resistance (x 10^3) ohms	Product (arbitrary units)	Relative Membrane Resistance
1.	0	1.95	33	64	1.0
	50	2.54	56	142	2.2
2.	0	1.90	15.8	30	1.0
	50	2.10	25.8	54	1.8
	200	2.17	31.0	67	2.24
3.	0	1.05	19.5	20	1.0
	12.5	1.24	27.4	34	1.65
	100	1.87	38.0	71	3.46
	200	1.90	40.7	78	3.78
4	400	2.12	41.0	87	4.24

ii) Comparative Observations on Other Muscles

A relatively few observations of the effects of nitrate on other crustacean muscles were made. None of the other anions was studied.

When nitrate solutions of 100 to 400 mM were perfused into the Nephrops closer muscle, the electrical responses to "fast" axon stimulation showed an initial increase in size followed by a gradual decline in magnitude below the level of response in normal saline (Fig. 130, a to c). The time constants of decay of the "fast" responses were increased in nitrate and remained larger than in normal saline as the response decreased in size. This was an indication that the membrane resistance was increased in nitrate, but that the output of transmitter substance was gradually decreased.

The fast mechanical response was often slightly increased at first by application of nitrate, but rapidly declined to a low level.

When high concentrations of nitrate (200 to 400 mM) were applied to the muscle, the "slow" electrical and mechanical responses showed a very rapid decline (Fig. 139, d, e). In addition, treatment with nitrate caused low frequency spontaneous firing of the "slow" axon.

Nitrate was observed to have an effect on membrane resistance similar to that observed in Carcinus, but less pronounced. Input resistances and time constants of nitrate-treated fibres were increased slightly above normal (Fig. 139, f, g).

In some cases the electrically excitable responses of

nitrate-treated fibres were smaller than normal, in spite of increased membrane resistance (Fig. 139, h, i). It is not certain whether or not this result was influenced by membrane damage due to prolonged impalement with microelectrodes.

Resting potentials were usually the same in nitrate as in chloride in this muscle. In some cases, however, a slight depolarization appeared in nitrate.

When nitrate (50 to 100 mM) was applied to walking leg opener and closer muscles of the crayfish, electrical and mechanical responses to "fast" closer axon stimulation, and to opener axon stimulation, were increased. In the closer muscle the fast twitch was increased and prolonged. In the opener muscle single stimuli sometimes gave rise to small twitch responses in nitrate. In several cases, however, the mechanical responses to opener axon stimulation were about the same in nitrate as in chloride.

It was found that nitrate markedly reduced the effectiveness of potassium depolarization in opener muscle preparations, as was the case in Carcinus. The amount of tension developed by a given concentration of potassium was consequently much less in nitrate.

In these crayfish muscles, resting potentials were not noticeably affected by nitrate application.

A series of observations was made to determine the effect of nitrate on the Cancer stretcher muscle. Application of nitrate resulted in small initial increases in both electrical and mechanical responses to indirect stimulation followed by a gradual decline in both. Hyperpolarization of 1

to 4 mV was typically produced by application of nitrate.

The input resistances of nitrate-treated fibres were slightly greater than those of the same fibres in normal saline (Fig. 140, a, e), although the length constants in some cases showed a decrease (membrane damage?). The mechanical responses of single muscle fibres to direct stimulation were similar in nitrate and in chloride (Fig. 140, b to g). No difference in the threshold for contraction could be detected in the two solutions. However, it was found that the tension per depolarization-length unit was slightly greater in nitrate in the four fibres examined. Results for one fibre are shown in Fig. 141. The results for Cancer fibres were thus different from those obtained from single Carcinus fibres.

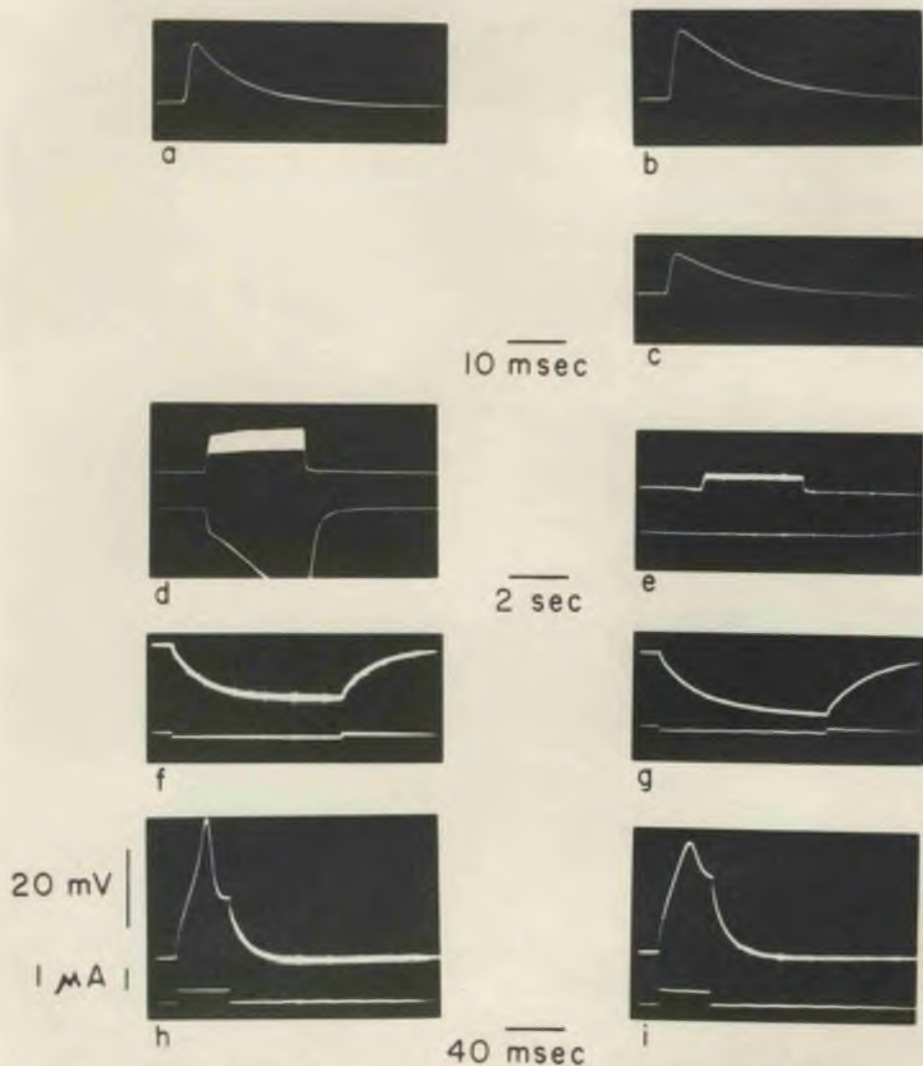


Fig. 139. Effects of nitrate on responses of Nephrops fibres to indirect and direct stimulation. (a) P.s.p. in response to a single stimulus applied to the "fast" axon. (b) P.s.p. of the same fibre after treatment with 200 mM NO_3 for 5 min. (c) P.s.p. from the same fibre 10 minutes later. (d) "Slow" responses in normal saline; "slow" axon stimulated at 90 per sec. (e) Responses to the same stimulation 5 min. after perfusion with 300 mM NO_3. (f, h) Responses of a distal muscle fibre to hyperpolarization (f) and depolarization (h) in normal saline. (g, i) Corresponding responses after treatment with 300 mM NO_3 for 5 min. In (d, e) tension of the whole muscle is shown (lower traces).

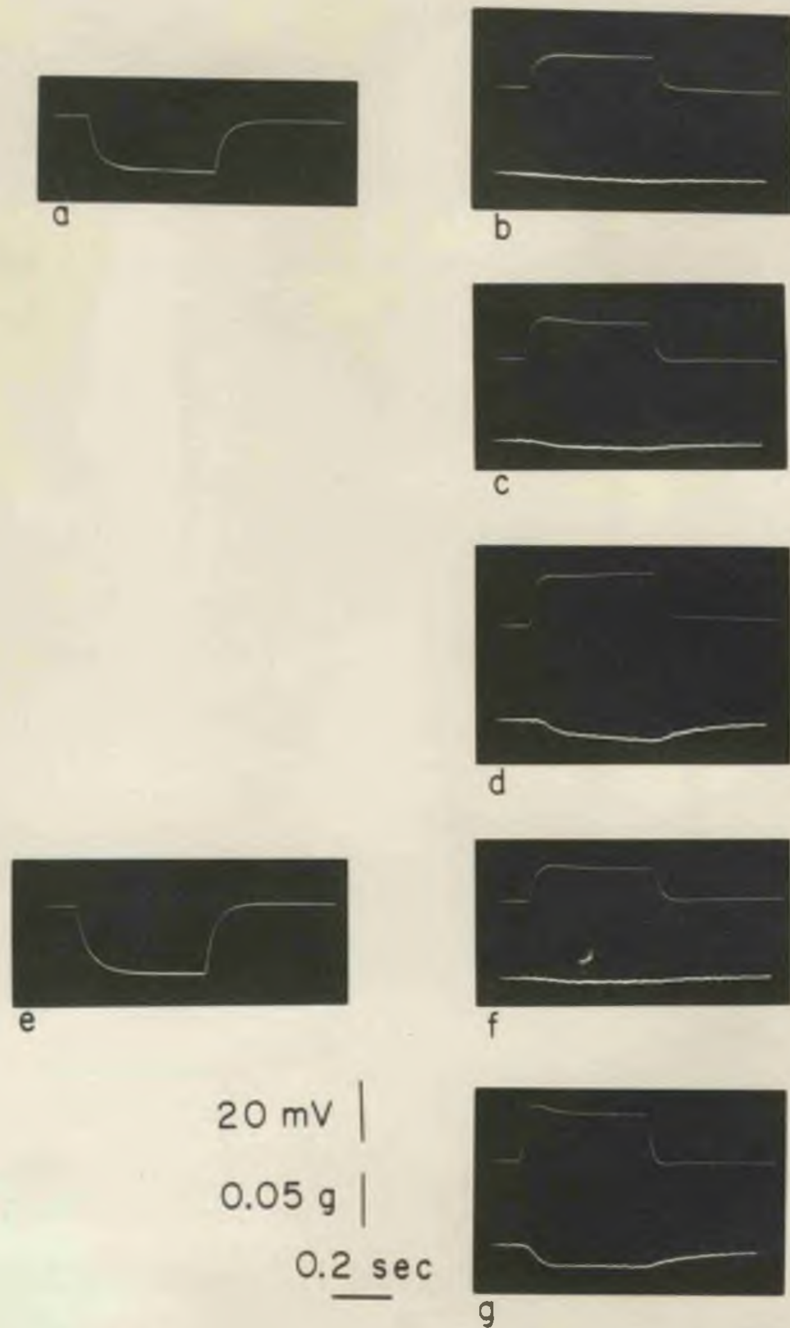
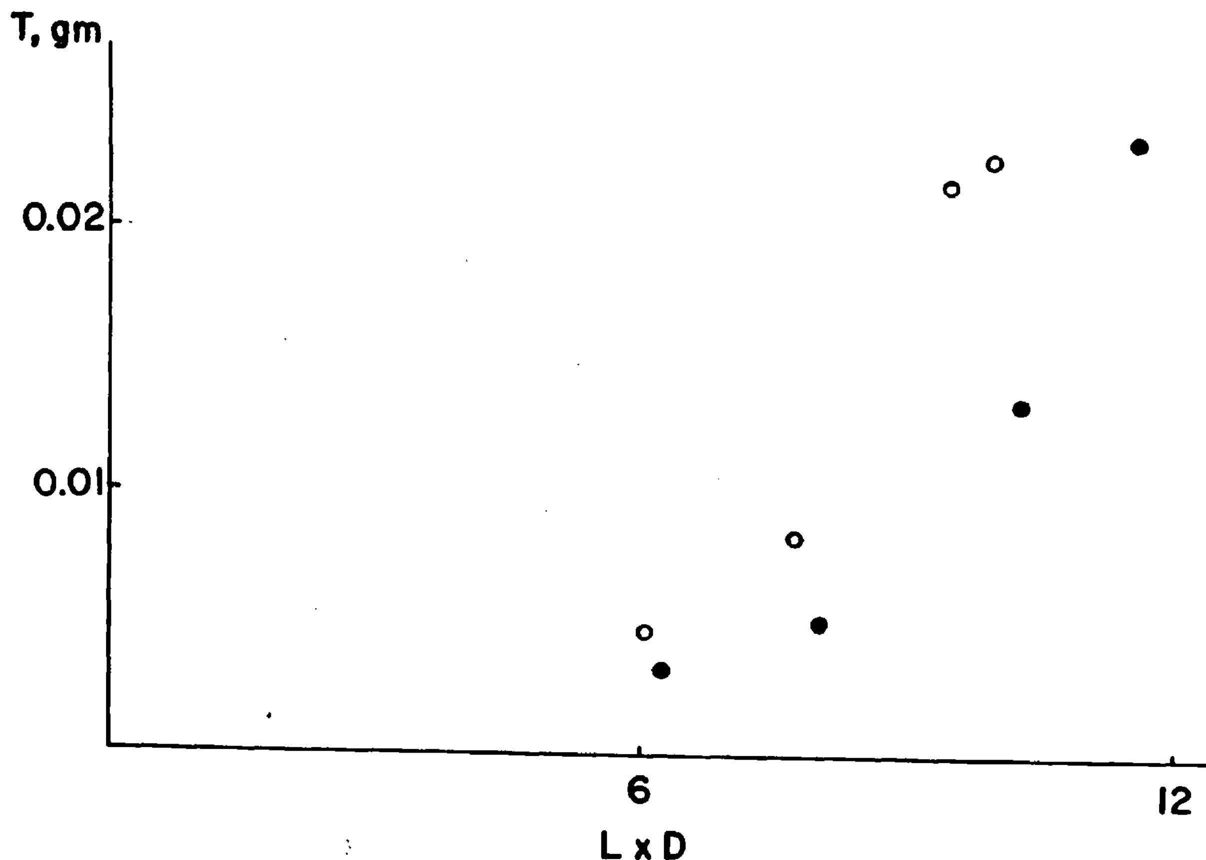


Fig. 140. Responses of a Cancer muscle fibre to direct stimulation with an intracellular microelectrode. (a) Response to a hyperpolarizing pulse in normal saline; r.p., 65 mV. (b, c, d) Responses to depolarizing stimuli; tension of the muscle fibre, lower traces. (e) Response to a hyperpolarizing stimulus after addition of 250 mM NO_3; r.p., 67 mV; current strength was the same as in (a). (f, g) Responses to depolarizing stimuli in 250 mM NO_3. In all cases the stimulating and recording electrodes were 0.5 mm apart. In (a) to (d), length constant was estimated to be 2.1 mm; in (e) to (g), 1.95 mm.



<u>Fig. 141.</u> Effect of nitrate on activation of a single <u>Cancer</u> muscle fibre. Ordinate and abscissa as in Fig. 136, except that the "threshold" depolarization was 57 mV. Filled circles: responses in normal saline. Open circles: responses after addition of 250 mM NO_3.

DISCUSSION

It cannot be claimed that the experiments reported in this study permit definite answers to be given to the questions posed in the introduction. However, suggestive evidence relating to certain aspects of excitation and contraction in crustacean muscles has been accumulated, and this evidence will now be discussed to determine whether or not any conclusions may be drawn from it.

a) "Fast" and "Slow" responses in Crustacean Muscles.

Previous to this study, no satisfactory explanation of the differences between "fast" and "slow" responses of crustacean muscles had been advanced. The suggestions offered, such as that of Hoyle and Wiersma (1958c), who accounted for "fast" and "slow" transmission in terms of separate transmitter substances, were at best, tentative.

In the present study evidence has been presented to show that in certain muscles, the main differences between "fast" and "slow" responses arise from two factors: 1) differences in the rate of release of transmitter substance from the two types of nerve ending, and 2) differences in the properties of muscle fibres innervated primarily by one or the other of the two axons. In the *Carcinus* muscle, this second factor appears to be of great importance, as it

probably is also in Pachygrapsus. In Nephrops, on the other hand, the first factor appears to be more important, because no outstanding differences between muscle fibres innervated primarily by one or the other of the two axons was apparent with respect to membrane properties and electrical excitability.

By the interplay of these two factors- differences in muscle fibre properties, and differences in the density of innervation by the two axons- it is possible to account for most of the varieties of electrical response observed in the muscles which were studied.

It was found throughout this study that the shapes of the electrical responses occurring in a particular muscle fibre could be related to the membrane properties of the fibre. Thus, Carcinus type B muscle fibres and Cancer muscle fibres, which had high membrane resistances and large time constants, showed prolonged p.s.ps. On the other hand, Carcinus Type A muscle fibres and Pachygrapsus "fast" fibres, which had low membrane resistances and small time constants, showed very rapid p.s.ps.

The fibres primarily innervated by "fast" axons (e.g., Carcinus Type A fibres, and Pachygrapsus "fast" fibres) often had very much lower membrane resistances than other fibres. The very large "fast" p.s.ps. produced in these fibres can only be accounted for by assuming that the fibres in question possess a much denser "fast" axon

innervation than other muscle fibres, thereby counterbalancing the effect of low membrane resistance.

The fibres innervated primarily by "fast" axons in Carcinus had other features distinguishing them from fibres innervated primarily by "slow" axons, including greater electrical excitability, higher resting potentials, and smaller sarcomere lengths. The same pattern appeared to be present in the Pachygrapsus closer, although it was not worked out in the same detail as in Carcinus. It is tempting to speculate that there may be specific "trophic influences" exerted by "fast" and "slow" axons on the fibres which they innervate, and that these specific influences may be partly responsible for the different membrane properties of muscle fibres predominantly innervated by one kind of motor axon. This type of mechanism has been shown to be present in cat muscles (Buller, Eccles, and Eccles, 1960), in which fast and slow muscles are differentiated after innervation by "fast" and "slow" motoneurones, and in which fast muscles can be converted to slow, and vice versa, by experimental changes in the innervation of the muscle.

However, in Nephrops it would seem likely that "trophic influences", if present, are not as specific in nature as may be the case in some crab muscles.

Recent observations by Mr. B.S. Dorai Raj, of the Department of Biology, University of Oregon, cast further doubt on the "trophic influence" theory as applied generally to crustacean muscles*. Mr. Raj has worked with the accessory flexor muscle of Cancer magister, which is innervated by a single excitor axon and a single inhibitor axon as are the homologous muscles of other crustaceans (cf. Wiersma, 1941). He has done experiments of the same sort as those done previously by the author and described in this thesis, and has found that "fast" and "slow" fibres with membrane characteristics similar in many respects to those found by the author in the Carcinus closer muscle, exist also in the accessory flexor muscle. Furthermore, the "fast" fibres are characterized by narrow sarcomere banding and by "Fibrillenstruktur" (Krüger, 1952); the "slow" fibres have broader sarcomere banding and "Felderstruktur". Since only one excitor axon innervates this muscle, the existence of "fast" and "slow" fibres cannot readily be attributed to differences in innervation by separate axons, unless perhaps the inhibitor axon has a very different distribution of nerve terminals than that observed for the excitor, or unless one axon can have different types of nerve endings (cf. Wiersma, 1951). However, the evidence as it exists at present indicates that "fast" and "slow" fibres in this muscle probably

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*The author is indebted to Mr. Raj for permission to quote his results in this Discussion.

cannot be attributed to the same mechanism as shown for certain vertebrate muscles. In other muscles, such as the *Carcinus* and *Pachygrapsus* closers, such a mechanism may exist.

In this connection a recent note by Girardier *et al.* (1962) is of interest. These authors have found that denervated crayfish muscle fibres have markedly different membrane properties (including higher membrane resistance) than fibres from neurally intact muscles. The change in membrane properties following denervation is very rapid. Innervation appears to exert a *general* influence on muscle fibre membrane properties, although specific influences of "fast" and "slow" axons, if any, remain to be described.

However "fast" and "slow" fibres originate in crustacean muscles, they certainly exist, and in such muscles as the *Carcinus* and *Pachygrapsus* closers it is very likely that the mechanical nature of "fast" and "slow" contractions is determined largely by the different contractile responses of these fibres*. Some evidence was found that even in Type C fibres of similar membrane properties, different muscle fibres showed a wide range of contraction speeds in response to the same type of directly applied depolarization (see Fig. 91). It was not found possible to record tension responses from individual Type A and Type B fibres, but it is probable that these are faster and slower, respectively, than the tension responses recorded from Type C fibres.

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*As in frog muscles; see Peachey and Huxley (1962), J. Cell Biol. 13, 177.

The usual fast twitch (Fig. 24,a) has a more rapid rise and fall than do the "twitches" recorded from most single Type C muscle fibres, which in general have time courses similar to recorded tension responses produced by single shocks to the "slow" axon (Fig. 24,c).

The probable variation in contraction speed in different muscle fibres can be attributed in part to the different membrane properties of the muscle fibres concerned. In Type A fibres the "fast" responses are often large, but comparatively brief. The briefness of these responses can be attributed to the short time constants of the muscle fibres concerned, and to the rapid repolarization following active membrane responses. Assuming for the moment that tension is dependent on membrane depolarization remains above a threshold level (cf. Orkand, 1962), it can be seen that the tension developed by Type A fibres will be large but of comparatively brief duration. Even if all muscle fibres had the same inherent contraction rates, the electrical responses of the Type A fibres, which are determined largely by the membrane properties, would impose a contraction on these fibres which would be briefer than that which could be elicited from Type B and Type C fibres. The fast twitches of Carcinus muscles can be attributed to the action of Type A fibres.

In Carcinus, the slow rise and fall of tension during "slow" axon stimulation can be linked in part to the longer

time constants of the fibres responding, coupled with smaller individual excursions of depolarization above the assumed tension threshold. The significance of the longer time constants is that coupled with the growth of the "slow" p.s.ps. by facilitation, they permit the establishment of depolarization "plateaus" yielding in turn greater products of time and depolarization above the tension threshold. At first glance it is more difficult to account for the very gradual relaxation of tension following "slow" axon stimulation. Slow repolarization imposed by large membrane time constants may provide a partial explanation, but all the fibres examined critically repolarized to resting membrane potential level, or within one or two millivolts of it, within 0.5 sec., whereas relaxation of "slow" tension took 3 to 8 seconds. It may be that rather slow contraction speeds occur in Type B fibres, and that these contraction speeds are slower than can be accounted for by slow electrical changes connected with large time constants. More work on single isolated muscle fibres is needed to clarify this point.

In Nephrops muscles a narrower range of muscle fibre membrane properties was found than in Carcinus and Pachygrapsus. Correspondingly, the "fast" and "slow" p.s.ps. had more uniform decay rates throughout the muscle. In addition, relaxation rates of "fast" and "slow" contractions

were usually much more similar than in Carcinus muscles. This suggests that contraction speeds of the muscle fibres may be fairly uniform throughout the muscle.

The evidence for the Nephrops closer muscle suggests that the characteristics of the "fast" and "slow" contractions in this muscle may be due primarily to differences in neuromuscular transmission at "fast" and "slow" nerve endings, much as has been suggested by Usherwood (1962 a,b) for the coxal muscles of the cockroach. In Carcinus and Pachygrapsus, on the other hand, certain definitive characteristics of the "fast" and "slow" contractile responses are probably determined by differences in the muscle fibres responding primarily to either "fast" or "slow" axon stimulation. However, in these muscles also, differences in neuromuscular transmission at different types of nerve ending may be important. In Carcinus Type C fibres, for instance, it was found that "facilitating" and "non-facilitating" electrical responses could be found in response to "fast" axon stimulation. Thus even a single motor axon may have nerve endings with different characteristics (cf. Wiersma, 1951; Wiersma and Bobbert, 1961).

None of the observations made on the muscles studied most critically provided any evidence for the existence of separate "fast" and "slow" transmitter substances. Although it cannot be stated whether or not separate transmitter substances are present until they are isolated and identified,

the evidence obtained in the present study provides provisional support for the hypothesis that "fast" and "slow" transmission processes are effected by the same transmitter.

b). Depolarization and Contraction in Crustacean Muscles.

In the present study efforts were made to determine whether or not contraction in crustacean muscles is initiated by membrane depolarization *per se*. This problem has remained unresolved since Hoyle and Wiersma (1958 c) produced evidence indicating that in crustacean muscles membrane depolarization may not be the factor which sets the contractile machinery in motion. As was pointed out in the Introduction, their evidence, while suggestive, cannot be considered a very firm proof of their contention.

It has been clearly demonstrated by Orkand (1962) and by the author (1962, and in the present study) that contraction of crustacean muscles does occur in response to depolarization of sufficient magnitude, whether produced by direct electrical means or by application of chemical agents. The main problem, therefore, is to determine whether nervous excitation utilizes this available depolarization-dependent mechanism to bring about contraction, and indeed whether different methods of depolarizing the muscle are equally effective in producing tension.

In the present study, an initial attack on this problem has been made by studying the contractile responses of single muscle fibres of the *Cancer* stretcher muscle. The

observations indicate that in the particular fibres studied, the threshold membrane potential for tension development appeared to be about the same for depolarization by nervous excitation as for initial depolarization by high potassium and by current passed through a microelectrode. No outstanding variations in the thresholds for different fibres of this muscle were noted. However, fibres from a rather limited region of the muscle were the only ones which could be examined by the techniques employed.

It was not satisfactorily determined whether the three methods used to depolarize the muscle fibres were equivalent with respect to the degree of tension produced per unit of depolarization. Problems associated with differences in the uniformity of depolarization over the length of the muscle fibre in different situations made difficult a comparison of the three types of depolarization. No concrete evidence indicating differences in effectiveness of the three types of depolarization was found. However, it was observed that potassium depolarization was less effective after a previous potassium-induced contraction.

If the assumption is made that fibres of this muscle must be depolarized past a "threshold" of 60 to 55 mV by nervous excitation before they will develop tension, it is clear that the electrical responses which have been recorded from the "edge" fibres of this muscle are large enough to initiate tension development at low frequencies of

stimulation (5 to 10 per sec.). At these frequencies of stimulation, responses in the "edge" fibres are 10 to 20 mV in magnitude, and since the resting potentials of the muscle fibres are 64 to 76 mV, the p.s.ps. exceed the "threshold" in many cases. It is probable that the majority of fibres elsewhere in the muscle, which have small electrical responses at frequencies of stimulation less than 20 per sec., do not develop tension until the frequency of stimulation is 20 per sec., or greater.

In *Carcinus*, *Nephrops*, and *Pachygrapsus* muscles, contraction can also be accounted for by assuming that nervous excitation activates a depolarization-dependent mechanism. For in all of these muscles, electrical responses exceeding the postulated thresholds for contraction can be found in at least some muscle fibres even at the lowest stimulation frequencies at which contraction can be detected.

If a mean threshold membrane potential for tension development of 55 mV is assumed for *Carcinus* muscle, depolarization of the muscle fibre membrane amounting to 10 to 20 mV (depending on the resting potential of the muscle fibre) is necessary to cause contraction. On this basis it is evident that the electrical responses to nervous stimulation are sufficient to activate a depolarization-dependent tension producing mechanism. For when the "fast"

axon is stimulated with single shocks, "fast" p.s.ps. of 15 to 20 mV are common in Type A muscle fibres, most of which were found to have resting potentials of 65 to 70 mV. These "fast" p.s.ps. generally exceed the assumed threshold of 55 mV and can account for the twitches elicited by single shocks to the "fast" axon.

When the "slow" axon is stimulated at frequencies of 5 to 10 per second, sufficient to cause a contraction, Type B fibres are often depolarized past the assumed threshold of 55 mV. The fibres of Figs. 9c, 11j, for example, had resting potentials of 61 and 64 mV respectively, and in both cases total depolarization was sufficient to exceed the postulated threshold of 55 mV during tension development. It can be postulated that the tension developed by Type B muscle fibres starts to level off at stimulation frequencies of 20 to 40 per second, because the total electrical response does not increase very rapidly in magnitude at higher frequencies.

Only at frequencies of stimulation greater than 15 to 20 per second do responses of Type C fibres become large enough to exceed the postulated threshold. In many cases only the slow electrical responses exceed the threshold at higher stimulation frequencies. It is possible, therefore, that most of the fast tension is developed by Type A fibres and a relatively small proportion of Type C fibres (some of which were observed to have electrically excitable membranes; see Fig. 22). However, the possibility that tension is also

developed by a method not dependent on depolarization, as has been suggested previously (Hoyle and Wiersma, 1958b,c) has not been ruled out. If contraction in Type C muscle fibres is depolarization-dependent, it is likely that many of them develop tension during "slow" axon stimulation at frequencies above 20 per second, but that only some of them develop tension during "fast" axon stimulation even at high frequencies.

It must also be borne in mind that muscle fibres which show small electrical responses to separate stimulation of "fast" and "slow" axons, as many Type C fibres do, can show considerably larger responses when both axons are stimulated simultaneously (Fig. 19). The animal may normally bring these muscle fibres into play only when extreme effort is required.

In Pachygrapsus only a few muscle fibres give large responses to "slow" axon stimulation at any frequency. These muscle fibres have responses rather similar to Carcinus Type B muscle fibres. This similarity includes the "levelling off" in magnitude of the electrical responses at frequencies of stimulation greater than 20 to 40 per sec. The mechanical response to "slow" axon stimulation also levels off at these frequencies (Fig. 59), and this indicates very strongly that "slow" tension is developed exclusively by the specialized "slow" type of fibre. The other muscle fibres probably develop tension only in response to

stimulation of the "fast" axon or to simultaneous stimulation of both motor axons. This situation contrasts with that in _Carcinus_, in which the "slow" tension does not level off at frequencies of stimulation of 20 to 40 per sec., as it presumably would if Type B fibres alone were effective in producing it, but continues to increase in magnitude with increasing frequency of stimulation (Fig. 26), thereby indicating activity of Type C fibres.

The spiking fibres of _Pachygrapsus_ account for the powerful "fast" twitches obtained from the closer muscle. Once again a contrast exists with the closer muscle of _Carcinus_, in which the "fast" twitches were relatively weak, and in which the specialized "fast" Type A fibres did not give rise to spikes when only one stimulus was delivered via the "fast" axon.

Comparison of _Carcinus_ and _Pachygrapsus_ closer muscles indicates that a basically similar mechanism has evolved in different directions in the two animals. In _Carcinus_ the "slow" system probably does most of the work, whereas in _Pachygrapsus_ the "fast" system predominates. It would be of interest to determine the extent to which "slow" and "fast" systems are used in normal activity in the two animals.

In the _Nephrops_ closer muscle the mechanisms appear to be rather different, but in this muscle also, the available evidence indicates that a depolarization-dependent mechanism

may be operative in tension production.

If a membrane potential threshold for tension development of 58 mV is assumed for Nephrops muscle, most of the muscle fibres must be depolarized by 10 to 20 mV (from resting potentials lf 65 to 78 mV) before they will develop tension.

Many of the proximal muscle fibres gave p.s.ps. of 10 to 20 mV in response to single shocks applied to the "fast" axon. The tension response of the muscle was a small twitch. The weakness of this twitch can be correlated with the absence of electrically excitable responses to single stimuli. Nevertheless, it appears that during "fast" axon transmission the electrical responses of some of the proximal muscle fibres exceed the threshold for tension development.

It was significant that the twitch response of the muscle to a single stimulus applied to the "fast" axon usually disappeared as the preparation became older. When this occurred, examination of the proximal muscle fibres showed that, although p.s.ps. of about 10 mV were common, very few of them exceeded the postulated membrane potential "threshold."

Tension in response to "slow" axon stimulation occurred usually only at frequencies of stimulation above 30 per second, and was very weak until frequencies of stimulation of about 50 per second were applied. When the frequency of

stimulation was less than 30 per second some of the distal fibres showed responses of up to 10 mV total magnitude, but almost none of these responses was large enough to exceed the postulated "threshold." When the frequency of stimulation was above 50 per second, total depolarizations of 10 to 20 mV could be observed in some distal muscle fibres. At higher frequencies of stimulation, superimposed twitches occurred in the tension records of some muscles, and electrically excitable responses could be seen in some of the distal muscle fibres.

The evidence obtained for the "fast" systems of the crayfish leg and claw indicate that a depolarization-dependent mechanism could be involved in "fast" tension production in these muscles. In the walking leg closer, spiking fibres were frequently encountered, while in the claw closer, large p.s.ps. of 10 to 20 mV were observed and it is possible that spiking fibres were present in this muscle as well. With an assumed threshold of 60 mV and resting potentials of 70 to 85 mV, the electrical responses to single shocks applied to the "fast" axon would be large enough in both muscles to exceed the "threshold" and give rise to tension responses in the form of twitches.

The relatively rapid decline of the twitch response with repeated stimulation in the claw closer may have been due to a rapid decline in transmitter substance release

(due to inadequate perfusion?) coupled with the relative scarcity of spiking fibres in this muscle. If the powerful twitch of this muscle were attributable to the combined responses of many fibres depolarized just past the threshold by p.s.ps. of 10 to 20 mV, a small decline in transmitter substance output affecting many fibres at once would result in a relatively large effect on the tension response. In the walking leg closer, on the other hand, spiking fibres were numerous, and small variations in transmitter substance output would have little effect on the tension response of this muscle.

The "slow" systems of the walking leg and claw closers, and the opener muscles, present problems for an interpretation of tension development based on membrane depolarization *per se*. In the opener muscle of the walking leg, potassium contracture does not occur unless the membrane potentials of the muscle fibres are lowered past a "threshold" of about 60 mV. However, the contraction produced by nervous excitation can be detected when electrical responses are considerably below the threshold.

It is possible that larger electrical responses to nervous excitation were present in this muscle and not discovered, but the small size of this particular muscle renders this possibility unlikely.

If larger indirectly produced electrical responses were not present, the conclusion must be that in this

muscle potassium depolarization acts in a different manner than does nervous excitation. It is possible that the transmitter substance acts on the contractile machinery without the mediation of membrane potential changes, and that the latter are to be regarded as unimportant "side effects", indicating the existence of nervous excitation but not contributing to the development of tension. It is known that certain drugs can cause contractions of muscle which are independent of membrane potential or changes in this potential (Evans et al. 1958; Axelsson and Thesleff, 1958). The transmitter substance of the crayfish opener muscle may have a similar action.

On the other hand, it is possible that both nervous excitation and potassium contracture act by depolarization of the muscle fibre membrane, but that the "thresholds" for these actions are different, that for nervous excitation being lower. Membrane depolarization _per se_ does not necessarily result in contraction; the manner in which the depolarization is produced is of crucial importance, if this hypothesis is correct. The transmitter action may have an additional action besides depolarization _per se_, such as facilitation of the entry of a "key" ion, perhaps calcium (Hoyle and Wiersma, 1958c); or the localization of the nerve endings near components of the sarcoplasmic reticulum may result in more efficient conduction of excitation to the fibre interior during transmitter action.

On the other hand, potassium depolarization may be accompanied by inhibition of some stage of the excitation-contraction coupling process. Potassium ions may act on the reticular components and make them less efficient in transmission of excitation to the fibre interior.

Several pieces of evidence give some support to the hypothesis linking indirect contraction to membrane depolarization rather than that accounting for contraction by a more direct action of the transmitter substance.

1) When the membrane potentials of the opener muscle fibres are lowered by application of potassium solutions of sub-contracture concentrations, the indirectly produced electrical responses are slightly attenuated, but the tension response is increased slightly (Fig. 108). This indicates that the transmitter action is not independent of the membrane potential level.*

2) Hoyle (personal communication) has found fibres in the closer muscle of Cancer which develop tension in response to a very small amount of depolarization applied by means of a current-passing microelectrode.** This observation suggests that crustacean muscle fibres vary considerably with respect to the "threshold" levels for

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*However, this observation is open to other interpretations. It is possible that the output of transmitter substance is increased in raised potassium and that the observed attenuation of the electrical response is due to decreased membrane resistance. The increase in the mechancal response could in this case, be due to the increase in transmitter substance.

**As was pointed out earlier, effects of this sort can be produced in depolarized fibres (p. 133). Additional investigation of this phenomenon is needed.

tension production. In the opener muscle of the crayfish walking leg, fibres of this "low threshold" type may be common.

In the experiments in which tension was recorded from single muscle fibres of <u>Cancer</u> and <u>Carcinus</u>, the thresholds for different types of depolarization were estimated to be similar. Nevertheless, it is possible that these thresholds may be different in other types of muscle fibre. It can be postulated that non-spiking fibres innervated primarily by the "slow" axon may have low thresholds for nervous excitation, but higher thresholds for potassium contracture.

On this basis the "slow" twitch of the crayfish claw could be explained. This "twitch" response could be due to the action of a number of "slow" fibres depolarized past threshold by very small electrical events. The slowness of the mechanical response may reflect the slow contraction speeds of these particular fibres, compared with the fibres responding primarily to "fast" axon stimulation.

The resolution of the problem of the different thresholds for different types of depolarization is dependent on further work with isolated single muscle fibres. At present, the available evidence favours the interpretation that many muscle fibres have similar thresholds to different types of depolarization, but that in some muscle fibres the threshold may vary with the method used for depolarization. The observation that single <u>Cancer</u> muscle fibres are less

effectively activated by successive potassium depolarizations is an indication that potassium depolarization may not be equivalent to the other methods of depolarization.

The underlying causes of possible differences in thresholds for different types of depolarization can only be guessed at. It is possible that potassium may have an inhibiting effect on tension development. On the other hand, the depolarization associated with nervous excitation may be coupled to some additional event, such as entry of a "key" ion as suggested by Hoyle and Wiersma (1958 c). Until experiments have been done to establish more definitely whether or not different types of depolarization are nonequivalent, further speculation is of little value.

c) Effects of Potassium on Crustacean Muscles.

In the present study it has been shown that potassium lowers the resting potential of crustacean muscle fibres and causes them to develop tension when the membrane potential has been lowered past a "threshold." The resting potential-potassium curve is influenced (at least in *Carcinus*) by the concentration of potassium inside the muscle fibre, which is subject to such factors as the previous thermal history of the animal, but the "threshold" for tension development apparently remains fixed.

The potassium contracture of *Carcinus* muscle is similar to that of frog muscle in its requirement for extracellular

calcium ions (cf. Frank, 1958). However, the rate of relaxation of the contracture of this muscle was slower than that of frog "twitch" fibres, with the exception of a rapid initial phase which was sometimes observed. It appeared likely that the contractures recorded from many Carcinus muscles represented responses of "fast" and "slow" muscle fibres, as in the frog (cf. Kuffler and Vaughan Williams, 1953).

Alteration of the potassium concentration of the perfusion fluid had profound effects on the electrical and mechanical responses to indirect stimulation. In most muscle fibres the size of the p.s.ps. was reduced in raised potassium, but in relatively excitable muscle fibres (as in Nephrops) electrically excitable responses were generated in high potassium solutions in response to stimulation which did not produce such responses in normal saline. This phenomenon was probably due to the fact that raised potassium lowered the membrane potentials of the muscle fibres towards the threshold of electrical excitability, so that a much smaller electrical response was required to generate electrically excitable responses.

In certain Pachygrapsus "fast" fibres the size of the p.s.p. became larger in high potassium, and it appeared likely that this was a manifestation of the type of electrical excitability demonstrated in certain Carcinus Type A fibres (see Figs. 7, 61, 66). Increased "fast" responses of

this type may counteract the effect of decline of electrical events in "spike" fibres as far as tension development is concerned.

In cases in which electrical activity was increased in high potassium, it is easy to see why the mechanical activity of the muscle was increased initially. However, in many muscles the electrical activity was reduced in high potassium, yet the mechanical activity was often increased initially. Examples include the crayfish opener muscles, the Carcinus and Pachygrapsus "slow" systems, the crayfish walking leg opener "fast" system, etc.

It is likely that these effects can be explained by the fact that the membrane potentials of the muscle fibres are lowered towards the threshold for contraction by high potassium. Therefore a smaller change in membrane potential is needed to exceed the membrane potential "threshold" for contraction. It is readily apparent that even though the indirectly produced electrical responses may be greatly reduced by high potassium, they may still exceed the "threshold" in partly depolarized fibres (cf. Orkand, 1962; and pp. 129, 133), whereas a much larger electrical response may not exceed the "threshold" in a fibre with a high resting potential. For example, the response of the Nephrops fibre of Fig. 102 (a) was about 10 mV in magnitude; the resting potential of the muscle fibre was 72 mV; hence the fibre was depolarized to 61 mV during the peak of the p.s.p.

In Fig. 102 (b), the response was 5 mV in magnitude; the resting potential was 60 mV; hence the fibre was depolarized to 55 mV during the peak of the p.s.p. If the estimated threshold of 58 mV applies to this fibre, and if membrane depolarization initiates tension, it is likely that the fibre develops tension in raised potassium but not in normal saline. The situation is probably analagous to that described by Orkand (1962) for direct excitation of crayfish muscle fibres in high potassium. In the latter case, current of a given strength produced a smaller electrical response in a potassium-depolarized fibre, but a larger mechanical response, because the threshold was effectively lower after potassium depolarization.

A number of observations cannot be interpreted easily in this way. In some muscles "slow" tension was depressed rather than enhanced by high potassium. Also, a return to normal saline after a short stay in high potassium often resulted in a marked depression of tension, particularly in the Carcinus closer muscle, even though electrical responses in such cases were restored to nearly their original size (Fig. 112). These observations contribute to the impression that high potassium may actually inhibit tension development, and that this inhibiting effect may remain after the extra potassium has been removed. The actual tension that is developed in excess potassium may depend on the relative effectiveness of the hypothetical

[illegible] inhibition and of the increased depolarization above the tension "threshold" during indirect stimulation. In most "fast" systems the electrical responses are fairly large (and may become larger in excess KCl due to the participation of electrically excitable responses); consequently the hypothetical "balance" is tipped decisively in favour of greater tension production. In many "slow" systems the "balance" is probably much more delicate because the electrical responses are relatively small and are often associated with electrically inexcitable muscle fibres. In these cases such factors as the condition and physiological state of the animal from which the muscle was taken may influence the delicate balance of the tension-producing mechanism and determine whether tension is reduced or increased in high potassium.

Another factor which could determine whether or not "slow" tension is depressed by high potassium in a particular muscle is the relative time constants of decay of the electrical responses in high and low potassium. The electrical responses decay more rapidly in high potassium, and this would presumably act to lower the tension produced. The delicate "balance" of the tension-producing mechanism in slow systems may thus be altered by changes in membrane time constants, and also by changes in length constants, which would affect the spread of depolarization and therefore the total depolarization attainable during a given type of stimulation.

However, the "potassium inhibition" hypothesis suggested above would have the advantage of explaining the facts that potassium depolarization is less effective than nervous excitation in eliciting tension in some muscles (such as the crayfish opener), and that single Cancer muscle fibres show an altered threshold after repeated applications of high potassium. This hypothesis could also be used to explain the decline of contracture tension with time after application of high potassium and the much slower decline of contractures elicited by sulphate and by sucrose (p. 108).

Hodgkin and Horowicz (1960b) have advanced an alternative explanation of the decline of potassium contracture. They attribute this decline to exhaustion of an "activator." However, Sandow and Kahn (1952) explained the decline in high potassium of the mechanical responses of frog muscles in terms of inhibition of the contractile mechanism by potassium, and pointed out that potassium inhibits ATPase activity (Perry, 1951). At present it cannot definitely be stated whether or not high potassium exerts an inhibition on the contractile apparatus of muscle.

d) Effects of Substituted Anions on Crustacean Muscles.

In Carcinus muscle it has been demonstrated that anions of the lyotropic series have profound effects on indirectly produced electrical and mechanical responses. These effects differ considerably from those found in vertebrate muscles

and even from effects in other crustacean muscles. This variety of response serves to emphasize once again the diversity of mechanisms in crustacean muscles.

In _Carcinus_ the foreign anions increased the membrane resistance of the muscle fibres examined, and this effect provides at least a partial explanation for the increased electrical responses to indirect stimulation. In Type A muscle fibres, the p.s.ps. to "fast" axon stimulation were often large enough to initiate electrically excitable responses at frequencies of stimulation which did not produce such responses in normal saline. This effect seemed to be due mostly to increase in size of the p.s.ps., resulting in depolarizations in excess of the threshold for electrically excitable responses. The anions tested had little effect on the electrically excitable responses to direct depolarization, although in some fibres thiocyanate increased these responses slightly. Therefore the main effect of the foreign anions on the indirectly produced electrical responses is probably due to the increased membrane resistance resulting from their application. It is unlikely that dramatic changes in potassium conductance, of the type described by Werman and Grundfest (1961) for barium-treated lobster muscle fibres, were involved in the action of these anions. It is more likely that a decrease in chloride conductance of the type described by Hutter and Padsha (1959) was responsible for the increase in membrane

resistence, and perhaps also for the hyperpolarizing actions of these anions.

It was observed that prolonged treatment with foreign anions resulted in a gradual decrease in magnitude of electrical [illegible] responses. Since the membrane resistances of most of the fibres examined did not show a similar decline with time (except in the case of some Type B fibres; Fig. 125), reduction in output of transmitter substance was probably responsible. In _Nephrops_ muscle this explanation seemed to apply also, except that electrical activity declined much more rapidly than in _Carcinus_ after treatment with similar concentrations of nitrate.

In spite of the increase in size of electrical responses to indirect stimulation, the associated mechanical responses were depressed by nitrate and bromide, and the "slow" mechanical responses were depressed by iodide and thiocyanate. In the case of nitrate the mechanical responses to direct electrical depolarization and to potassium depolarization appeared to be depressed also. Results for other anions were less clear-cut, but it is possible that they also had a slight inhibiting effect on tension development. The nature of "nitrate inhibition" can only be guessed at, but it is possible that nitrate may interfere with the action of calcium in the excitation-contraction coupling process (cf. Peterson and Feigen, 1962). It is also possible that foreign anions

affect components of the sarcoplasmic reticulum concerned with excitation-contraction coupling. It has been suggested by Girardier et al. (1962) that a tubular membrane effectively in series with the cell membrane and selective for chloride ions may mediate the conduction of excitation from the cell surface into the muscle fibre. Foreign anions may alter the properties of this membrane, causing it to be less permeable to chloride, and thus interfere with excitation-contraction coupling.

In the case of thiocyanate, the "inhibition" (if present) was not as pronounced as with nitrate. However, both thiocyanate and iodide produced hyperpolarization, and this may have been largely responsible for the decrease in "slow" tension, by effectively raising the threshold for contraction. In the case of "fast" responses, the electrical activity is increased to such an extent that small changes in threshold or in effectiveness of depolarization would not have much effect on the tension output which, as one would expect, is much greater than in normal saline.

The responses of crayfish and _Cancer_ muscle fibres after treatment with nitrate did not conform to the pattern observed for _Carcinus_. No "nitrate inhibition" was observed for the former muscles (apart from, in _Cancer_, gradual decrease in the electrical response as in _Carcinus_ and _Nephrops_, probably due to decline in transmitter substance output). In crayfish the mechanical responses (particularly the "fast"

twitch) were enhanced by nitrate as in frog muscle. In <u>Cancer</u>, membrane resistance was increased, as in <u>Carcinus</u>, but direct depolarization was not less effective in initiating contraction. Indirect responses, bothelectrical and mechanical, were slightly increased in nitrate at first in the <u>Cancer</u> stretcher muscle.

These rather different results for <u>Cancer</u> and <u>Carcinus</u> may indicate specific differences in those parts of the membrane concerned with excitation-contraction coupling. It was found that fibres from the two muscles have different internal resistivities. Perhaps other fundamental differences also exist.

In none of the muscles examined critically were foreign anions found to have an appreciable effect on the membrane potential threshold for contraction. This result is different from that obtained by Hodgkin and Horowicz (1960c) for frog muscle. Nitrate and other anions were found to lower the membrane potential threshold in single frog muscle fibres.

Clearly there is much more to be learned about the action of anions on crustacean muscles. Why are muscles of different species not affected in the same way by these ions? What membrane sites are influenced by treatment with foreign anions? Only further work with single fibres and perhaps with the electron microscope can resolve these problems.

e) Concluding Remarks

Most of the results of this study support the view (Fatt and Katz, 1953c) that membrane depolarization is a link in the normal excitation-contraction sequence in crustacean muscles. In all of the "fast" systems which have been examined in this study, and in most of the "slow" systems, the indirectly produced depolarizations are sufficient to exceed the estimated thresholds for tension production. If the observations made on crayfish muscles were excluded from consideration, the conclusion of this study would probably be that membrane depolarization *per se* is the agent which initiates contraction. However, in crayfish muscles the threshold for tension production by potassium depolarization is apparently different from the threshold for tension production by nervous excitation, and the possibility must be considered, therefore, that the manner in which depolarization is produced has an important bearing on the resulting tension response.

Depolarization can be rendered less effective as a contraction-producing agent by treatment with certain ions, such as nitrate (in the case of *Carcinus* muscle) or barium (in the case of *Nephrops* muscle). The possibility that excess potassium may inhibit tension development in crustacean muscles also exists.

The mechanical responses of crustacean muscles have been found to be governed in some muscles by "fast" and

"slow" types of muscle fibre, responding primarily to "fast" and "slow" axon stimulation respectively. These fibres probably have different contraction speeds as well. The discovery of the importance of the properties of the responding muscle fibres in determining the electrical and mechanical responses of the muscle supports the view which has been presented in the present study that a single excitatory transmitter substance may be involved in excitatory neuromuscular transmission in crustaceans.

SUMMARY

1. Muscles from the walking legs of several decapod crustaceans (*Carcinus maenas*, *Nephrops norregicus*, *Astacus pallipes*, *Cancer magister*, *Pachygrapsus crassipes*) were studied with the object of determining the mechanisms involved in the production of tension by nervous excitation.

2. In the closer muscle of *Carcinus*, which is innervated by two excitor axons, "fast" and "slow," it was found that three different types of muscle fibre could be distinguished. Muscle fibres of the first category (Type A) showed large electrical responses to stimulation of the "fast" axon, but small (or no) responses to "slow" axon stimulation. Fibres of the second category (Type B) gave large electrical responses to "slow" axon stimulation, but small responses to "fast" axon stimulation. The third type of fibre (Type C) gave responses of an intermediate size to stimulation of both "fast" and "slow" axons.

3. Analysis of the electrical properties of the muscle fibre membranes with intracellular microelectrodes showed that the three types of fibre had different membrane properties. Type A fibres had small membrane time constants, low membrane resistances and electrically excitable membranes; Type B muscle fibres had large membrane time

constants, high membrane resistances and electrically inexcitable membranes; and Type C fibres had intermediate membrane properties.

4. The electrical responses to indirect stimulation via the excitor axons could be explained in terms of the different membrane properties of the responding muscle fibres. The time constants of decay of the indirectly produced electrical responses were related to the membrane time constants of the responding muscle fibres. The occurrence of electrically excitable responses during indirect stimulation was limited to muscle fibres with electrically excitable membranes.

5. An additional factor determining the nature of "fast" and "slow" electrical responses in individual muscle fibres was the density of innervation of the muscle fibres by the excitor axons. It was pointed out that Type A fibres must possess a much denser innervation by the "fast" axon than the other types of muscle fibre.

6. The differences between the "fast" and "slow" mechanical responses recorded from the whole muscle could be largely attributed to the different electrical responses of fibres responding primarily to stimulation of "fast" or "slow" axons (Type A and Type B muscle fibres).

7. In the _Pachygrapsus_ closer muscle, specialized "fast" and "slow" muscle fibres were also present, and the

mechanisms of "fast" and "slow" contraction appeared to be very similar to those found in *Carcinus*.

8. In the *Nephrops* closer muscle, fibres responding primarily to stimulation of "fast" or "slow" axons were present, but fibres throughout the muscle had similar membrane properties. Differences between "fast" and "slow" contractions in this muscle were due mainly to differences in "fast" and "slow" neuromuscular transmission.

9. The electrical and mechanical responses of *Carcinus* muscles to treatment with solutions containing excess potassium chloride were investigated. It was found that *Carcinus* muscle fibres behaved as imperfect potassium electrodes. The immediate response to potassium chloride was different from the response after a prolonged exposure.

10. Muscles of animals exposed to low temperatures were more easily depolarized by potassium than muscles from animals maintained at a higher temperature. The probable explanation of this behaviour was that muscles of cold-acclimated animals contained less potassium.

11. Tension was developed by *Carcinus* muscles when the average depolarization of the muscle fibres produced by potassium exceeded a membrane potential "threshold" of 55 mV. The potassium contracture did not occur in the absence of external calcium ions. Strontium ions, but not barium

ions, could support a potassium contracture in the absence of calcium ions.

12. In *Astacus* and *Nephrops* muscles, contraction occurred when potassium depolarization exceeded "thresholds" of 60 mV and 58 mV, respectively.

13. The electrical and mechanical responses of single muscle fibres of *Carcinus*, *Cancer*, and *Pachygrapsus* muscles stimulated with an intracellular current-passing electrode were investigated. In these muscles, membrane potential "thresholds" of the muscle fibres studied ranged from 60 mV to 45 mV.

14. Tension was developed by single *Cancer* muscle fibres when depolarization produced by stimulation of the motor axon exceeded a "threshold" of 60 to 54 mV. The "threshold" for potassium contracture in single *Cancer* fibres was estimated to be 60 to 55 mV for an initial application of high potassium, but the "threshold" was increased for successive treatments.

15. In the walking leg opener muscle of *Astacus*, potassium chloride depolarization was less effective than nervous excitation in producing tension.

16. In all other muscles studied, the electrical responses during indirect stimulation were large enough to exceed the estimated membrane potential "thresholds." It was concluded that in many muscle fibres the "thresholds"

for production of tension by different types of depolarization are the same, but that in other cases these thresholds may be different. Most of the evidence indicated that membrane depolarization is a link in the excitation-contraction coupling process in crustacean muscles.

17. In muscles of Nephrops, Astacus, Carcinus, and Pachygrapsus, application of solutions containing excess potassium ion concentrations usually increased the mechanical responses to both "fast" and "slow" axon stimulation initially, apparently by lowering the membrane potentials of the muscle fibres towards the threshold for contraction and the threshold for initiation of electrically excitable responses. However, certain observations suggested that potassium ions may also have an inhibitory effect on muscular contraction.

18. Indirectly produced electrical and mechanical responses of Carcinus and Nephrops muscles were increased initially by barium-containing solutions. In Nephrops the threshold for production of electrically excitable responses was lowered by barium treatment. The effect was not evident in Carcinus muscle fibres. In Nephrops the tension declined with time to zero although electrical responses remained large. Barium apparently brought about gradual inhibition of contraction in this muscle.

19. The anions bromide, nitrate, iodide, and thiocyanate, when applied to Carcinus muscle preparations, increased the electrical responses to stimulation of the excitor axons. This change was accompanied by increased membrane resistance of the muscle fibres.
20. The mechanical responses of Carcinus muscles to both "fast" and "slow" axon stimulation were decreased by addition of nitrate. Tension recordings from single muscle fibres showed that the tension-producing effectiveness of directly applied depolarization was reduced in nitrate.
21. In iodide and in thiocyanate solutions, "slow" mechanical responses were reduced, but "fast" mechanical responses were increased. The former effect was partly attributable to hyperpolarization produced by the foreign anions, which effectively increased the membrane potential "threshold" for tension development. The increase in the "fast" mechanical response was attributable to increased electrical activity of Type A muscle fibres, which showed large electrically excitable responses in thiocyanate solutions.
22. Evidence from studies of the effects of ions on electrical and mechanical responses of crustacean muscles indicates that tension is probably produced by a depolarization-dependent mechanism during nervous excitation, but that the effectiveness of depolarization can be altered by foreign ions.

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