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INVESTIGATIONS INTO THE STRUCTURE AND FUNCTION
OF NERVE AND SKELETAL MUSCLE OF ANISOPTEROUS
ODONATA, WITH SPECIAL REFERENCE TO AESCHNID NYMPHS

by

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GREY COLLEGE.

Being a thesis presented in candidature for the degree
of Doctor of Philosophy of the University of Durham.

January 1968



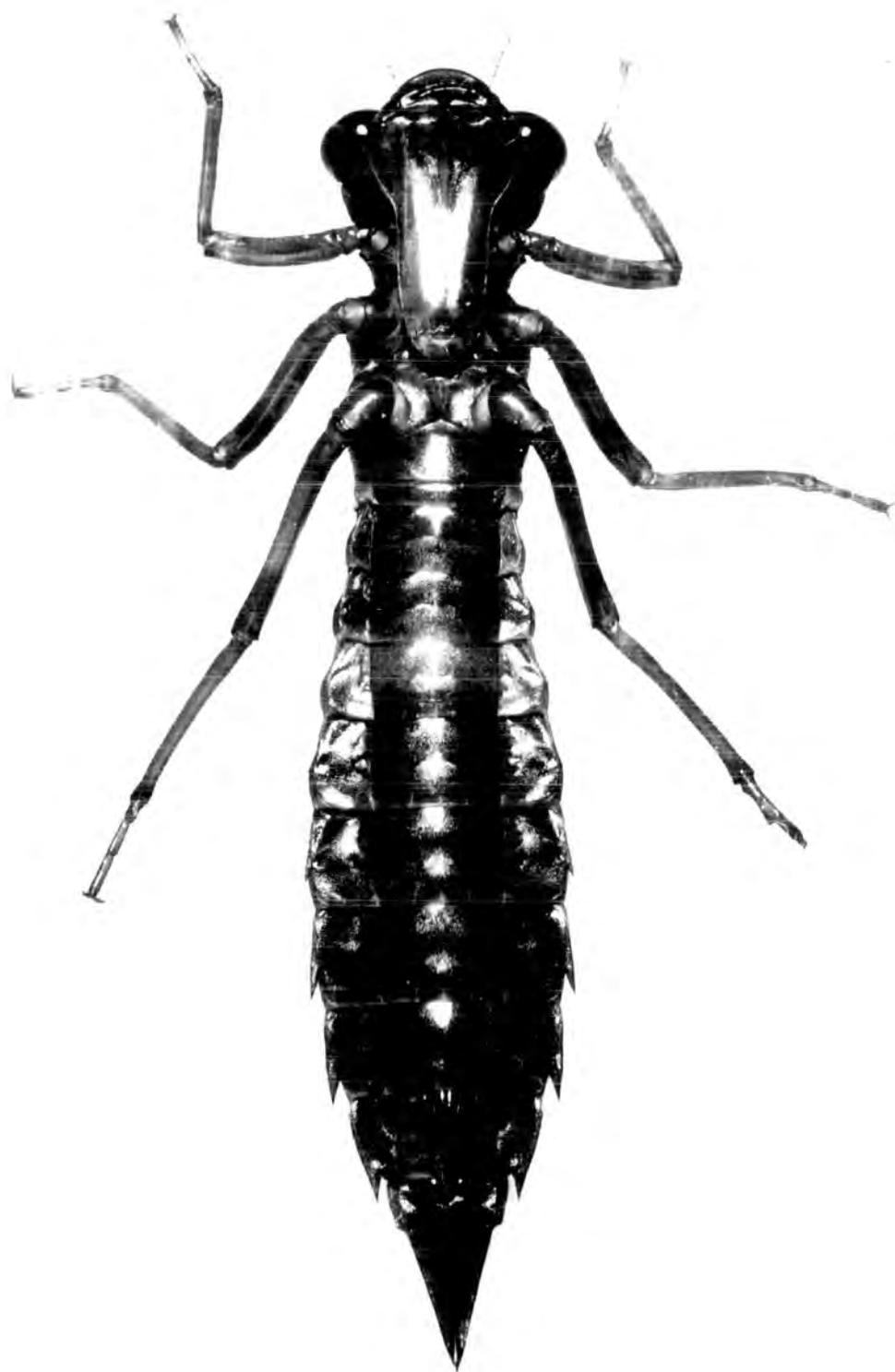
PUBLICATION

A preliminary report of some of this work, comprising the subject matter of Chapter 4 and part of Chapter 7, has been published under the title of "Electrical and Mechanical Responses of the skeletal muscle of a primitive insect" in Nature 215, 991 - 992.

The majority of Chapter 6 has been accepted for publication by Comparative Biochemistry and Physiology under the title "Fatigue-dependent facilitation of the slow skeletal muscle response of dragonfly nymphs".

FIGURE 1.

A late-instar larva of a species of Aeschna,
probably A. juncea. Ventral surface. X 4.



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CHAPTER 1

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GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

The science of quantitative neuromuscular physiology is approximately 20 years old, dating from the invention of the intra-cellular micro-electrode (Graham & Gerard, 1946; Ling & Gerard, 1949). In spite of the numerical superiority of the invertebrate animals it is perhaps not surprising that the great majority of experimental work has been carried out either on vertebrate animals or on invertebrate tissues such as the giant axons of squids and other molluscs. It was already clear by the early 1950s that hopes of a unified theory of the action of excitable tissues were to be disappointed, with the discoveries by such workers as Fatt & Katz (in crustaceans) and Hoyle (in insects) that invertebrate muscle membranes do not show all-or-nothing electrical responses to neural stimulation, and that some types do not even show an active membrane response. As a result of these and other discoveries, the fields of vertebrate and invertebrate neuromuscular physiology have to a large extent separated, with many common techniques, but with few concrete theoretical links.



It is now clear that the overwhelming majority of synaptic junctions of animals with discrete nervous systems operate through chemical mediation; only in a few animals does any possibility of essentially ephaptic transmission exist; the neuromuscular junction of skeletal muscle is, of course, simply a chemically-mediated synapse, that is to say the presynaptic impulse causes release of a chemical agent or transmitter substance which stimulates the post-synaptic membrane to produce a post-synaptic impulse. This mechanism, and the evidence for it, are fully set out by Katz (1961).

It has also become apparent that the basic mechanisms that underlie nerve action in all animals are similar; the ionic hypothesis (Hodgkin, 1951) can be applied to giant axons of squids as well as to the myelinated nerve fibres of frogs and mammals, and recent work indicates similar processes in the cockroach (Narahashi, 1965); even in herbivorous insects, which have apparently hostile ionic concentrations in their haemolymph, similar processes are at work, although details of the mechanism whereby the charge is transported across the membrane during activity may be different, and active components in extra-axonal sheaths may also be involved (Treherne, 1966; Treherne & Maddrell, 1967).

It is in the mechanisms of muscle action that the great differences between vertebrate and invertebrate lie. These differences seem to originate from two causes, the relative inexcitability and leakiness of the invertebrate muscle fibre membrane (e.g. Fatt & Katz, 1953) and the virtual absence in invertebrates of all-or-nothing responses - either mechanical or electrical (Hoyle, 1957; Wiersma, 1961).

Early workers on invertebrate material, such as Pringle (1939) and Wiersma & van Harreveld (1938) established the presence of two distinct types of muscle responses; these were defined as "fast" (or "twitch") and "slow" (or "tonic"), the definitive factor being the time-course characteristics of the mechanical response. With the advent of the intracellular recording technique it has become apparent that many invertebrate muscle fibres exhibit both types of response, being innervated by more than one axon per muscle fibre (see, e.g. Hoyle, 1957; Wiersma, 1961).

In vertebrates both types of response can also be found, but occur in completely separate muscle fibres; multiple axon innervation of a single fibre is not found. The picture which has emerged of neuromuscular transmission in vertebrate twitch muscle fibres (the vast majority of

vertebrate fibres) shows a pattern which is fairly uniform within the vertebrates, but unlike any responses found elsewhere. Katz (1961, 1966) gives a full description of the standard transmission type found in vertebrate twitch systems.

The non-propagated responses of vertebrate slow fibres, first described by Kuffler & Vaughan Williams (1953a & b), are much more like those of invertebrates, and for this reason are often thought primitive. Typical vertebrate slow responses are shown by Bach-y-Rita & Ito (1966). Unlike the twitch fibres, slow fibres of vertebrates are usually multiterminally innervated, i.e. the innervating axon synapses with the muscle fibre at many points, or end-plates. This type of multiple innervation is normal in invertebrate fibres, which are often multi-axonally innervated as well.

The similarity between vertebrate slow muscle fibres and the fibres of invertebrates is underlined by the recent discovery that vertebrate slow muscle fibres have a membrane capacity and resistance several times greater than do vertebrate twitch fibres (Adrian & Peachey, 1965); invertebrate muscle fibres also have relatively large membrane capacities and resistance (e.g. Fatt & Katz, 1953).

Within the phylum Arthropoda no coherent picture of neuromuscular transmission has appeared comparable to that seen in vertebrates. It is generally thought that the arthropods, a rather diffuse grouping, represents an adaptive radiation and evolution of types from a basic metamericly-segmented annelid-like form or forms. If this is true, it is perhaps not surprising that a multiplicity of modes of physiological action has evolved. In the case of transmission of nerve impulses it is becoming apparent that mechanisms have been evolved, where necessary, to adapt axonal activity in detail, rather than fundamentally, where hostile environments are provided by, e.g., exotic haemolymphs (Treherne & Maddrell, 1967). In the case of the action of the muscle membrane, however, present knowledge shows such a variety of forms that the conclusion that some of the basic mechanisms have changed, or have evolved separately, is inevitable.

Two major arthropod groups have been studied in depth - the classes Crustacea and Insecta. The Crustacea show a great deal of adaptive radiation in functional morphology, but because the vast majority of species have remained marine, it is perhaps not surprising that there is some uniformity between species in neuromuscular

transmission properties; there is, however, considerable variation in muscle fibre properties between different muscle blocks and fibres of each species. In the insects there is much more environmental variation, but the evolutionary development is generally thought to have been rather more sequential than occurred in the crustaceans; consequently there would seem to be a basic insect pattern, as exhibited by the orthopteran orders - such insects as locust and cockroach - with various developments in higher forms for such specialised functions as flight, and in varied haemolymphs.

The basic differences between the crustacean and insect types will be dealt with in detail in Chapter 4; the following is a preliminary summary of those differences which appear most fundamental; documentation of the statements which follow appearing in the detailed discussion (Chapter 4).

The crustacean muscle fibre is invariably innervated by an axon which evokes a response which is either slow, or can be referred to the tonic type of contraction; multi-axonally innervated fibres usually, though not invariably, receive a fast axon, and often are innervated by an inhibitory axon. The insect muscle fibre is virtually always innervated by a fast axon, and this may be the sole

innervation; often only a minority of the fibres of a muscle block are slow-innervated, and a very few fibres receive an axon, the function of which is arguably inhibitory. The insect fast response usually shows an active membrane response component in addition to the end-plate potential (e.p.p.) produced by transmitter action. The crustacean fast response, with certain highly evolved exceptions, consists only of an e.p.p., any active component being small, late and abortive.

Insect slow electrical responses facilitate and summate to a very great extent, often producing a tetanic potential change which completely depolarises the membrane. The slow responses of crustaceans also facilitate, though to a much lesser extent, and summation is much more limited.

At the beginning of the study set out in this thesis it was hoped that the application of well-tried neuromuscular techniques to insects more primitive than those forms at present documented might reveal response patterns different from those found in higher insects; it was thought possible that any different forms discovered might resemble crustacean patterns, and thus indicate a closer relationship than at present appears. For the reasons set out in Chapter 2 larvae of the genera Aeschna

and Libellula, which are dragonflies of the order Odonata, sub-order Anisoptera, were chosen as experimental subjects. In Chapter 2 an account is given of those aspects of the general biology of the animals, the knowledge of which is a pre-requisite for neurophysiological experiments to be carried out. Chapter 3 contains a brief description of the electrical properties of the dragonfly muscle fibre membrane; a comparative description of the responses of dragonfly muscle fibres follows in Chapter 4.

These three chapters collectively fulfil the original objectives of the study, at least as far as one type of primitive insect is concerned; the remainder of the thesis is concerned with aspects of the properties of neuromuscular transmission in dragonflies which were seen in passing to be interesting during the initial stages of the study. Chapter 5 covers the normal functioning of the electrical part of transmission, and Chapter 6 discusses in some detail an interesting part of this. In Chapter 7 some aspects of mechanical functioning of the muscle are investigated, and Chapter 8 reports preliminary findings from a study on the importance of concentration and balance of haemolymph divalent cations.

General experimental methods and the treatment of results are described in Chapter 2; specific techniques

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are set out in the relevant chapter, and in appendices.

Where results appear in two forms - tabular and diagrammatically - the tables have been removed from the text into Appendix A.

CHAPTER 2

THE EXPERIMENTAL ANIMAL AND ITS PREPARATION FOR
EXPERIMENTATION

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EXPERIMENTATION

INTRODUCTION

The initial objective of the study was to find an animal whose neuromuscular response patterns are markedly different from those of other known insects, in a way which could be construed as primitive. Various insects have been investigated by other workers, and are documented to a varying extent, but the main body of insect neuromuscular literature centres upon two groups, the cockroaches (Blatteria; Dictyoptera) and the locusts and grasshoppers (Acrididae; Orthoptera). The great majority of other insects which have been investigated are either closely related to these, or are from more advanced groups. All investigated insects, therefore, have an organisation at least as advanced as the orthopteroid orders.

Imms (1957) suggests that the orders of insects can be evolutionarily separated as follows :-

- (1) The apterygotes - primitively wingless insects.
- (2) The palaeopteran pterygotes - Dragonflies and Mayflies.
- (3) The neopteran pterygotes, comprising :-

- (a) the orthopteroid orders
- (b) the hemipteroid orders
- (c) the endopterygotes.

The Orthoptera and Dictyoptera seem therefore, despite their exopterygote development, to be considered as quite advanced insects. The Apterygota are generally thought to be the most primitive; indeed, the position of some of these wingless forms in the class Insecta has been called into doubt many times.

Unfortunately the apterygotes commonly available in temperate climates are rather small for detailed neuromuscular investigation by conventional methods. The largest apterygote available was Petrobius maritimus (Thysanura) but its occurrence was seasonal and variable; in addition, the only muscles of any size were thoracic inter-segmental longitudinal muscles, and their nerve trunks were almost too short to be seen. It was therefore found necessary to consider whether the evolutionarily lower orders of the Pterygota might contain a group which was both sufficiently primitive and suitable for experimentation.

Martynov (1925) divided the Pterygota into two groups which he named the Palaeoptera and the Neoptera. Palaeopteran forms are mainly extinct, only two orders remaining in the modern fauna - the Odonata (dragonflies

and damselflies) and the Ephemeroptera (Mayflies). Palaeopteran orders are distinguished by the absence of a wing-flexing mechanism; they are therefore primitively unable to fold back the wings over the abdomen at rest. Imms (1957) considered the two surviving Palaeopteran orders to be relics of the Palaeocene fauna, though it is evident that some evolution has proceeded since that period, notably in the adoption by both groups of an aquatic larval stage. These two orders, therefore, may represent relics of a form ancestral to all modern (neopteran) forms.

Some interesting evidence helps to confirm this impression. Dragonflies have retained more distinct abdominal segments than have other pterygote insects. Of the eleven segments plus telson of the hypothetical ancestral insect abdomen, ten are distinctly recognisable, and vestiges of the eleventh and of the telson are visible (Heymons, 1904); there are eight abdominal ganglia. Recent investigations with the electron microscope have revealed that the tracheal system of the dragonfly nymph does not penetrate the fibres of the flight muscle (Smith, 1961); the surface only is tracheated. In addition, the muscle fibres are organised rather more simply than in higher forms, the fibrils being arranged radially (Smith, 1961, 1966). One physiologically primitive feature is

that the wing muscles beat synchronously, that is to say the muscles contract once for a single efferent impulse (Pringle, 1957).

Either dragonflies or mayflies seemed, therefore, a reasonable choice in a selection for presumed primitiveness; the most direct evidence for primitiveness seemed to relate to dragonflies. Since neither order had been investigated before, and since dragonfly larvae of various sorts were available for most of the year from aquarium dealers (Messrs. Haig), it was decided to carry out a preliminary study of late-instar nymphs of two genera of the sub-order Anisoptera - Libellula and Aeschna. It soon emerged that there was little quantitative difference between the two, and the great majority of work has been carried out upon the genus Aeschna. Various species, between which no differences have been found, have been used, chiefly A.juncaea and A.cyanea. A key for identification of larval anisopterans is given by Corbett, Longfield & Moore (1960), but identification of species before adult emergence is difficult; certain identification is impossible.

The remainder of this chapter gives the details of the general experimental methods used in the study, and of the investigations necessary to discover the various pieces of information required for an electrophysiological

investigation - anatomy and innervation, haemolymph ionic content and the formulation of a satisfactory experimental saline, and the design of a suitable preparation of the whole or part of the animal.

THE ANATOMY OF THE MESOTHORACIC LEG

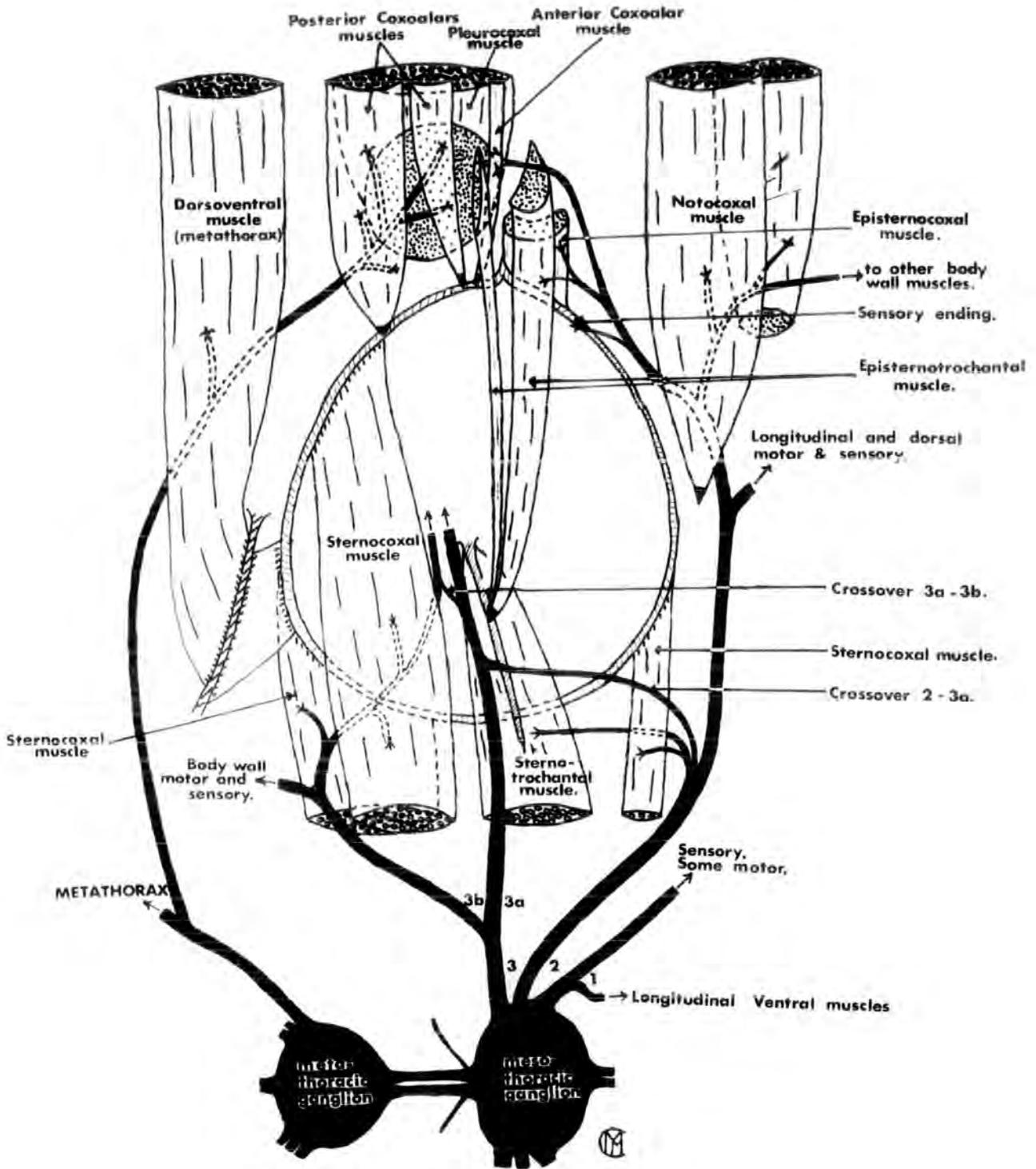
Most studies on the neuromuscular systems of invertebrates have been carried out on limb muscles, chiefly because the longest muscle fibres in the animal usually occur in the leg, because the innervating nerve trunks are long and clearly defined, and because the leg muscles usually lend themselves to linking with mechanical transducers. For this reason a study was made of the muscles of the thorax associated with leg movement, and also of the musculature of the coxa; the layout of the motor innervation was also observed.

For anatomical dissection the live animal was opened mid-ventrally and immersed in a weak solution of methylene blue in 0.8% saline (NaCl solution) for a few minutes. The timing of this process was not found to be critical. The blue colouration which penetrated various tissues could be differentiated, if necessary, with 70% alcohol, and the colour fixed (it slowly dissolves in aqueous baths) with saturated ammonium molybdate solution.

After this treatment the nerves appeared pale blue or white, muscle fibres appeared dark blue, as did connective tissue, and intra-pleural fat was almost black.

Evidence from dissection was confirmed by analysis of serial sections; tissues were fixed by the method of Murray (1937) or by that of Petrunkevitch (1933), both of which use phenols in solution to soften the hard and brittle cuticle. The latter method produced the least distortion of all methods tried in an effort to trace the pathways of individual axons - others being such traditional fixatives as Bouin's and Gilson's and also a method used by Fielden (1963) involving 0.2% osmic acid in saturated picric acid. However, no fixative has yet been found in which sufficient detail is retained to enable light microscope differentiation between axon and glial cell, and to allow the tracing of axon. Fixed tissues were double-embedded in celloidin and paraffin wax, sectioned at 10μ and stained with Heidenhain's haematoxylin.

The innervation of the muscles of the mesothorax associated with coxal movement is shown in Fig.2, and the structure and innervation of the coxa appears diagrammatically in Fig.3. The organisation of the metathorax seems to be very similar to that of the mesothorax; the prothorax is somewhat more simply organised, because of the absence



The Musculature of the Coxal Region
of the Mesothorax of
Aeschna. (Diagrammatic)

TERMINOLOGY FROM CLARK 1940

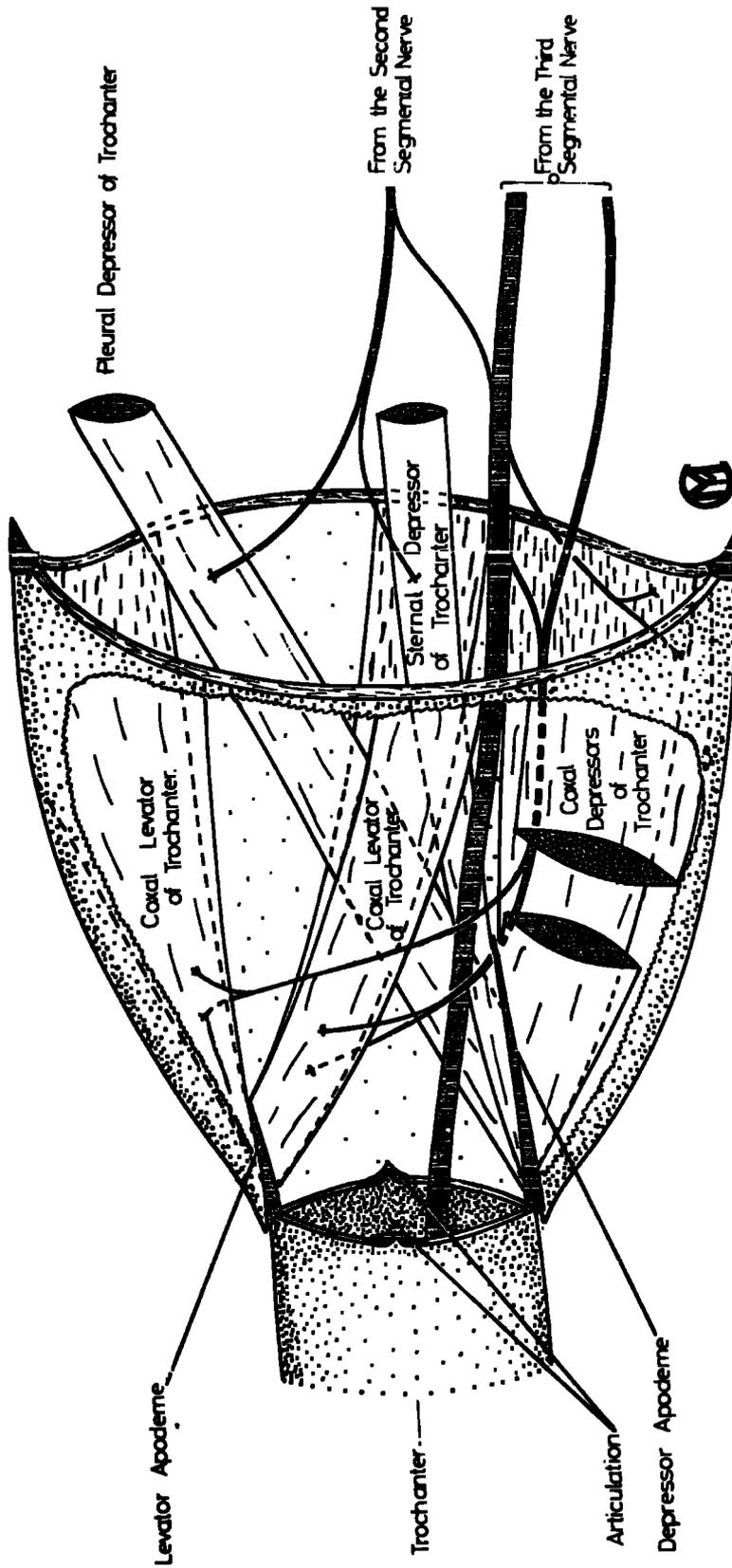


Figure 3.

The Organisation and Innervation of the Coxal Muscles

of flight muscles, and there are only two segmental nerves, the anterior of which clearly corresponds to segmental nerve 1 of the meso- and metathorax, and the posterior of which combines the connections of segmental nerves 2 & 3 of the meso- and metathorax.

It is rather surprising that there is so little information in the literature concerning the internal anatomy of the Odonata in view of their size and the ease with which they can be obtained. Apart from a paper by Rogosina (1928), mostly concerned with sensory nerve bodies and innervation, but including several diagrams showing "multiterminal motor nerve endings" (almost certainly tracheae), the main works on the anatomy of the Odonata are those of Clark (1940), of Zawarzin (1912, 1924), of Mill (1965), of Knights (Fielden) (1960, 1965) and of Smith (1961, 1966). Of these, only Clark is concerned with muscle organisation of the thorax; Zawarzin's classic works concern the organisation of neurones within the ganglia, Smith's the ultrastructure of flight muscle, and Mill's the musculature and innervation of the abdomen. Fielden's work also has mainly involved the abdomen.

Clark studied the musculature of the thorax in some detail in relation to function. Although his work was carried out upon adult dragonflies, the present study

of larval musculature has revealed no qualitative differences; consequently Clark's nomenclature, which he based on function, has been adopted here. Innervation is shown for the first time. The structure of the coxa has not therefore been elucidated, as far as the author can determine; the names are therefore original, but are based upon rules set out by Snodgrass (1935) and follow closely the principles upon which Clark allotted names.

It is regrettable that histological studies have met with a conspicuous lack of success, and that no information is available concerning the detailed innervation of the muscle fibres. Such studies as have been carried out tend to confirm the suggestion of Wigglesworth (1959) and the observation of Fielden (1963) that there is a structural differentiation of the segmental nerves (by axon diameter) between dorsal (motor?) and ventral (sensory?) regions. Otherwise, only negative results - the lack of complete success of any fixation process tried - can be reported. However, physiological evidence of the number of axons involved in the innervation of those muscles used has been obtained, and is set out in Chapter 4.

ANALYSIS OF THE HAEMOLYMPH

Considerable knowledge of the constituents of

dragonfly haemolymph can be obtained by a search of the literature. The main works in which analysis of at least one radical of dragonfly haemolymph is made are those of Duchateau, Florquin & Leclercq (1953), of Sutcliffe (1962), of Schoffeniels (1960), of Bone (1944), and of Clark & Craig (1953). Details of their analyses appear in Table 1.

It was decided to undertake re-analysis of the main constituents in order to obtain statistical confidence limits for all the major inorganic ions. In all methods analysis was carried out on a 10 μ l sample of haemolymph, obtained by allowing thorough drying-out of the animal's cuticle, then making a puncture of the articular membrane between two abdominal sternal plates and squeezing gently to produce a droplet of haemolymph from which an accurate sample could be withdrawn with a micro-pipette. The methods used were as follows :-

Sodium. Sample diluted by addition of 1ml distilled water, and analysed with an EEL flame photometer, using for calibration full-strength standard solutions similarly diluted.

Potassium. By the same method as sodium.

Calcium. By the same method as sodium, but using standard solutions containing, in addition to the standard amounts of calcium, the correct amounts of the other ions, as

TABLE 1

Results of Haemolymph Analysis, and Comparative Data
from the Work of Other Authors

ION	AESCHNA	LIBELLULA	AUTHORITY
<u>Sodium</u>	133.4 \pm 2.8 142 \pm 2.1 135 179 143	142.0 \pm 3.1 - 78 - 52	Original Sutcliffe (1962) Duchateau et al (1953)
<u>Potassium</u>	3.6 \pm 0.2 8 \pm 0.7 4.3 5.4 4.5	5.8 \pm 0.8 - - 3.8 -	Original Sutcliffe (1962) Duchateau et al (1953)
<u>Calcium</u>	10.3 \pm 1.1 16 20.4 7.5 -	8.4 \pm 1.0 16 18.5 - 8	Original Duchateau et al (1953) Bone (1944)
<u>Magnesium</u>	7.2 \pm 0.6 3.0 6.1 -	3.6 \pm 0.6 6.0 - 5	Original Duchateau et al (1953) Clark & Craig (1953)
<u>Chloride</u>	121.0 \pm 3.1 112 \pm 4.9 91	117.2 \pm 1.8 - 77	Original Sutcliffe (1962) Schoffeneils (1960)
<u>Inorganic Phosphate</u>	1.9 \pm 0.4	-	Original
<u>Bicarbonate</u>	15 \pm 1.0	-	Sutcliffe (1962)
<u>Free amino acid as glycine.</u>	34 \pm 4.1	-	Sutcliffe (1962)

The figures are given in mM/l., \pm one standard error

determined by other analyses. This was necessary because of the interactions of other ions with the calcium flame.

Magnesium. The method of Heagy (1948) using titan yellow as a quantitative colorimetric indicator, and stabilising the colour with polyvinyl alcohol. It was found necessary to adopt a standard time procedure after addition of the titan yellow, since the colour changes slowly in light. The colour was analysed by an EEL colorimeter; the sample was placed in the colorimeter $5\frac{1}{2}$ minutes after addition of titan yellow, the colorimeter switched on immediately and the reading taken after a further $1\frac{1}{2}$ minutes.

Phosphate. (Total acid radical). Determination in an EEL colorimeter of the blue coloration of stannous chloride solution. The method was an adaptation by Snell & Snell (1949) of the method of Fiske & Subbarow (1926).

Chloride. Volhard back-titration of excess silver nitrate against ammonium thiocyanate, according to the method of Wigglesworth (1938), but using the microburette of Shaw (1955) for titration, and adding the drops of liquid to each other on an unwettable polyflourethylene tile.

The results of this analysis and comparative results from the work of others appears in Table 1.

The following "balance sheet" can now be drawn up :-

<u>Anions</u>		<u>Cations</u>	
Chloride	120mM/l.	Sodium	136mM/l.
Bicarbonate	16mM/l.	Potassium	4mM/l.
Phosphate as	2mM/l.	Magnesium	6mM/l.
H_2PO_4		Calcium	10mM/l.

Total anion - 138mM/l. Total cation - 156mM/l.

This type of inbalance, showing excess cation, is the normal result of the analysis of insect haemolymph. It is usually accepted that the inbalance represents the presence of amino acid radicals - amino acid measured as glycine equivalent was reported by Sutcliffe (1962) to be 35mM/l. - and other organic anions.

The pH of the haemolymph was determined with a Pye-Ingold micro-pH assembly, which enables repeatable results to be obtained from samples of 20 - 25 μ l. The mean value from seven animals was found to be 7.8 ± 0.2 (s.e.), range 7.6 - 8.1. This value is unusually alkali by comparison with other insects (Boche & Buck 1942).

THE FORMULATION AND TESTING OF AN EXPERIMENTAL SALINE

The figures set out above were used in the formulation of an experimental saline; they are largely based on the results of haemolymph analysis by the methods described, but take into account the results of other workers,

especially where low-concentration ions are concerned.

The saline was made up from "AnalaR" chemicals as follows :-

Sodium Chloride 124mM/l.

Sodium Dihydrogen Orthophosphate 2mM/l.

Sodium Hydrogen Carbonate 12mM/l.

Potassium Hydrogen Carbonate 4mM/l.

Magnesium Chloride 6mM/l.

Calcium Chloride 10mM/l.

This saline was then tested experimentally to discover whether it was capable of supporting life in the muscle tissues of the dragonfly larva. A specimen was pinned ventral side uppermost to a plasticine bed in a "perspex" bath, and a piece of the ventral region of the cuticle removed to reveal part of the coxal musculature. The experimental saline was added to cover the preparation and the resting potential of a number of fibres determined with microelectrodes by methods to be fully described in subsequent sections of this chapter. The resting potential was further sampled at intervals of one hour to observe whether any change had taken place. It was found that the resting potential of fibres in this saline fell within one hour to a very low level.

The pH. of the saline was found to be about 7.0, but any attempt to adjust it to the correct value of about 7.8 resulted in the formation of a white precipitate

which rough analysis showed to be mostly calcium phosphate. Since haemolymph analysis had shown total measured phosphate to be very low in all experimental animals, and absent in some, it was decided to omit phosphate from the saline, with a consequent increase in the level of Na Cl to 126 mM/l. to maintain the correct sodium level. It is likely, in fact, that almost all the phosphate of the living animal is intracellular.

As a result of this adjustment the pH rose to 7.9, well within the confidence limits of the observed value. This saline was also unable to maintain the resting potential, however.

Wood (1957) found it necessary to add fairly large amounts of sucrose to his saline for Carausius morosus; he considered that the reason sucrose was required was osmotic rather than metabolic. When either of the above dragonfly salines was used, some distortion of the fibres became noticeable after a time. A rough experiment, in which a quantity of the phosphate-free saline was shaken up with a quantity of sucrose, showed a great improvement in the maintenance of resting potential. A series of quantitative experiments was therefore carried out with various amounts of sucrose dissolved in standard phosphate-free saline (which from the pH point of view is clearly the

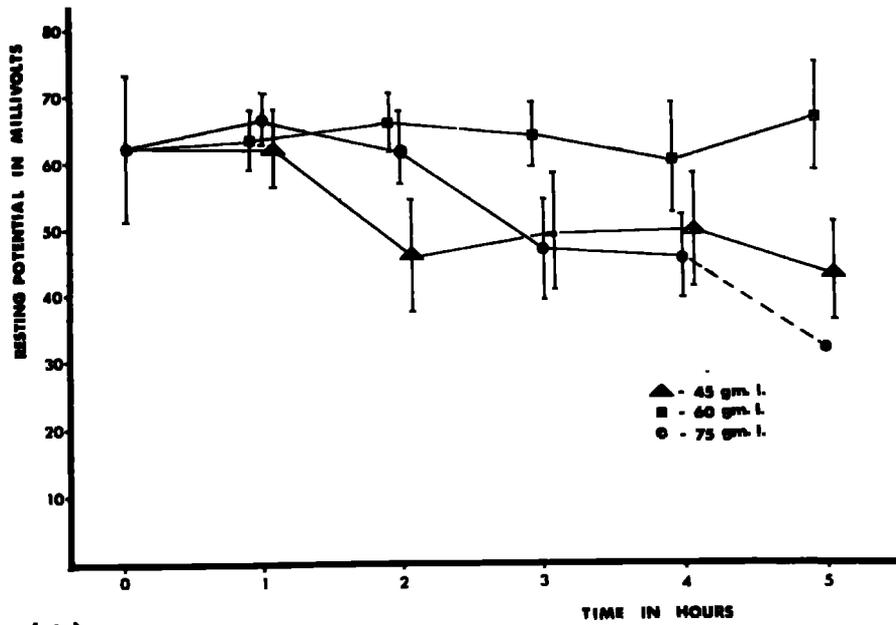
better one), observing the changes with time in the mean resting potential of ten randomly-impaled fibres. The results are tabulated in Table 5, Appendix A, and are shown graphically in Fig.4.

It will be seen from these graphs that a sucrose concentration of the order of 50 - 60 gm/l. is sufficient to maintain resting potential over a period of six hours. The level is not apparently very critical, and this is probably a reflection of the tissue's ability to withstand some variation of osmotic concentration in the living animal. It was decided, fairly arbitrarily, to make up standard saline by dissolving 55 gm sucrose in phosphate-free saline, making the saline quantity up to one litre. This gives an approximately 160mM/l. concentration of sucrose.

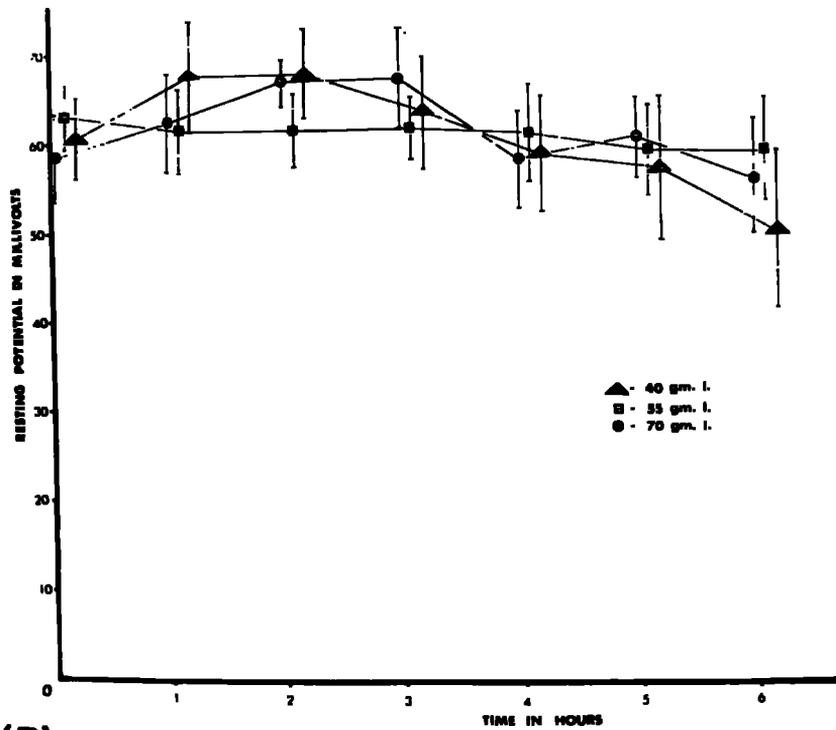
This saline has performed satisfactorily throughout the study, and has on several occasions supported activity and full resting potential for more than 18 hours.

THE EXCISED PREPARATION

It has generally been accepted that insect preparations must be dissected with great care because damage to the tracheal supply greatly affects the muscle fibres. Huddart (1965), for example, states that the activity of various lepidopteran muscles was much reduced by damage of the tracheal supply, and this was often



(A)



(B)

The Effect of Addition of Sucrose to Normal Saline upon Resting Potential.

Figure 4.

accompanied by a fall in resting potential. Several early experiments on dragonfly muscles along the lines of those described in the preceding section of this chapter suggested that the leg muscles of dragonflies were indifferent to severing of the main tracheal supply to the leg. This may reflect the primitive nature of the tracheation. It was decided, therefore, to develop and test a fully excised preparation of a single functional unit of leg musculature.

Like the legs of most other insects, those of dragonflies are dominated by two segments which between them produce all the muscular power of the leg - the coxa and the femur. Although the leg is multiply jointed and segmented, the joints of these two segments with their distal neighbours (the first trochanter and the tibia respectively) correspond in function to the thigh and knee joints, respectively, of the vertebrate. The other articulations are relatively weak. In dragonflies the femur contains much the longest muscle fibres (up to one centimetre long) but the coxa contains many more fibres. In the coxa the muscles are divided functionally into two groups - those which depress the trochanter and consequently raise the body, and those which are levators of the trochanter. The largest muscle, occupying most of the ventral half of the coxa, is the depressor, and it is this muscle from which the

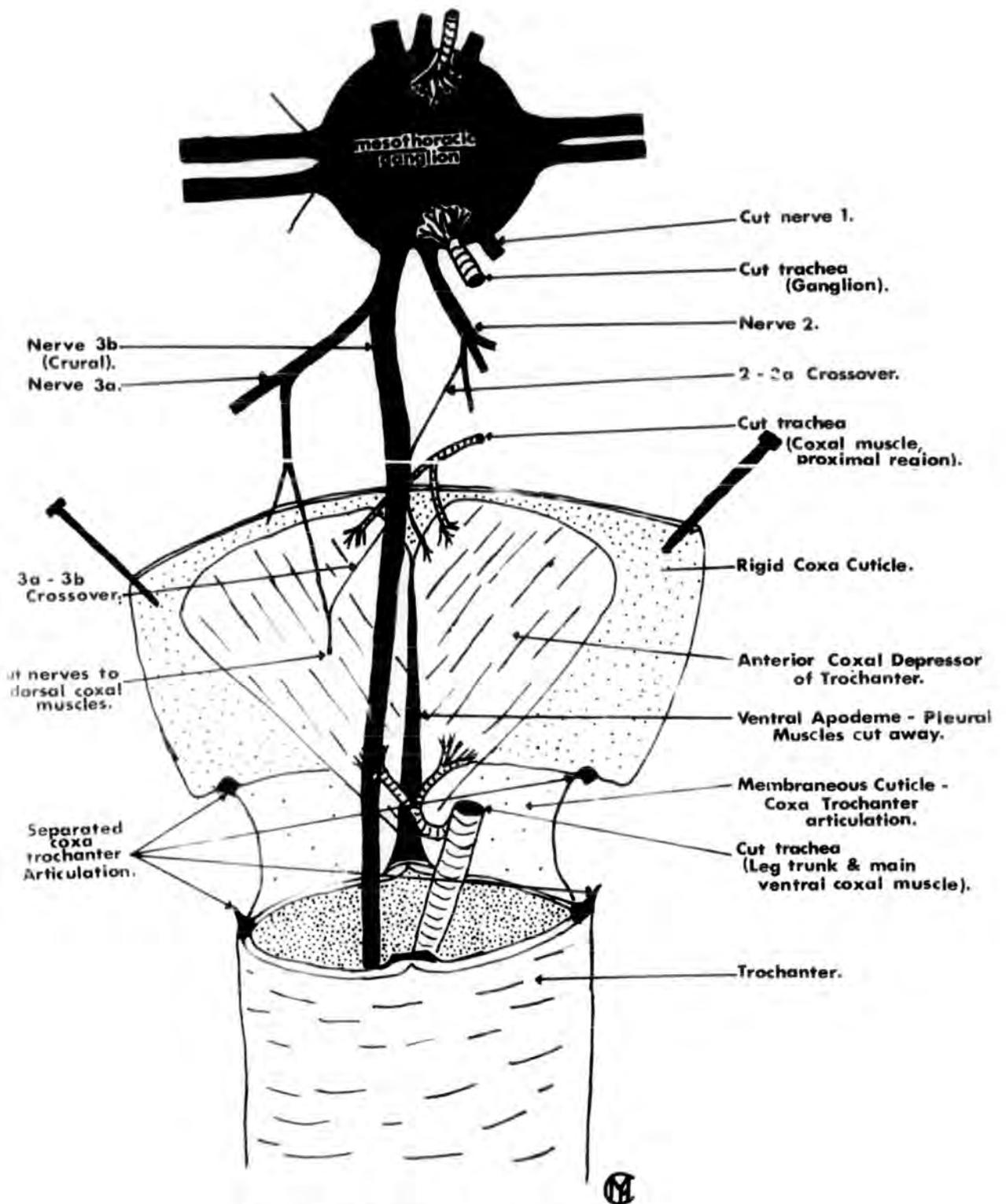
excised preparation was developed, and upon which the majority of the work here reported has been carried out.

The preparation is shown as it appears under the dissecting microscope, after completion, in Fig.5. It is obtained from the whole animal as follows :-

The animal is attached with "plasticene", ventral side uppermost, to the wax bed of a "perspex" bath with its legs well splayed out, and the mesothoracic legs particularly well immobilised. The animal is covered with saline; the ventral articulation between mesothoracic coxa and thorax is freed with a scalpel point, and a large piece of the sternum, bounded laterally by the meeting with the coxa, is cut away, leaving intact proximal to the mid-line the apodeme into which the sterno-coxal musculature is inserted (see Fig.2 for nomenclature). This musculature is cut away from the ventral margin of the coxa, and the crural nerve can now be seen. A further strip of cuticle is removed from the sternum, revealing the ganglion; care has to be taken to disentangle the crural nerve from the remains of the sterno-coxal muscles. When the ganglion is revealed all nerves from it (except the relevant crural nerve) can be cut. As little strain as possible must be placed on the crural nerve; this is best achieved by cutting the other nerves in the order :- segmental nerves

FIGURE 5.

Excised preparation of the Coxal Depressors of Trochanter
muscle. Diagrammatic.



Excised Preparation of
the Coxal Depressors of Trochanter

1 & 2 ipsilateral, 1, 2 & 3 contralateral, anterior interganglionic commissure, posterior interganglionic commissure, unpaired dorsal nerves. At the same time nerve 3b can be cut off near its origin from nerve 3. The whole leg is now freed from the "plasticene" by which it is pinned to the wax bed, pulled gently away from the animal, and all tracheal trunks and remaining muscle connections cut, together with the articular membrane between coxa and thorax and the dorsal articulation. The isolated leg is placed in the experimental bath (described below) and fixed by passing two pins through the femur, holding the leg with its still-attached ganglion, ventral side down to the wax bed. A piece of the coxal cuticle, representing about a third of the whole cylinder (or, more strictly, truncated cone) which forms the exoskeleton of the coxa, is cut away, as is the levator apodeme at its meeting with the trochanter; these two excisions remove the majority of the levator musculature. The depressor musculature is now revealed as appears in Fig.5. The two lateral articulations are now cut and the remaining cuticle flattened down to the wax bed and pinned.

A similar preparation was made of the flexor tibialis muscle of the femur; the complete isolated leg was pinned ventral side uppermost by the tibia, the nerve dissected free from the coxa and trochanters, and the femur

opened simply by slitting the ventral surface and pinning back; the very large pinnate flexor tibialis muscle, which occupies the great majority of the space in the tibia, is thus revealed.

Both of these preparations were adaptable for use with the mechano-electronic transducer, described below, when required. The articular membrane between the coxa (or femur) and the trochanter (or tibia) was cut away, together with most of the distal segment, and the pin of the transducer was pushed through either the remains of the segment or the apodeme (see, e.g., Fig.6).

Neither of these preparations showed any reaction to the severance of the tracheal supply. This may be related to the normal respiratory habit of the living animal which respire through rectal gills and exchanges oxygen and carbon dioxide as solutes in the aquatic medium; the tracheal system of the (aquatic) nymph is, however, fully formed and gas-filled even in the earliest instars, so internal distribution of respiratory gases may be in the gas phase, not in the solution. Consequently the tissues may well be adapted to a low oxygen-tension environment, and the result of excision and immersion in a saline bath with a large gas-exchange surface no handicap.

At the time that this preparation was developed it was apparently unique among insect preparations in

withstanding tracheal isolation, but Hoyle has recently (1966a) reported an isolated preparation of locust coxa. It would seem likely, therefore, that with care isolated preparations can be made from many insects; perhaps the more advanced endopterygotes will prove the most difficult. None-the-less, dragonflies seem to be extremely easy to study in isolated preparations; in no experiment has any side effect attributable to tracheal damage or to oxygen-lack been observed.

OBSERVATION AND DISPLAY OF MECHANICAL AND ELECTRICAL RESPONSES

Organisation of the basic apparatus, all or part of which was used in each group of experiments, is illustrated in Fig.6. The preparation was placed in a saline bath constructed from "Perspex"; the bath was divided by a partition into a large experimental chamber and a smaller sump, into which excess saline from the experimental chamber could overflow and then escape via a drain tube in the bottom of the sump. This bath is illustrated in Fig.7. The experimental chamber contained a thick black wax bed, into which pins could be inserted; the black coloration improved reflected-light visibility by reducing the background glare. Into the wax bed of the chamber were inserted three silver/silver chloride electrodes which were connected to wander-plug sockets in the side of the

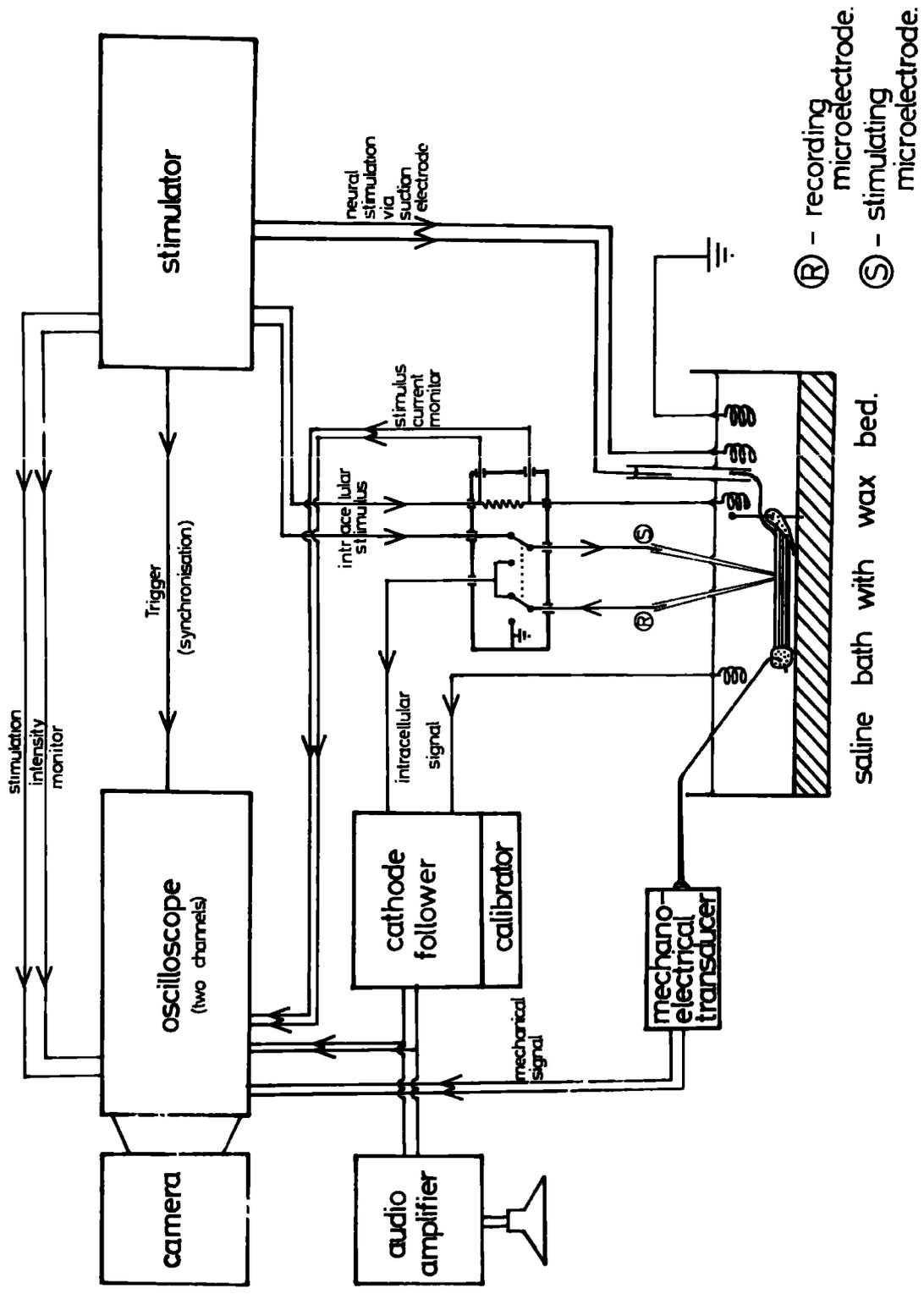
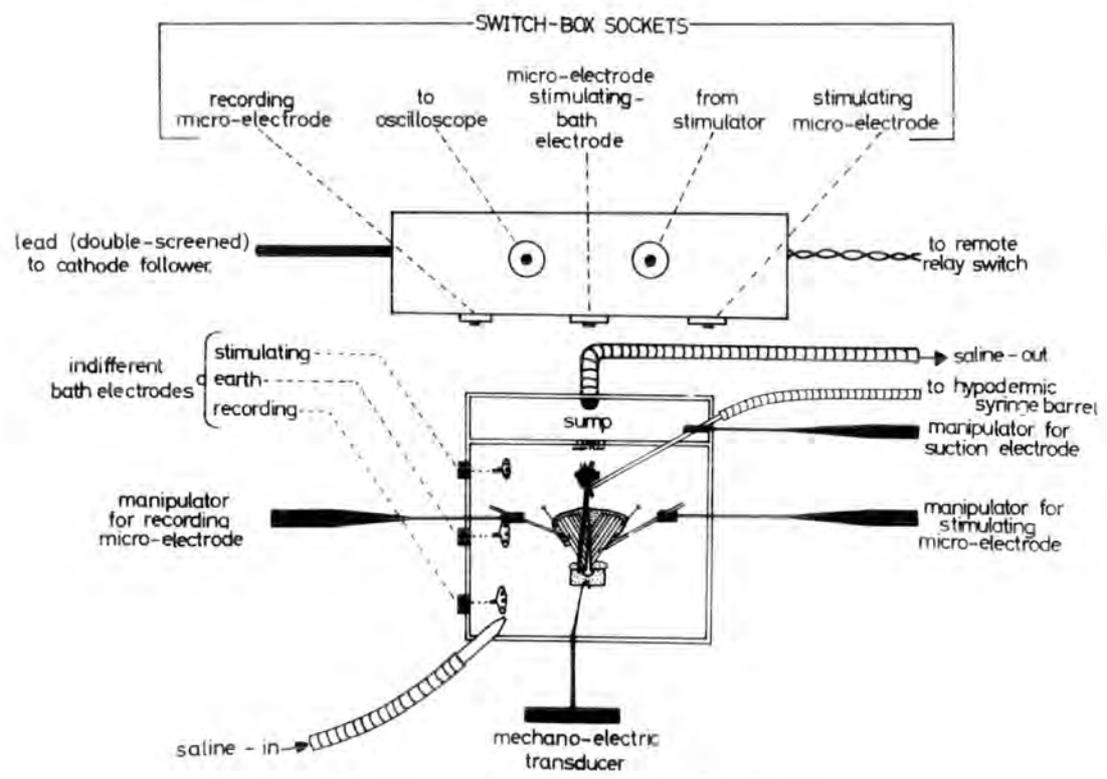
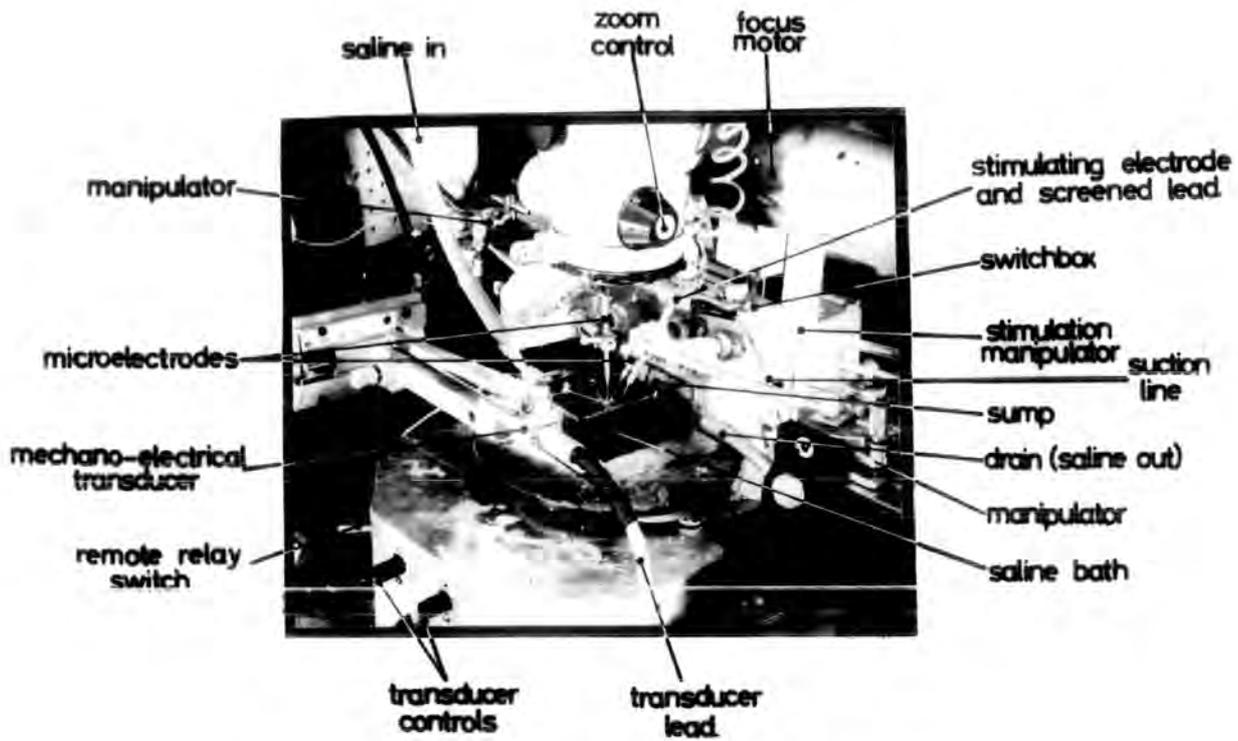


Figure 6.

THE ORGANISATION OF THE APPARATUS

FIGURE 7.

The experimental chamber and the arrangement of the apparatus grouped around it.



The Arrangement of the Apparatus.

chamber. These acted as indifferent bath electrodes for several purposes in different experiments. Saline was added, rapidly if necessary, to the experimental chamber via a tube, the end of which was placed beneath the surface of the bath saline; excess saline flowed into the sump and away down the drain without altering the depth of the bath saline. The consequence of these precautions was that the focus of the observing microscope was not affected throughout saline changes.

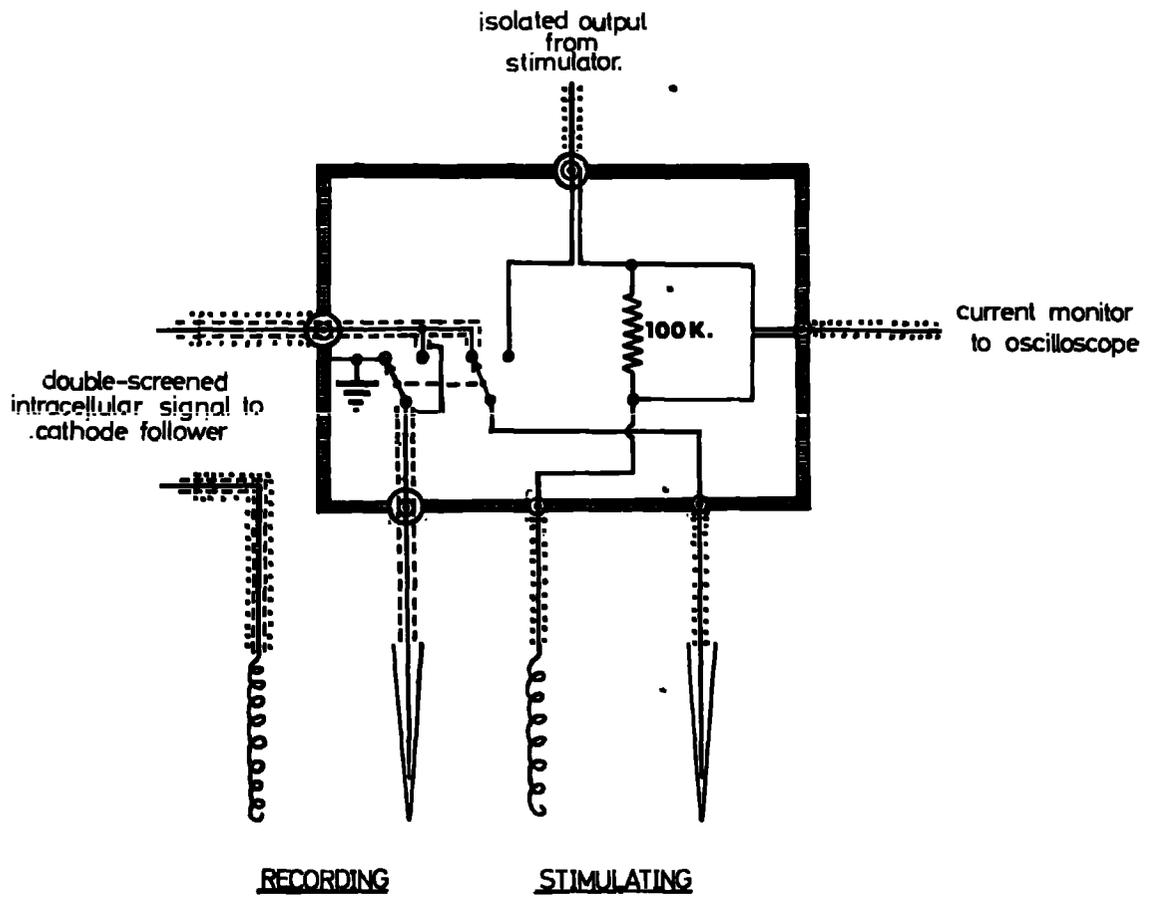
The preparation was pinned to the wax bed at its proximal end with stainless-steel 1" insect sprigs, as shown in Figs. 5 & 7; when mechanical responses were to be recorded, the distal neighbouring segment was cut down in size and fixed to the needle of the mechano-electronic transducer (see Figs. 6 & 7).

The transducer was based on the R.C.A. 5734 valve, the anode of which is mechanically linked to an external pin; this pin is free to move within narrow limits, and causes a change in the conductance characteristics of the valve; in a suitable circuit, this can be observed as a change in anode potential. The circuit used was that of Talbot, Lillenthal, Beser & Reynolds (1951), and the valve was placed in an assembly built from drawings kindly supplied to Dr. D.W. Wood by Dr. E. Bulbring, F.R.S., the design of which was based on that of Talbot et al (1951). This

assembly acted as a mechanical link between the preparation and the valve pin, and also acted as a mechanical sensitivity attenuator. The transducer mechanism was so adjusted that responses could be read to an accuracy of about $2\frac{1}{2}$ mgm, the limiting factors being drift and the "noise" produced by seismographic response to vibrations of the surrounding apparatus, bench etc.

Electrical activity of the preparation was monitored via glass microelectrodes, a full description of which is given below. The electrodes were manipulated by means of a Zeiss Sliding-plate Micromanipulator, and were clamped by means of a brass chuck and nylon grub-screw into the needle holders of the manipulator. The electrodes were connected to a switch-box in the way detailed in Fig.7; the circuit of the switch-box appears in Fig.8. ~~The function of this switch-box appears in Fig.8.~~ The function of this switch-box was to switch either of two micro-electrodes into the input of the display apparatus, and to use one of the two electrodes as a current-passing, direct membrane stimulator; the current is monitored as a voltage drop across the 100K resistor. The use of this switch-box is detailed in Chapters 3 & 6.

From the switch-box, the intracellular signal was taken through double-screened cable to the cathode follower input. The purpose of the cathode follower is to match the



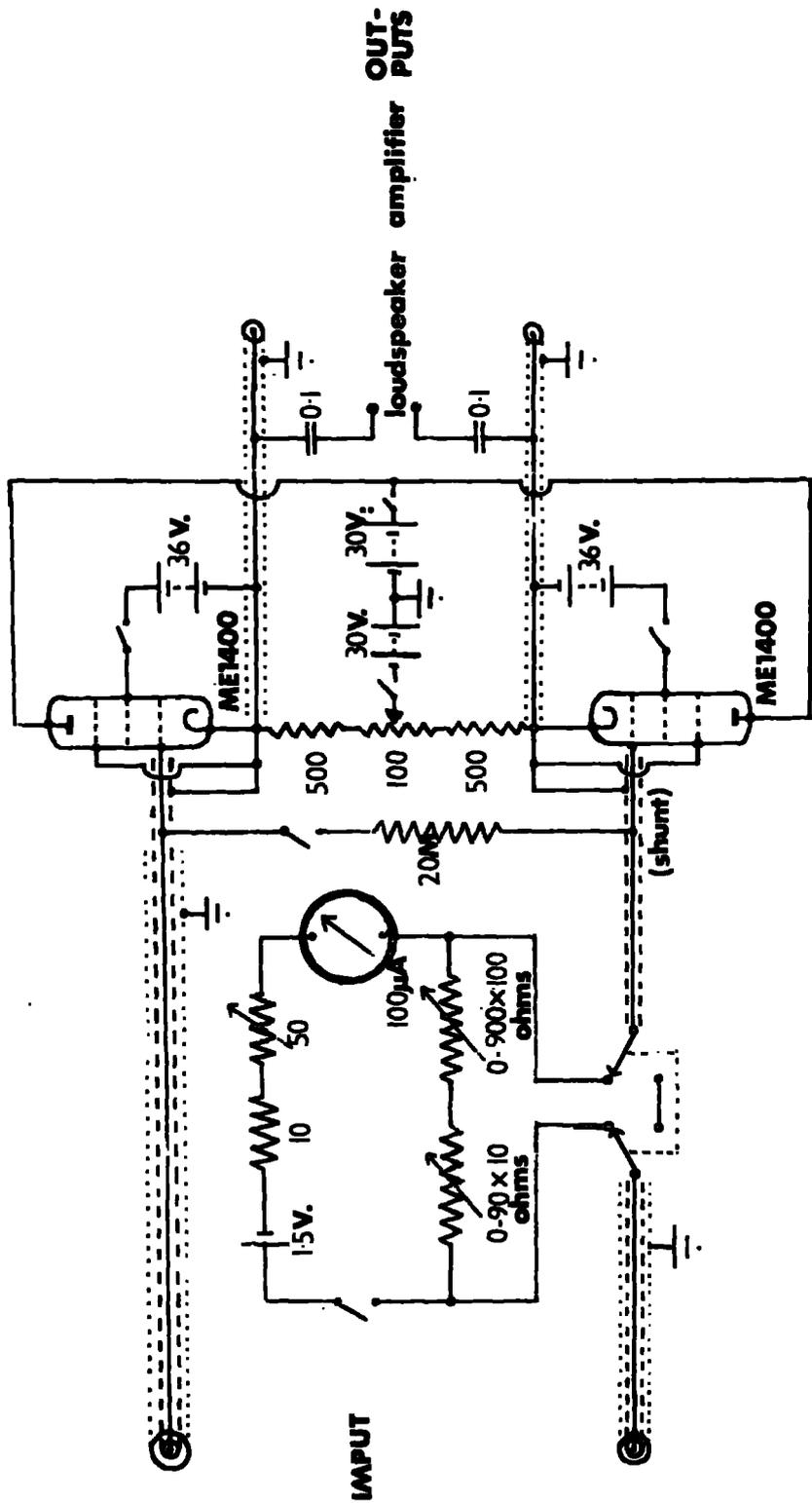
THE ARRANGEMENT OF THE SWITCHBOX

The two-pole change-over switch is remotely operated by a 6-volt D.C. relay

Figure 8.

high electrical impedance of the electrode. It was constructed from the circuit of Bishop (1949), but using Mullard ME1400 electrometer valves instead of the 954 acorn valves used by Bishop. Into the inputs of this cathode follower was incorporated a 20 megohm shunt, the use of which is described below; also included was a simple calibration unit based on Ohm's Law, comprising a battery, setting, potentiometer, ammeter and decade resistor boxes. When the current flow was set at $100\mu\text{A}$, switches of 10 and 100 ohms in the resistor boxes produced d.c. changes of 1 and 10mV respectively in the cathode follower input. This enabled the display oscilloscope to be recalibrated, a step made necessary by the negative amplification (about 0.97) produced by the cathode follower. The complete circuit of the cathode follower and calibrator appears in Fig.9.

From the cathode follower, the signal was taken to the input of one of the plug-in vertical amplifiers, type CD1442 (high-gain differential), in a Solartron CD1400 system oscilloscope; the screen display produced was photographed with a Shackman oscilloscope camera, type AC2/25, using Ilford 5G91 trace film. The signal from the cathode follower was also lead to a simple loud-speaker amplifier (Donaldson, 1958); auditory monitoring of electrode sensitivity, particularly during penetration of a cell, was often helpful, and the sound of a square pulse injected into the



cathode follower and calibrator

After BISHOP, 1949

resistance in kilohms, capacity in microfarads.

Figure 9.

micro-electrode became, with experience, a very good rapid test of an electrode's quality.

Because the camera, in position, obstructed free vision of the oscilloscope screen, signals tapped from the outputs of the oscilloscope vertical amplifiers were fed, via the D.C. potential adaptor shown in Fig.10 to the input of a Cossor 1949 oscilloscope; this provided a monitor of the events displayed on the Solartron screen. This rather unwieldy and unsatisfactory arrangement was made necessary by the lack of provision by the manufacturer of "slave" facilities for the CD1400 series oscilloscopes.

The preparation was spot-illuminated and viewed with a Zeiss stereo microscope; the use of x10 and x25 eye-pieces, combined with the zoom objective of the microscope, gave a viewing magnification continuously variable between x10 and x100, adequate for all purposes, including initial dissection.

The complete apparatus, including the stimulation units described below, is shown in Fig.11. The microscope and experimental bath are at lower centre of the photograph; the controls below the microscope stage are those of the mechano-electronic transducer except the right-hand switch which is the switch-box relay operator. The transducer itself is in front of the microscope stage, and it is manipulated by means of the Palmer rack-work on the left of

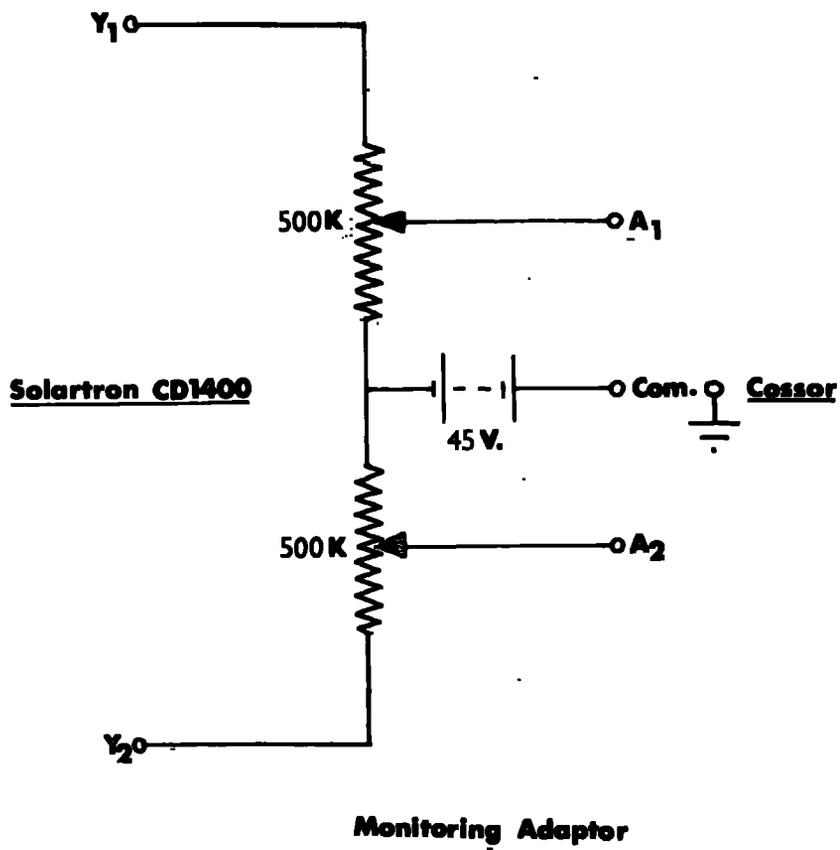
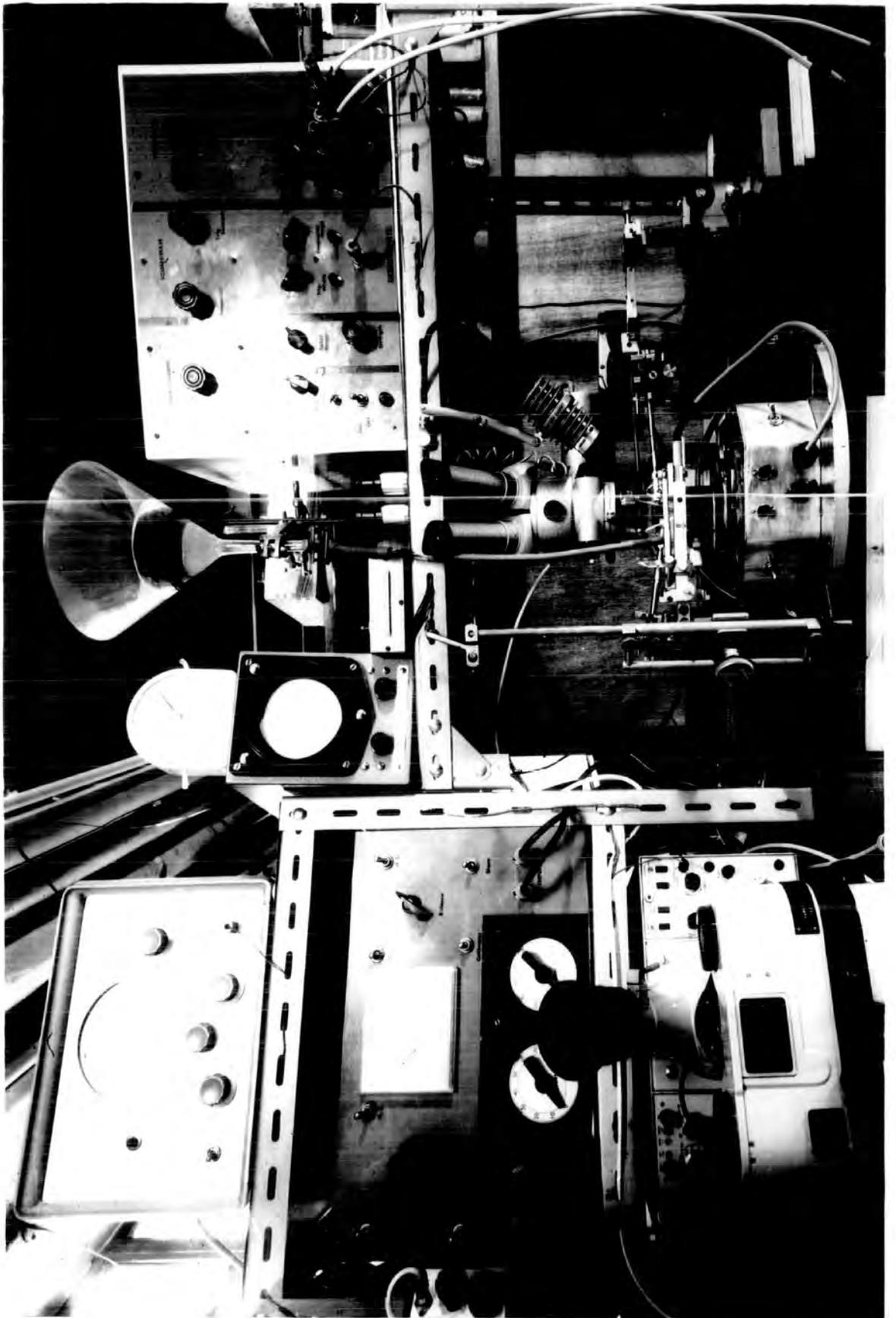


Figure 10.

FIGURE 11.

A. general view of the apparatus used in the investigations.

For a full description, see text.



the stage. At lower right is the oscilloscope and camera; next above is the cathode follower, and at top right is an Advance signal generator type HI, used for time calibration. Top centre are the monitoring screen, and a large funnel which acts as a saline reservoir and header tank and is joined by tubing to the experimental chamber. Top right is the stimulator; the white cables at its lower right-hand corner are the operating switches, mounted on cables to decouple them mechanically from the preparation. The loudspeaker amplifier is concealed, but its controlling knob is at extreme left, centre.

THE GLASS MICRO-ELECTRODE.

Micro-electrodes were first made and employed by Peterfi (1925), but these were very crude and gave poor results; the technique was taken over and refined by a group of American workers, who developed both practical electrodes and the necessary associated electronic circuitry; the refined techniques were initially published in 1946 (Graham & Gerard) and most modern workers have based their technique on that of Ling & Gerard (1949).

The micro-electrode is a fine-tipped pipette, filled with an electrolyte solution which serves to make electrical contact with the interior of the cell and to reduce the trans-tip membrane. Typical micro-electrodes,

of tip diameter less than 0.5μ , have d.c. resistances greater than 10 megohms. Resistances of greater than 20 megohms are difficult to use because of air-borne electrical interference, the amplitude of which is proportional to circuit resistance. On the other hand, it is evident that the smaller the tip diameter (and thus the greater the resistance), the less damage and disturbance is incurred in inserting the electrode through the membrane of a living cell. In general, a tip size of 0.5μ , having a d.c. resistance of 5 - 15 megohms, is a good compromise. Katz (1966) discusses the theory of micro-electrode recording at some length.

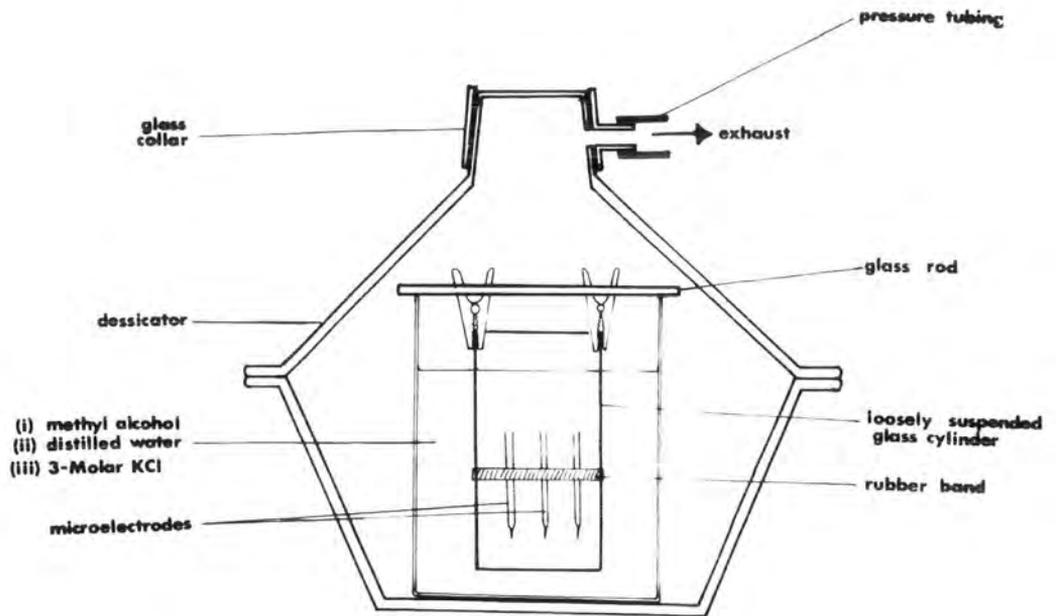
The electrolyte used to fill the electrode originally was 3-M.KCl, and this remains the most common electrolyte still. There are, however, certain problems involved in this type of recording as a consequence of electrolyte leakage by diffusion from the electrode tip. Because of this, many workers have experimented with alternative electrolytes, such as solutions of potassium sulphate or potassium citrate (e.g. J.C. Eccles & co-workers), or with additives to a basic KCl solution (e.g. Agin & Holtzman (1966)). Diffusion from the tip leads to d.c. potentials being developed across the tip, due to different diffusion velocities of different ions; the value of these potentials is a function of external ionic concentration. Changes in

concentrations of ions around the tip such as are involved in the insertion of the tip into a cell thus lead to spurious changes in addition to real ones; and d.c. values are therefore to some extent inaccurate and distorted. This subject is discussed at some length by Katz (1966), by Coombs, Eccles & Fatt (1955) and by Adrian (1956), among many others.

The techniques used in this study were as follows :-

Micro-electrodes of tip diameter circa 0.5μ were pulled from 2mm "Pyrex" glass on a Palmer Electrode Puller, type H 101. They were filled by initial immersion under reduced pressure in methanol; the pressure was reduced sufficiently to bring about boiling of the methanol. The methanol was replaced by distilled water at normal pressure to avoid KCl precipitation and the water in turn replaced by 3-M.KCl under reduced pressure. KCl replacement must be performed under low pressure in order to remove dissolved atmospheric gases from the solution. This method of filling is based on that of Tasaki, Polley & Orego (1954). The apparatus used for filling is shown in Fig.12.

The electrode was coupled via a silver/silver chloride liquid/metal junction to the cathode follower and calibrator unit previously described, together with a second silver/silver chloride electrode acting as indifferent bath electrode. A rough indication of the tip (junction)



Electrode Filling.

Figure 12.

potential (see above) was obtained by comparison of the d.c. level of the oscilloscope trace produced by direct contact between the electrode connecting silver wire and the bath, and with the micro-electrode included; electrodes with tip potentials greater than 10mV, or with unstable tip potentials were rejected.

In the early part of the project each electrode was tested before use by means of an injected square pulse, using the 20 megohm shunt incorporated in the cathode follower. Electrodes were rejected unless :-

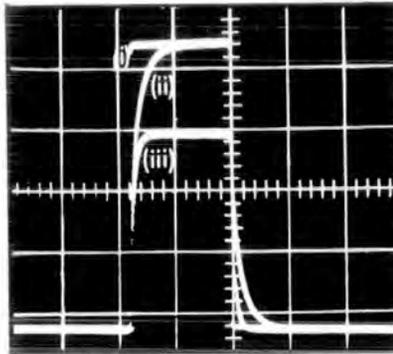
(a) the square pulse was transmitted without loss of amplitude, and with a minimum of distortion (see Fig.13a).

Gross distortion is a product of a blocked electrode tip or imperfect filling, but a little distortion in the form of slow rise and decay of the square wave is inevitable; this is a consequence of the high electrode impedance being combined with some inevitable stray capacitance in the connecting wires. (See Fig.13b).

(b) The d.c. impedance was greater than 5 - 7 megohms. This was tested with the shunt resistor by the method of Donaldson in which the inclusion of a shunt of known resistance reduces the size of an injected square wave by a proportion which is a function of electrode resistance (Fig.13c).

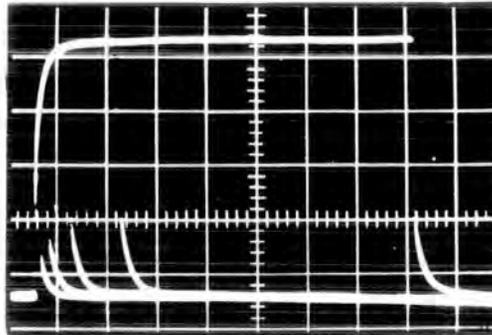
FIGURE 13.

Characteristics of glass micro-electrodes.



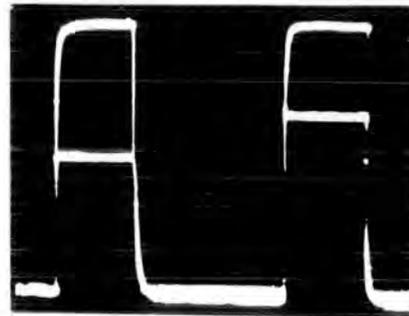
THE ELECTRODE PERFORMANCE

- a
- (i) Electrode shorted out.
 - (ii) Electrode in.
 - (iii) Shunted electrode.



b ELECTRODE RESPONSE

Pulses of duration 0.1, 0.2, 0.7, 1.75 & 10 msec.



c 20 M. RESISTOR 10 M.
SHUNT IN
OUT

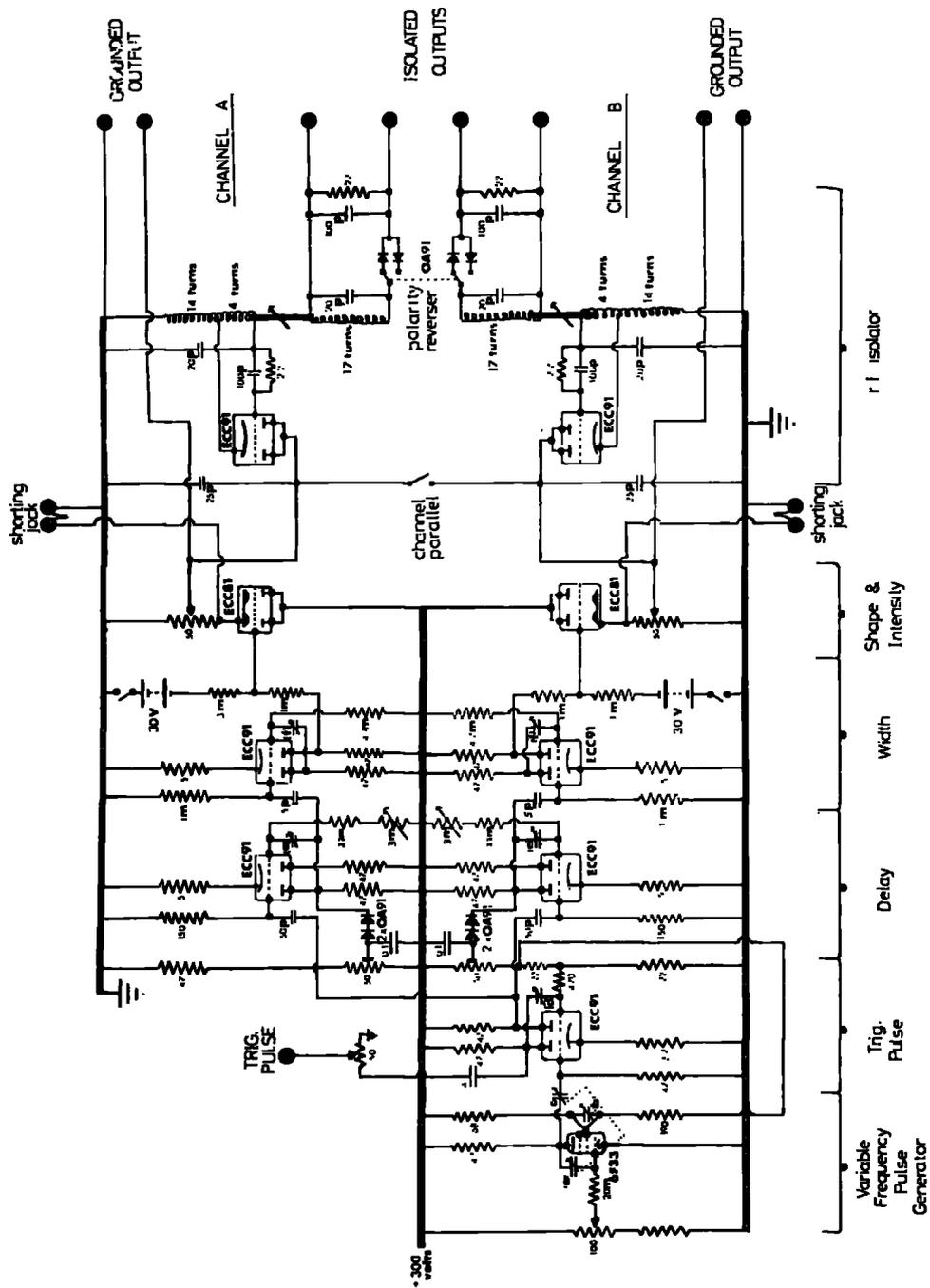
Electrode Characteristics.

With experience it was generally found possible to gauge the quality of the electrode without exhaustive testing; one good test was to listen to an injected square wave with the audio amplifier - a sharp click indicated a poor or broken electrode which allowed through the square wave almost undistorted, a plop indicated a good electrode, an indistinct noise indicated that the electrode was not transmitting well. It was found to be quicker, in the long run, to adopt such measures as these, together with a cursory examination of the tip by microscope after pulling, than to test at every stage. As a result, two out of five filled electrodes were rejected just before they would have been used.

The electrode circuits used, and the experimental procedure adopted, were basically those of Fatt & Katz (1951).

STIMULATION TECHNIQUES

A stimulator of complex design was specially constructed. The circuit diagram is given in Fig.14; the pulse generating section of this is that of Donaldson (1958), except that it was found necessary to change the output stage and the values of some resistors; the R.F. isolation stage is based on a design of Schmidt & Dubbert (1949). Facilities were incorporated in the stimulator to reverse the polarity of the square-wave which is generated,



Dual Channel Square-Pulse Stimulator and R.F. Isolator

After Donaldson (1958) & Schmidt & Dubbert (1949)

Resistance in Kilohms; Capacity in Microfarads (unless otherwise stated)

Switched Capacity Values:- (a) 01, 001, 0001, 100p. (b) 01, 001, 0001, 300p. (c) 001, 0001, 100p. 22p. (d)(e) 1, 01, 001, 0001 (f) 01, 001, 0001, 0003, 0001, 330, 100p

Figure 14.

to operate the output via remote switches through the shorting jacks (giving a mechanical decoupling from the apparatus, to reduce vibration effects), and to parallel the channels (enabling pairs of stimuli to be produced by differential setting of the delay controls).

The stimulator operates at frequencies from 750Hz to one stimulus every 32secs; pulses are from 0 - 25V, pulse width up to 55msec; pairs of stimuli can be generated at intervals of up to 180msec, and this is also the maximum delay available between production of the trigger pulse (which is used to synchronise the sweep of the oscilloscope with events caused by stimulation) and production of the stimulus. Positively-polarised square pulses, referred to earth, are also produced, at up to 60V.

Two types of electrical stimulation are available to the neuro-muscular physiologist - directly via the membrane and indirectly via the nerve. The former technique is used in Chapter 3, where a full description is given. The great majority of experiments, however, involve examination under various conditions of the response evoked in the muscle fibre by stimulation of the innervating nerve trunk.

The classical technique of nerve stimulation has been to pass a square-wave current pulse through the nerve via a pair of metal electrodes, first lifting part of the nerve out of the (conducting) saline medium. Considerable

sophistication of this method has been achieved by way of selective stimulation (see, e.g., Kuffler & Vaughan Williams, 1953a). This method was attempted on whole-animal preparations of dragonflies, with very poor results. It was confirmed that the apparatus was producing a sufficient strength of stimulus by testing it on a preparation of a cockroach (Periplaneta americana), on which the technique is known to work (Wood, 1961). This failure was later attributed to the presence around the crural nerve of a thick and poorly-conducting sheath.

The isolated preparation was, in any case, innervated by so short a length of nerve as to make this technique practically impossible, even using the refined method of passing the current between a single electrode onto which the nerve was hooked and an indifferent bath electrode. A technique was developed using the single electrode/bath electrode method in which the ganglion was pinned, holding the nerve fairly taut, and a drop of paraffin oil placed on the ganglion (which was under the surface of the saline bath). The single metal electrode - made either of silver/silver chloride or tungsten wire and insulated, except at the tip, with varnish - was then placed with its tip in contact with the ganglion, or with a part of the crural nerve inside the oil-drop. When a

stimulus was applied, current surged between the oil-encased ganglion and the bath by the only route open to it - the wet surface of the crural nerve.

This technique worked adequately, but the short length of the crural nerve made the adding of the oil-drop slow and difficult. A much more sophisticated technique, which is none-the-less much easier to use, was therefore developed using suction electrodes.

Invented many years ago, the suction electrode technique is now widely used for detection of nerve impulses, superseding wire external electrodes (e.g. Easton, 1960; Strickholm, 1961); Florey & Kriebel (1966) suggested their use as stimulating electrodes. Basically, a quantity of bathing fluid is drawn into the end of a pipette in which it makes contact with an electrode; by production of a pressure gradient from outside to inside of the pipette the tissue under experiment is drawn against the opening of the tip and held there, and extracellular electrical signals can be detected by an electrode in the pipette, with reference to an outside indifferent electrode.

Using a similar system, but applying a square wave to the electrode inside the pipette, gives a very convenient stimulating system, delivering a concentrated and closely directed current pulse at the pipette tip. Experiments have shown that threshold stimuli are created

in the region of the required axon by suction electrodes at much lower outputs of power from the stimulator than is required when stimuli are delivered conventionally. There is some complication, consequential to the presence of the thick, poorly-conducting sheath around the crural nerve. It has been found by experiment that maximum stimulation efficiency is not obtained when the nerve fits very tightly around the electrode tip, but when it is sufficiently loose to permit some current surge in the saline around it. This is, of course, the exact reversal of optimum condition for extra-cellular recording, where the tighter the fit, the better the results obtained (Florey & Kriebel, 1966).

Stimulating suction electrodes were manufactured for this study by drawing out 2mm "Pyrex" glass tubing to tips of suitable diameter; the tips were fire-polished with a hot-wire forge to remove sharp edges (a refinement which is not essential for stimulation). A silver/silver chloride wire was placed between the end of the glass tube and the length of rubber tubing into which this end was inserted; this joint was sealed with paraffin-wax. The other end of the tubing was connected to the barrel of a 10ml hypodermic syringe, which was used to create the necessary pressure gradients.

The suction electrode was mounted on a Prior

manipulator, the silver wire connected to the stimulator,
and one of the experimental bath contacts used as an
indifferent electrode.

CHAPTER 3

THE PROPERTIES OF THE MUSCLE FIBRE MEMBRANE.

CHAPTER 3THE PROPERTIES OF THE MUSCLE FIBRE MEMBRANEINTRODUCTION

Nerve and muscle tissues depend for their action on the properties of excitable membranes. In each of these tissues the membrane is in a state of dynamic equilibrium in relation to the ion contents of the cytoplasm on one side of the membrane, and of the extra-cellular fluid on the other. In the process of excitation this equilibrium is momentarily upset but soon returns to its original balance.

The classical formulation of the ionic theory of nerve and muscle action is due to Hodgkin (1951, 1958, 1964). In the 1958 paper Hodgkin suggests that the dynamic balance is brought about by two distinct systems; one of these brings about concentration differences by absorbing potassium ions into the cell and ejecting sodium ions; the other allows these two ions to diffuse at varying rates down their concentration gradients. The excitable membrane is thus permeable to a varying and controllable extent to both these ions, but at rest will transport them up concentration gradients. It is the second system that is affected by any processes of excitation; ion permeabilities increase momentarily, and as a result equilibrium concentration values are temporarily changed.

This theory was erected, and has evolved, on evidence derived mainly from experiments on various types of nerve fibre; its principles seem to apply to any known excitable membrane, however, but the details may differ; other ions may be involved in the active secretion of diffusion systems, for example (Keynes, 1962; Huddart, 1967). A large proportion of investigated tissues, particularly vertebrate tissues, follow the theory very closely.

In the natural workings of the animal, the excitable membrane is stimulated by one of two methods. The first, stimulation by action of a transmitter substance or neurohumour, will be discussed in Chapter 4. The second method depends on electrotonic spread of charge from one part of the membrane to another.

When a region of the membrane has been activated, the changes in ionic balance, which are reflected in observable changes in electrical charge distribution, affect ionic balance in neighbouring parts of the membrane. These regions may respond to the resulting imbalance by undergoing changes in permeability which further increase the ionic imbalance in that region. This is an active membrane response. In some cases, notably in vertebrate twitch muscle fibres, this response can result in regions becoming fully permeable to the unbalanced ions, producing

a maximal, all-or-nothing response.

Responses of this type, whether amplifying or not, can be investigated by direct electrical stimulation of the membrane. This is achieved by passing current across the membrane between an intra-cellular micro-electrode and the bathing medium, producing a charge build-up.

With very small applied currents stimulation can be so restricted that electrotonic spread of charge does not evoke any active membrane response. It is possible to use such stimulation to determine the values of certain parameters of the membrane. Because the membrane is permeable to charged particles - ions - it gives the appearance of an electrical conductor. Hodgkin & Rushton (1946) have described quantitatively the behaviour of the membrane in electrical terms, showing it to act as a resistor-capacitor network in the conduction of charge, and establishing a theoretical basis for the determination of the membrane parameters, or constants.

This chapter describes the effect of direct stimulation of the muscle fibre membrane, and gives a preliminary indication of the order of magnitude of the parameters of the muscle membrane of dragonflies.

METHODS

Two micro-electrodes, one for passing current, the other for recording evoked responses, were used in conjunction with the switchbox (Fig.8); this contained a two-pole change-over switch, remotely operated by a relay. In the switch position shown in Fig.8, the micro-electrode used for recording is earthed, while the one used for stimulation is switched into the recording circuit inputs. In the other position the recording electrode is switched into the display input and the stimulating micro-electrode is connected directly to the stimulator output. The bath electrode with which the stimulating circuit is completed is connected to the stimulator via a fixed 100K resistor; when current flows during stimulation it passes through this resistor, and the voltage drop associated with this is monitored by a direct-coupled oscilloscope channel. An Ohm's Law substitution allows current flow to be calculated within the accuracy of the resistor value. This method is based on that of Fatt & Katz (1951).

The switch arrangement enables the stimulating micro-electrode to be inserted normally, identifying successful penetration by the appearance of the resting potential. Once the stimulating micro-electrode has been successfully inserted it can be switched from display to

stimulating mode, bringing into the display circuit the recording micro-electrode, which is then used normally to detect responses.

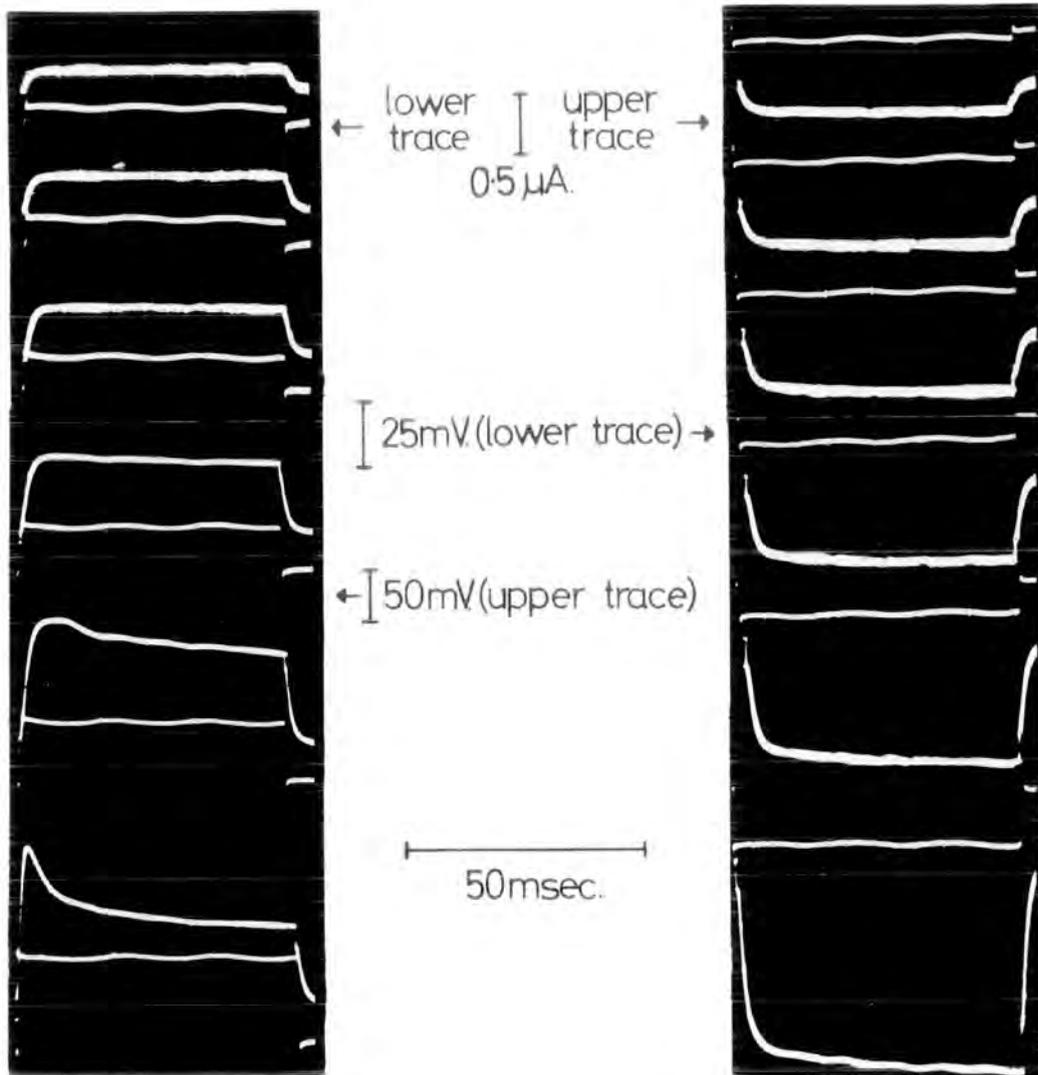
For measurement of membrane parameters the method of Fatt & Katz (1953) was adopted. The stimulating micro-electrode was inserted initially, about a quarter of the fibre length away from one end of the fibre. The recording micro-electrode was then inserted at two different distances from the stimulating electrode along the fibre, the distances being measured with a micrometer eye-piece. At each insertion both depolarising and hyperpolarising pulses were passed through the stimulating electrode and the size and time-course of the response recorded.

RESULTS

The effect of direct stimulation of the membrane is illustrated in Fig.15; in this illustration the traces presented were obtained with the recording micro-electrode inserted within about 75μ of the stimulating micro-electrode. Thus the response recorded here was very close to that evoked at the tip of the stimulating electrode. It will be seen from Fig.15 that the response follows the stimulus very closely except at very high intensity depolarising currents, when a small active response is produced. The response is not maintained, but decays to a plateau level which seems

FIGURE 15.

The responses of the membrane of coxal muscle fibres to directly-applied hyperpolarising and depolarising current pulses.



Depolarising.

Hyperpolarising.

THE DIRECT STIMULATION OF THE MUSCLE MEMBRANE

The recording microelectrode was within 100μ of the current-carrying (stimulating) microelectrode

to represent the passive response component. The threshold level for evocation of the response is very high indeed; in this case the membrane is practically depolarised (the resting potential being about 80mV), and the response represents a considerable overshoot of the zero-potential line. At recording distances greater than 100 μ the active membrane component cannot often be seen, and the size of the observed response is much less; clearly the active response is not propagated, and the passive response decays rapidly away. The time-courses of the rise and decay phases are much expanded.

The stimulator used in this experiment was not of either constant-current or constant-voltage type, so results for hyperpolarisation cannot be easily compared with those for depolarisation. However, the bottom trace of each column in Fig.15 shows the responses evoked by maximum power output of the stimulator. It can be seen that there is less current flow to hyperpolarisation; this presumably reflects higher resistance to outward current flow. The voltage evoked by hyperpolarising current at full power is much less than that evoked by depolarising current, and there is of course no active component.

These results are quantified when two or more penetrations of the same fibre by the recording micro-electrode are used to calculate the membrane constants. These calculations were made using the adaptation of the theory and methods of Hodgkin & Rushton (1946) by Fatt & Katz (1951), who used micro-electrodes instead of external wick electrodes. The method of calculation of the constants from the final equations of Fatt & Katz is set out in Appendix B.

In this study it was not intended to obtain absolute mean values and ranges of the parameters, since this topic was a side-issue to the central objectives. It is clearly valuable, however, to have some comparative knowledge of the membrane characteristics. The following results are therefore only an indication of the probable order of magnitude of the parameters.

The space constant, λ , was determined in one fibre using both hyperpolarising and depolarising pulses, and the values obtained were 0.28mm and 0.30mm respectively; there is therefore clearly no essential difference between the two methods, so long as the current intensity is well sub-threshold. In another fibre λ was 0.53mm.

The effective resistance between inside and out of the fibre, R_e , was found to be 5.7×10^5 ohms using hyperpolarising pulses, and 2.6×10^5 ohms using depolarising

pulses. This would seem to reflect an asymmetry of the membrane permeability, there being reduced impedance to inward current flow, i.e. to outward flow of cations.

The determination of fibre diameter by electrical means, using equation 9 of Appendix B, produced two different results when hyper- and depolarising pulses were used. Optical measurement, although not very accurate, none-the-less showed that the true fibre diameters - in the range 30 - 60 μ , lay between experimentally determined values. It seems clear from this that the Hodgkin-Rushton theoretical treatment involves an effective rather than a real fibre diameter. The different results obtained are probably a consequence of the different permeability of the membrane in the reverse direction.

Using the electrically-determined effective cell diameter, values for the specific membrane resistance of 278 & 214 ohms/sq.cm. were obtained in one fibre for hyperpolarising and depolarising currents respectively; a comparable value from a rather larger cell (53 μ effective diameter, actual value about 50 μ) using a depolarising pulse was 530 ohms/sq.cm.

Only one successful evaluation of the time constant and of the specific capacity was made. The values obtained were 2.5msec and 47 μ F'. Qualitative observations suggested

that the time constant, like the space constant, is independent of direction of current flow in the determining experiment, but that capacity to outward current, like resistance, is somewhat higher. This is in agreement with membrane cable theory, where space and time constants do not depend on transverse "resistances" (permeability channels).

Results obtained in these experiments are tabulated in detail in Table 2.

DISCUSSION

The effect of directly-applied hyperpolarising pulses is exactly the same as has been observed in all other animals - simple following of the applied charge, the only distortions being a consequence of the leaky-capacitor nature of the membrane.

To depolarising pulses, the membrane of dragonfly coxal muscles behaves as a normal, if rather insensitive, electrically excitable invertebrate muscle membrane, which shows no sign of all-or-nothing, self-regenerative behaviour. Similar observations to those reported here have been reported in work on other insects by Cerf, Grundfest, Hoyle & McCann (1959), by del Castillo, Hoyle & Machne (1953) and by Werman, McCann & Grundfest (1961) in various locusts, and by Usherwood (1962) in locust and cockroach. In crustaceans,

TABLE 2

The Detailed Results of Membrane Constant Determination

	<u>EQUATION</u>	<u>FIBRE 1</u>	<u>FIBRE 1</u>	<u>FIBRE 2</u>
Current pulse polarity	-	H.	D	D
Space constant (λ). mm.	7	0.28	0.30	0.53
Effective resistance between inside and out (R_e). ohms.	8	5.7×10^5	2.6×10^5	2.3×10^5
Longitudinal resistance of the fibre/unit length (r_l). Ohms/cm.	10	4.07×10^7	1.73×10^7	8.7×10^6
Effective fibre diameter (d). μ .	9	28	42	53
Specific resistance of the membrane (R_m). ohms/sq.cm.	11	278	214	530
Time constant (τ). msec.	12	-	-	2.5
Specific capacity of the membrane (C_m). μF /sq.cm.	12	-	-	47

Equation numbers refer to the relevant parts of Appendix B.

H - Hyperpolarising pulse applied.

D - Depolarising pulse applied.

Fatt & Katz (1953), Atwood (1963a&b), and Dorai Raj (1964) have all observed similar phenomena. Dragonfly coxal muscle fibres thus seem to be quite normal in their response to direct stimulation, as compared with other arthropods.

The values obtained for membrane constants are also comparable to others, although those of R_m (effective membrane resistance) and of R_i (which is a best estimate) are a little higher than the only other known arthropod figures, in crustaceans (Fatt & Katz, 1951). The only exception to this is the value obtained for the length constant, λ , which is considerably shorter than found elsewhere. The observed values indicate a likely average size of 0.4mm, probably with a variation of \pm 0.2mm (this estimate is based on several qualitative observations as well as the quantitative ones reported above). Fatt & Katz (1953) obtained values for λ of 0.95mm - 1.3mm in Carcinus and 0.6 - 0.83mm in Portunus (both decapod crustaceans); Usherwood (1962a) reports values of 1.6 - 2.0mm in Romalea microptera and 1.0 - 1.1mm in Schistocerca (both orthopteran insects). The length constant is therefore considerably shorter than the average value of most arthropods, and is outside the normal (very variable) range. It is, however, similar in magnitude to the space constant of locust wing muscles (Hagiwara & Watanabe, 1954), which are, of course, somewhat specialised

and not perhaps comparable with skeletal muscle fibres.

In all other measured parameters the observed values are within the range of the figures for various crustaceans given by Fatt & Katz (1953). Little two-electrode work has been carried out on insect material, but those few results reported are of the same order of magnitude (Usherwood, 1962a). The very short space constant is probably, therefore, a consequence of the relatively small diameter of the fibres, to which it is directly related (see equation 9, Appendix B).

It is interesting to briefly compare these values with the comparable ones obtained on vertebrate material, e.g. frog sartorius muscle fibres (Fatt & Katz, 1951). The differences are reflected in the values of specific resistance and capacity; vertebrate twitch muscle fibres have resistances up to two orders of magnitude greater than the figures for dragonfly reported here, the range of resistances of frog sartorius fibres being 3000 - 4000 ohms/sq.cm; capacities, at 8 - 10 μ F/sq.cm, are almost an order of magnitude smaller. Clearly the invertebrate muscle fibre membrane is much leakier to passage of ions; the increased capacity may indicate a much larger region of ion (charge) store on or within the membrane, or it may be explained in terms of complex membrane shape increasing the effective area of the dielectric layer.

CHAPTER 4

THE NORMAL PATTERN OF MUSCLE RESPONSE

CHAPTER 4

THE NORMAL PATTERN OF MUSCLE RESPONSE

INTRODUCTION

The ionic theory of the action of excitable membranes has been set out in the preceding chapter. Activation of the membrane in the normal animal is achieved over the surface of each nerve or muscle cell by the mechanisms already described. Activation of one cell by another is achieved through synaptic transmission in which the activating, or pre-synaptic, cell secretes a neurochemical agent, the transmitter substance, which in turn activates the post-synaptic cell (Eccles, 1964).

Muscle cells are activated by the nerve cell axons which innervate and control them via a special type of synapse, the neuromuscular junction. Terminal processes of the axon come into close apposition with a specialised area of the muscle cell membrane; the structure of this synaptic region, the end-plate, is of greatly varying complexity in different animals.

Vertebrate twitch muscle fibres usually bear one or two end-plate only. Even in these cases, however, there is no suggestion of control being effected by more than one motor nerve fibre. Vertebrate slow muscle fibres also receive only one axon, but this axon may terminate in many

end-plates on each fibre (Bach-y-Rita & Ito, 1966). In invertebrates, each muscle cell receives several end-plates from each of one or more axons, of which there may be as many as five.

When the brief nerve impulse reaches the motor axon end-plate terminals it initiates the release of a quantity of transmitter substance which diffuses across the synaptic cleft (the gap between pre- and post-synaptic membranes). Katz & Miledi (1965) have demonstrated that acetylcholine (the transmitter substance in many vertebrate synaptic systems) is released from the presynaptic membrane of frog sartorius muscle junctions between 0.25 and 1msec after arrival of the nerve impulse, and that the post-synaptic membrane activation process is maximal within 1.5msec of impulse arrival, delay by diffusion across the synaptic cleft being of the order of 1msec. Delays from these two causes are probably similar in the end-plate synapses of most animals. Transmitter is thus released very briefly; after diffusion across the synaptic cleft it is immediately attacked by specific enzymes associated with the post-synaptic membrane which destroy the transmitter's excitation properties; its action on the post-synaptic membrane is thus extremely brief. Before it is inactivated the transmitter causes specific alterations of membrane permeabilities; after its inactivation the membrane potential reverts by ion movement to its

original level along an exponential time course. This potential excursion is called the end-plate potential (e.p.p.), but it is sometimes referred to in invertebrates as the junctional potential.

The post-synaptic membrane, having been stimulated locally in the end-plate region, excites the neighbouring areas of the muscle fibre membrane by the processes described in the previous chapter. In vertebrate twitch fibres this excitation is always amplificatory and produces an all-or-nothing response; in invertebrates propagation is almost always decremental - the further a potential is observed from the initiating end-plate the smaller it appears; only the presence of many end-plates on each fibre enables roughly equal depolarisations over the whole membrane surface to be achieved. In some invertebrate muscle fibres the action of transmitter released at the end-plate as a consequence of fast axon activity may initially give rise to an active membrane response (a.m.r.), a non-linear component added to the basic e.p.p. (e.g. Hoyle, 1957). Because this response is only rarely all-or-nothing in invertebrate fibres, it is not maximal, neither is it propagated non-decrementally.

Three types of axon innervate the skeletal muscle of invertebrates and exercise peripheral control. Two of these are excitatory, the other inhibitory. The difference between fast and slow excitatory axons were defined by

Pringle (1939) and by Wiersma and van Harreveld (1938) in terms of the time-course characteristic of the mechanical response. The response to the fast axon is a rapid increase in tension, and twitches can usually be evoked by the application of single stimuli to the fast axon. No such twitch can be evoked by stimulation of the slow axon, and tetanic responses gain tension relatively slowly. The slow tetanic response, unlike that evoked by the fast axon, is relatively fatigue-free, and can be maintained for considerable periods of time.

The main differences between the two main groups of arthropods, the classes Insecta and Crustacea, lie in the organisation of the innervation, and absolute size of the e.p.p.s. and the presence or absence of the a.m.r.s.

In those insects so far investigated it has been found that all muscle blocks, and virtually all fibres of each block, are innervated by a fast axon. Some of the fibres (usually a minority) also receive a slow axon, and a few fibres are innervated by a third type of axon, the function of which is arguably inhibitory (Hoyle, 1955b & c, 1957, 1966a & b; Wood, 1957, 1958; Cerf, Grundfest, Hoyle & McCann, 1959; Huddart, 1966; Usherwood, 1962b; Usherwood & Grundfest, 1965). The insect fast response, with very few observed exceptions, consists of an a.m.r. superimposed on the basic e.p.p. The slow responses

which have been observed have mostly been small or even below the noise level of observing equipment when evoked by single stimulation, but repetitive activity leads to massive facilitation and summation.

The occurrence of fast and slow innervation in crustaceans is much more difficult to establish because of the tendency of muscle fibres of the more advanced decapods to evolve distinct, apparently fast or slow mechanical responses to activity of nerve fibres which elsewhere produce opposite effects. Such fibres are usually referred to one of two types - tonic or phasic - on the basis of their mechanical responses, and either type can be innervated by either a slow or a fast axon. In general, however, innervation by slow fibres seems to be normal, and when muscle fibres are singly innervated the response patterns seem to indicate a slow innervation, even where muscle fibre responses have apparently greatly evolved. When crustacean muscle fibres are either dually- or multiply-innervated, one of the axons is usually of the fast type, the remainder being slow. Not all fibres of a dually- or multiply-innervated muscle fibres receive all the axons, and slow-only or fast-only fibre innervations are common. In addition to these excitatory axons, many crustacean muscle fibres receive an inhibitory innervation. (Hoyle & Wiersma, 1958a; Atwood, 1963a & b, 1965a & b; Kennedy & Takeda, 1965a & b;

Dudel, 1963; Wiersma, 1961).

Electrical responses in crustacean muscle fibres are greatly varied; the majority of fast and slow responses consist simply of e.p.p.s., occasionally with minor, abortive a.m.r.s. Fast responses may include a large spike component, but these are relatively rare, and are generally found in some fibres of such specialised muscles as closer muscles (e.g. in Pachygraspus) or abdominal muscles of crayfish. In these muscles the fibres which develop the large spikes are often innervated only by a fast axon. Spiking, including all-or-nothing spiking, can be induced in many fibres by repetitive stimulation (Furshpan, 1955; Dorai Raj, 1964; Atwood, 1965b) and also by direct depolarisation of the membrane (Fatt & Katz, 1953; Atwood, 1965b).

The neuromuscular responses of Aeschna described in this chapter would seem to indicate that spiking activity has been separately evolved from simple e.p.p.-like responses in insects and in crustaceans, and that the distinction between types of innervation can be drawn only between the more advanced members of each group.

METHODS

Preparations of both femoral and coxal muscles, with intact central connections, were mounted in the experimental bath. The preparations were stimulated with a large-

tipped suction electrode, a loop of the nerve being drawn into the end. In addition the ganglion was stimulated via the cut end of the posterior inter-ganglionic commissure, which was drawn into the electrode; this achieved stimulation of the crural nerve indirectly.

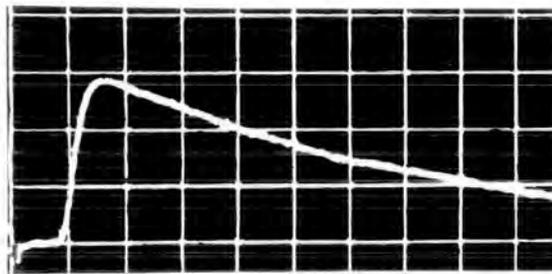
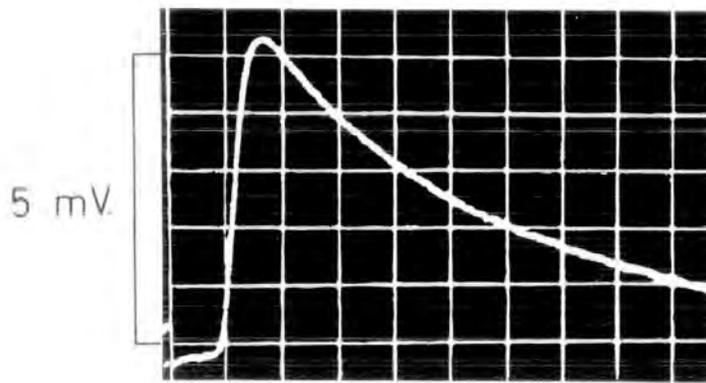
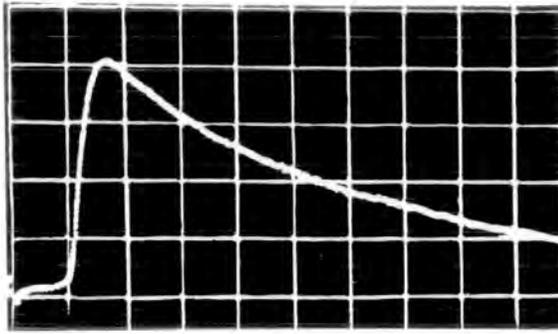
Normal saline was used throughout; the transducer was attached to the preparation to observe mechanical responses, and electrical responses were observed through a single recording micro-electrode.

RESULTS

Single indirect (neural) stimulation of the coxal muscle preparation failed to evoke any visible mechanical response. Observation of electrical events, however, revealed that stimulation above a threshold intensity evoked electrical responses by the muscle membrane. These are shown in Fig.16 where it can clearly be seen that the evoked responses (action potentials) are purely end-plate potentials, with no a.m.r. components. Graphical plots of individual action potentials show that decay phase is a purely exponential event, and examination of the rising phase on a greatly magnified time base shows no discontinuity of potential change. The three illustrated responses of Fig.16 were evoked successively, without moving the micro-electrode; it can be seen that the size of the action

FIGURE 16.

Three successively-recorded muscle fibre responses of the coxal muscle to single neural stimuli.



10 msec.

The Normal Action Potential
in the Coxal Muscle

potential, as observed at one locus, is subject to variation. The variation is, in fact, a random change from the statistical mean, and plots of the sizes of large numbers of successively-evoked responses reveal a normal distribution.

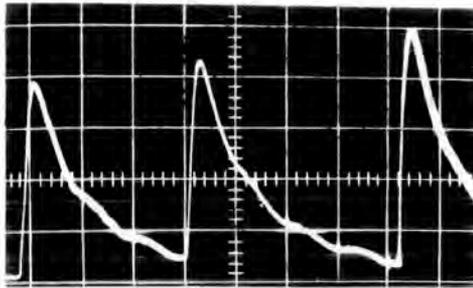
Several preparations of coxal muscle showed some tendency to spontaneous activity for a time after excision - presumably an injury response caused by depolarisation through injury of some afferent fibre or cell. Fig.17 shows comparative traces of spontaneously-evoked and stimulus-evoked action potentials, and it can be seen that there is no difference between them in size, shape or time-course.

Since single neural stimulation evokes an electrical response but no mechanical activity, the nerve would seem to be of the arthropod slow type. This is confirmed by application of a train of stimuli which evokes tetanic contraction; this does not decline significantly through fatigue when maintained.

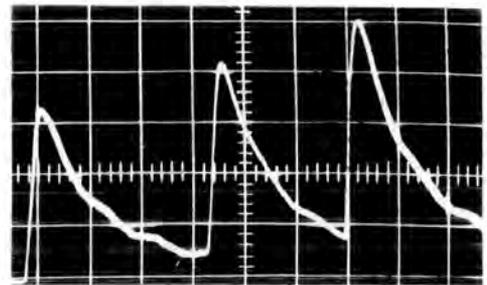
By carefully advancing the stimulus intensity from sub-threshold levels, it can be established that all fibres of the coxal muscle are incorporated in the response at the same level of stimulation; there is no step-wise involvement of a few fibres at a time. It can therefore be concluded that the coxal muscle is innervated by a single axon which is of the slow type and controls every axon.

FIGURE 17.

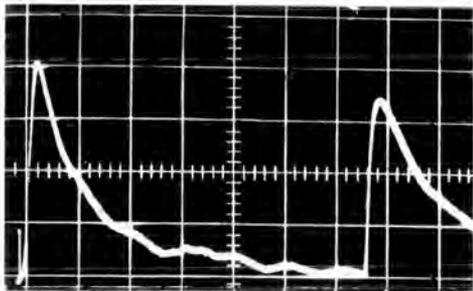
Spontaneous and stimulated activity in coxal muscle fibres.



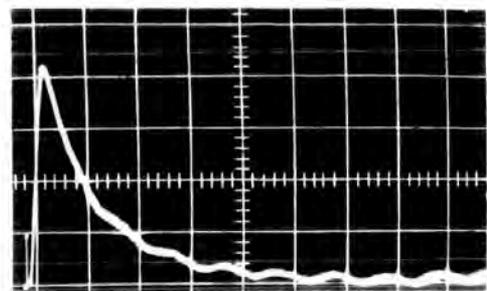
(a)



(b)



(c)



(d)

ELECTRICAL RESPONSES FROM COXAL MUSCLE

spontaneously generated by the ganglion -

(a), (b), & r.h. response of (c)

evoked by neural stimulation - (c) (l.h. response)

& (d).

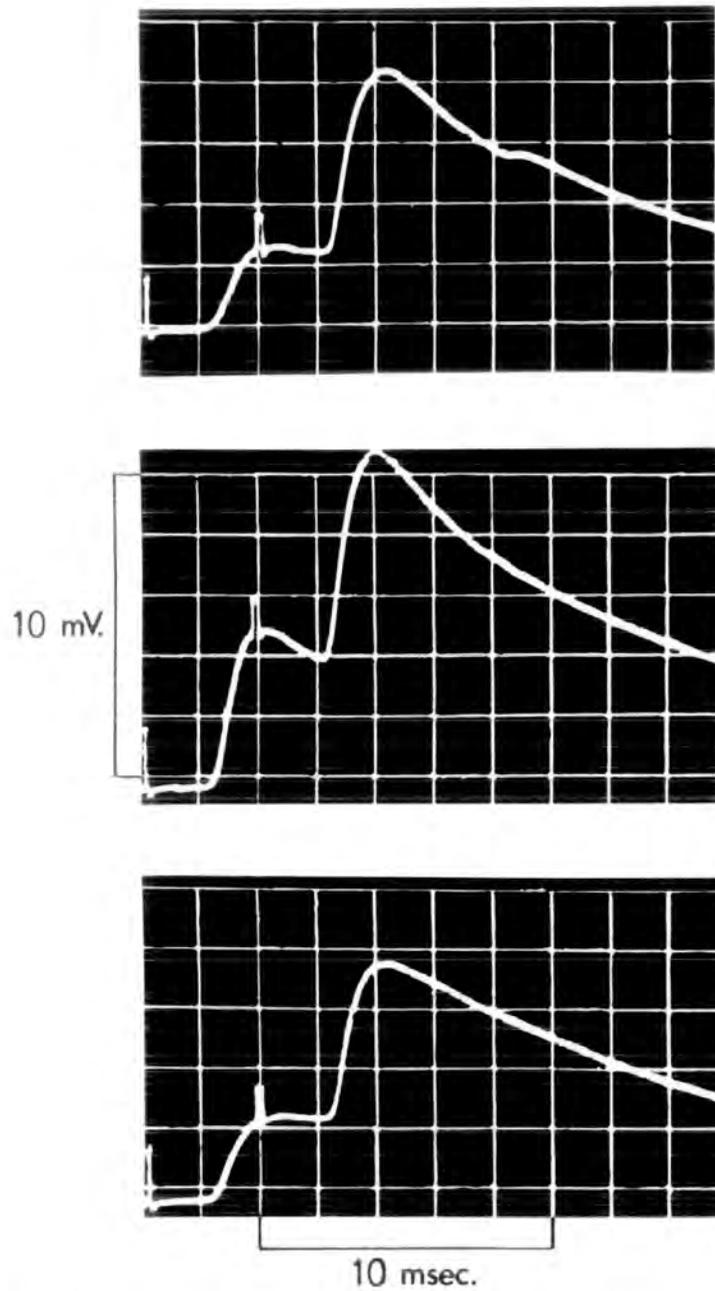
grid:- 2mV vertical, 5msec horizontal

In Fig.18 the electrical response to a pair of stimuli separated by a 5 msec interval (hereafter referred to as a 5 msec stimulus gap) is shown. As in Fig.16, the three illustrated responses were evoked consecutively from a single locus. As a result of the random variation in size, the second response can exceed the first in size, or it can be smaller. However, the results of many experiments have lead to the conclusion that on average the second potential is not significantly larger than the first; when repetitive stimulation is applied, a train of action potentials is evoked in which no one potential is significantly larger than any other. When the second of two action potentials is larger than the first, the first potential is said to have facilitated the second; this process is clearly absent here.

Summation of electrical responses to repetitive stimulation is completed within three or four responses at most frequencies. At first each potential is initiated at a point on the decay phase of the preceding potential at a level which is somewhat less polarised than that at which the preceding potential was initiated (Fig.37, Ch.7). Within three or four responses, however, each potential excursion commences at about the same membrane potential. Summation is thus very restricted in this muscle.

FIGURE 18.

Three successively-evoked muscle responses of the coxal muscle to paired neural stimuli.

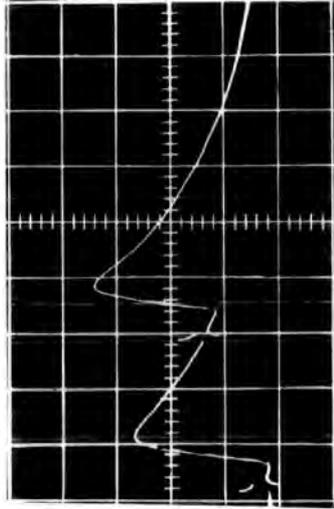
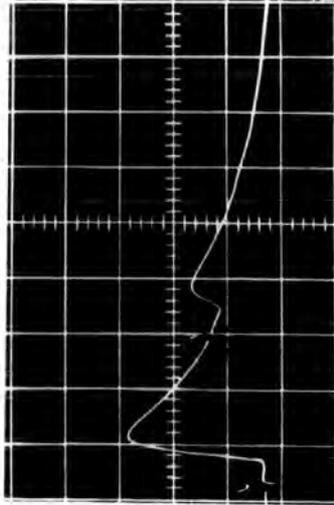
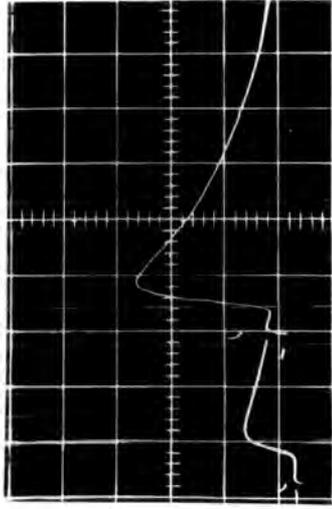
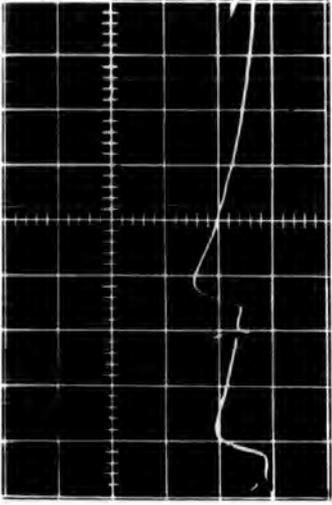


The Electrical Response of Coxal Muscle to Paired Stimulation.

In order to ascertain whether the coxal muscle was typical of other leg muscles, the femoral muscle preparation was evolved and stimulated either pre-ganglionically via the posterior commissure or post-ganglionically via the crural nerve direct. Single stimulation of the crural nerve evoked a large twitch in the flexor tibialis muscle; a fast innervation was clearly present. Observation of electrical events in the muscle membrane, and a careful adjustment of the stimulus intensity, showed that fast electrical responses such as those illustrated in Fig.19 could be evoked in every fibre. In some preparations a slow innervation could also be stimulated, at an intensity sub-threshold to that required to stimulate the fast axon, and a slow response very similar to that seen in coxal muscle could be observed in some, but not all, fibres of the flexor tibialis. The use of the suction electrode for stimulation was thought to be the cause of the failure to stimulate the slow axon alone in some preparations, since stimulus intensity falls off away from the electrode tip and the fast axon may be much nearer to the tip than is the slow. In the responses illustrated in Fig.19, the fast action potentials are much larger than the slow, but in other observations fast action potentials have been observed which approach the size of the smaller, slow potentials.

FIGURE 19.

Tonic and twitch electrical responses from the flexor tibialis muscle.



TONIC AND TWITCH ELECTRICAL RESPONSES FROM THE
FLEXOR TIBIALIS MUSCLE

Grid:- 10mV (vertical); 5msec. (horizontal)

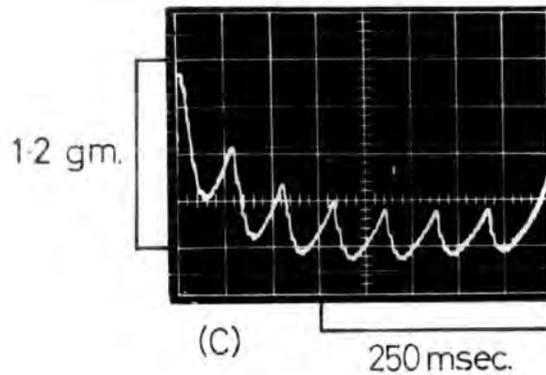
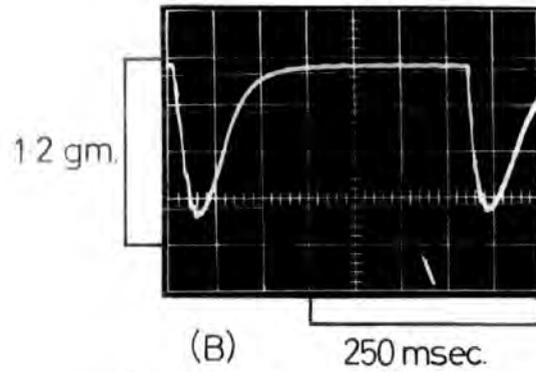
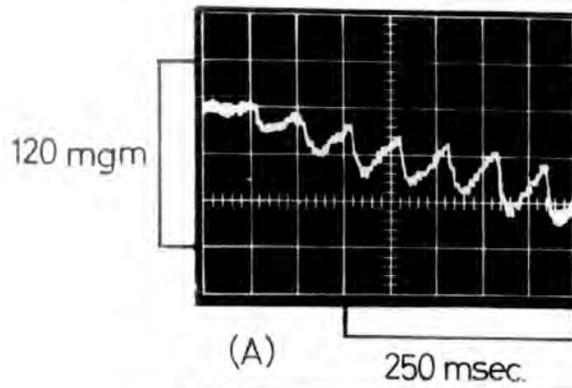
The sizes of both fast and slow action potentials vary not only between successive evoked responses at the same locus, but also very considerably from observation to locus to locus in the same and in different fibres. Slow action potential sizes were observed to range in size between 0.5 - 25mV, though relatively few observed potentials exceeded 20mV depolarisation. Far fewer observations of the fast response have been carried out, but the size range appears to be 20 - 55mV. This variation is examined quantitatively in the next chapter, where possible causes are suggested.

The mechanical responses of the flexor tibialis muscle to stimulation of fast and slow axons is illustrated in Fig.20. The slow axon was stimulated pre-ganglionically; attempts to stimulate the slow axon in the crural nerve directly usually resulted in incorporation of the fast axon after three or four impulses. It was found impossible to stimulate the fast axon pre-ganglionically.

It can be seen from Fig.20a that, in response to the first stimulus of the applied train (effectively a single stimulus) a very slight mechanical response is evoked by the slow axon. Stimuli were applied to the preparation of Fig.20 approximately on each vertical grid line, and there is a very small response after the first (left-most) vertical line in Fig.20a). This "twitch" to single slow stimulation is very tiny or absent in the flexor tibialis muscle; in the coxal

FIGURE 20.

Tonic and twitch mechanical responses from the flexor
tibialis muscle.



MECHANICAL RESPONSES FROM THE FLEXOR
TIBIALIS MUSCLE

- (A) stimulation of the slow axon at 20/sec.
- (B) single stimulation of the fast axon
- (C) stimulation of the fast axon at 20/sec.

muscle, where all fibres are slow-innervated, it can be somewhat larger, but no preparation has yet generated more than 20mgm tension. The response of the flexor tibialis to single stimulation of the fast axon (Fig.20b) is a twitch, at least three orders of magnitude greater in tension than the corresponding slow response. The size of the fast twitch is subject to rapid fatigue.

In response to repetitive stimulation of the slow axon, both preparations exhibit a response pattern similar to that of Fig.18a. There seems to be a process of mechanical facilitation at work, for examination of Fig.20a will show that there was a significant response to the second stimulus (response to the first being almost insignificant); to the third stimulus the individual response was larger than to the second, as well as summing with it, and there was a similar increase in size, plus summation in subsequent responses as well. The pattern of response is studied in detail in Chapter 7.

Fig.20c shows the comparable mechanical response to repetitive stimulation of the fast axon. It can be seen that the response to single stimulation was not maximal, and that every subsequent stimulus evoked an individual contraction.

DISCUSSION

It is clear from these observations that the electrical responses of Aeschna are very variable indeed. It is also clear that the innervation of the coxal muscle is unlike that of any other insect muscle block previously described, and that the fast responses of the flexor tibialis are unlike those of any other reported insect in the complete absence of any active membrane response.

In a review (1957), Hoyle was unequivocal in stating that all insect muscles are innervated by a fast axon; no investigation since then on any new insect material has invalidated this statement. Some muscle blocks have been described which receive only one excitatory axon, e.g. a coxal muscle of locust (Hoyle, 1966a), but this axon is clearly of the fast type.

Recent work by Usherwood & Grundfest (1965) has indicated that not all fibres of a muscle block are fast innervated - 20% of the fibres of the metathoracic extensor tibia muscle of locust are not. None-the-less, the coxal depressor of trochanter muscle of Aeschna is the first insect muscle reported to be completely without fast innervation to be reported. Occurrences of slow-only innervated muscle blocks in crustaceans are well documented (e.g. Hoyle & Wiersma 1958a).

The pattern of innervation of the flexor tibialis of Aeschna is very similar to that described by Hoyle (1955b) in the extensor tibialis of the locust. The absence of an active component of the fast electrical response in any fibre of the muscle is completely unique among insects, however. Hoyle (1966a) has reported obtaining responses from one or two fibres of Schistocerca coxal muscle in which there is no a.m.r., but the great majority of fibres from this muscle (anterior coxal adductor) show the normal type of insect fast response.

The end-plate potential evoked by the fast axon of most insects is of the order of 30mV depolarisation, and most insect fast e.p.p.s fall within the size range 15 - 40mV (Hoyle, 1955c; Becht, Hoyle & Usherwood, 1959; Wood, 1958; Huddart, 1966). In crustaceans the action potential is almost entirely an e.p.p., except in specialised cases, and the maximum size in any but these cases is 20mV; sizes of 2mV or less are commonly seen (Hoyle & Wiersma, 1958). Although the absence of an active component in Aeschna fast responses indicates affinities closer to the generalised crustacean than to other insects, the size of Aeschna fast e.p.p.s (20 - 55mV) is much more insect-like.

The slow responses of Aeschna, however, have little in common with those of other insects. The size range (0.5 - 25mV) includes responses larger than seen elsewhere,

where to single stimulation depolarisations greater than 10mV are rare - the largest reported responses were found in the locust (Hoyle, 1955c, 1957), in Carausius (Wood, 1958) and in Bombyx mori (Huddart, 1966). An exception to this is the response of certain specialised fibres of locust jumping muscles where large slow responses often include an active component (Hoyle, 1955c). The size range of crustacean slow e.p.p.s is very similar to that found in Aeschna. There is another important similarity between crustacean and Aeschna responses, for the text-figures of Hoyle & Wiersma (1958a & b) and of Atwood (1965b) show that the phenomenon of base depolarisation (see Chapter 7) to a low plateau level (rapidly attained in response to repetitive stimulation) occurs in crustaceans. The main difference between the repetitive responses is that there is no facilitation of the action potentials of Aeschna. It is equally clear from text-figures such as those of Hoyle (1957) or of Huddart (1966) that slow responses of insect muscle fibres are not fundamentally different; in the insects, facilitation processes are carried much further, as is summation, and when the plateau level of depolarisation is reached, the size of the individual e.p.p.s, if distinguishable, is very much smaller in relation to the basic depolarisation than is that of crustaceans - or of Aeschna.

The electrical responses of Aeschna leg muscles would seem to show some affinities with both insects and crustaceans, but closest affinities would seem to be with a generalised crustacean pattern. At the same time these fibres show certain unique properties, most notably the absence of facilitation. It is clear that the intermediate nature of the electrical responses further establishes the unlikelihood of there being any fundamental differences between the arthropods. The variety of responses reflects the long evolution of types and physiological make-ups within the Arthropoda.

CHAPTER 5

THE VARIATION OF THE SIZE OF THE SLOW ACTION POTENTIAL

CHAPTER 5

THE VARIATION OF THE SIZE OF THE SLOW ACTION POTENTIAL

INTRODUCTION

Variation is a basic property of living systems; few properties of cells which can be expressed quantitatively are invariable from animal to animal of a species, or even between cells of the same animal. For this reason the great majority of measurements carried out on animals are expressed statistically as means, and an indication is given of the confidence limits.

Muscle responses of invertebrates are particularly subject to variation. The size of the invertebrate action potential is likely to be more variable than that of the vertebrate muscle fibre, since the latter is an amplified, all-or-nothing event, subject only to the variability of the muscle membrane. The invertebrate action potential is governed not only by the transverse properties of the membrane, but also by the longitudinal properties, since it spreads decrementally from discrete points of origin; it is also a function of the amount of transmitter released, and of the affinity of the membrane towards the transmitter. Many authors have noted the variability of action potential size in both insects and crustaceans, in particular Fatt & Katz (1953) and Hoyle (1966a).

It is in crustaceans that variability comparable to that of Aeschna, briefly described in the last chapter, is found. Variability from fibre to fibre is detailed by Hoyle & Wiersma (1958); no published work has commented quantitatively on variability at one locus, but several published traces of action potentials recorded successively (e.g. Dudel & Kuffler, 1961) indicate that such variation is common among crustaceans. Because of the development of a.m.r.s in insect fast responses, and the process of facilitation of insect slow responses, variation of responses is to some extent distorted. It is particularly difficult to observe any variation of response size at any one locus, but there is evidently variation in size as observed in different fibres, both in slow (Hoyle, 1957) and fast (Hoyle, 1966a) responses.

Because of the great variability in observed action potential size observed in Aeschna it was decided to conduct surveys within single muscle blocks (using the coxal muscle preparation) to attempt to discover any pattern of variation, and to see if it was possible to erect any theoretical model by which potential size could be predicted at any one locus.

METHODS

The coxal preparation, with ganglion removed, was bathed in normal saline. A single micro-electrode was

carefully selected; it was required that the electrode should penetrate the membrane with great ease, so that there should be an absolute minimum of damage. This enabled an accurate trace to be obtained, not only of the size of the action potential, which would probably not be much affected by a little damage, but also of the decay phase and of the resting potential.

The muscle was sampled across the fibres, first traversing the proximal ends of the fibres, then the centres, and finally distally; in each traverse the electrode was moved far enough between insertions to be certain that no fibre was penetrated twice in each traverse. No attempt was made to avoid penetration of the same fibre in different traverses, and it is likely that some fibres appear more than once in the table of results, having been sampled at one or both ends, as well as centrally.

At each penetration the nerve was stimulated and five successive action potentials were recorded; the principle of recording successive potentials was strictly adhered to. After these had been recorded, the resting potentials were measured by photographic comparison of d.c. level on the oscilloscope screen before and after removal of the electrode from the fibre.

Variability of the size of the action potentials

along single fibres was investigated using the two-electrode technique used for measuring membrane parameters (see Chapter 3). The "stimulating" electrode was inserted at one end of the fibre, an action potential recorded, and then the "recording" electrode was inserted at different loci along the fibre, further potentials being recorded. Identification of the fibre, to ensure that both electrodes were in the same one, was made by observation with the "recording" electrode of a small hyper-polarising current passed through the other.

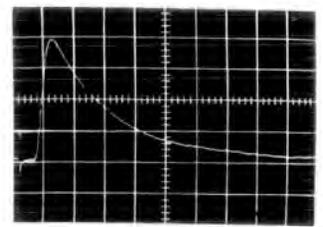
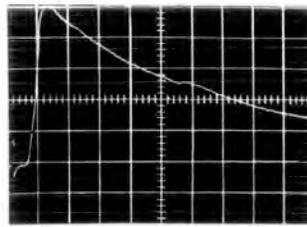
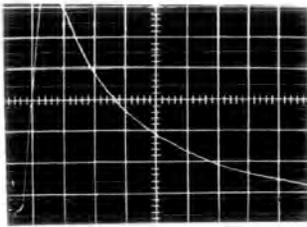
RESULTS AND CALCULATIONS

The results of using the two-electrode system to follow the size of the action potential along a single fibre confirmed the impression that had been obtained from random penetrations, namely that there is variation of at least $\pm 50\%$ along each fibre. The variation is almost certainly much greater than this, since the difficult technique of making many penetrations of one fibre was only partially successful. The variation is much greater than that found in various crabs by Fatt & Katz (1953), and is undoubtedly a product of the extremely short length constant (see Chapter 3) which produces a rapid decay of electrotonically spreading events.

The traces in Fig.21 are typical of those observed in the survey of responses obtainable from a single muscle

FIGURE 21

Neurally-evoked responses evoked from a single muscle block.
Two responses were successively recorded at each locus
(see text).



(i)

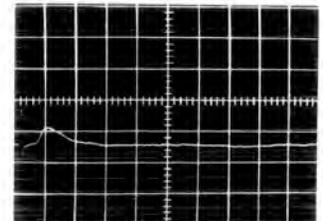
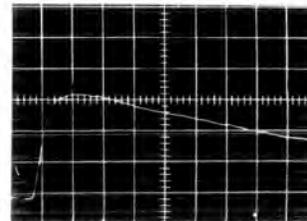
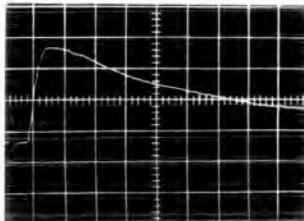
r. p. = 80mV.

(ii)

r. p. = 92mV

(iii)

r. p. = 81 mV.



(iv)

r. p. = 73mV.

(v)

r. p. = 87mV.

(vi)

r. p. = 85mV.

THE VARIABILITY OF THE NEURALLY - EVOKED SLOW MUSCLE RESPONSE

Two successive responses from six different fibres of Coxal muscle.

Grid:- 2mV (vertical) 5msec. (horizontal)

block. The results which appear in Fig.21 were in fact specially recorded for the figure, all being observed at the same oscilloscope sensitivity; in the actual surveys sensitivity and sweep speed were set at the maximum possible, in order to measure size and times with the greatest accuracy. As a result, all results to be reported in this chapter have roughly the same percentage accuracy, however small or large the observed potential.

It is clear from Fig.21 that variation occurs not only in the sizes of the action potential and resting potential, but also in the rate of decay. The two traces which are shown from each cell are successively recorded and give an indication of the variability of the response. It is not so clear from Fig.21 that the rate of rise of the action potential is also variable, but measurement shows this to be the case.

After a "pilot" survey had been studied it was decided to measure the following parameters at each locus of penetration :-

- (i) The size of the action potential (mV).
- (ii) The mean rate of rise of the action potential (mV/msec, equivalent to V/sec). The total time that elapsed between the first excursion of the membrane potential from its resting level and the peak level of the action potential - an S-shaped

potential change - was measured directly and divided into the action potential size.

(iii) The quarter-decay time of the action potential (msec).

This is an exponential function of the action potential size, like the half-decay time, and was used in preference to the half-decay so that both it and the rise time could be accurately measured on the same trace photograph.

(iv) The resting potential (mV).

From the parameters measured in photographic records two other functions were calculated - the rise factor and the decay factor. It is evident that the rates of rise and decay of an action potential are governed by the magnitude of that potential to some extent; in other words, a larger action potential will have longer rise and decay times, all other factors being equal. In order to obtain factors describing the rising and decaying phases in a way numerically independent of the size of each potential, rise and quarter decay times of each potential were divided by the size of that potential. The resulting rise and decay factors have the dimensional forms of milliseconds per millivolt, therefore; this is numerically equal to seconds per volt.

The data measured directly from the photographic records of each survey - action potential sizes, rise times, quarter decay times and resting potentials - was punched on to paper tape and the calculations necessary to derive the mean values for each variable, and also of the two factors, was carried out by electronic computer. A note on computer methods appears in Appendix E. The computer produced a print-out of the results in table form; the results from that survey which contained the most samples, and which forms the basis of all the diagrams in this chapter, appears as Table 6, Appendix A.

Experiments in which extended sequences of potentials were recorded from one locus and then averaged in various groupings have shown that the mean value for the potential observed at any one locus in these surveys is likely to be no more than 10% different from the best estimate of the true mean; that is, the standard error is usually about 5% of the mean for samples of 5; probability of an error greater than 10% is 0.05. The two places of decimals given by the computer are, of course, meaningless, and confidence limits of $\pm 10\%$ should be placed on each value. The exception to this is the resting potential, which was directly measured and is not a mean value; the accuracy of resting potentials is probably within $\pm 2\%$, the accuracy of

the display equipment; they were in fact measured to the nearest millivolt.

The results presented in Table 6 (Appendix A) were used in the construction of the histograms and scatter diagrams that appear in Figs.22 - 28. Results from other surveys present very similar patterns, and overall mean values for resting and action potentials from all observations in each survey were not significantly different from survey to survey (i.e. from animal to animal). The mean values for this survey were :-

Resting potential - $81.6\text{mV} \pm 1.1$ (s.e.).

Action potential - $8.30\text{mV} \pm 0.50$ (s.e.).

Frequency-distribution histograms of the resting and action potentials appear in Figs.22 & 23a respectively. It can be seen from these histograms that the resting potential has a positively-skewed distribution - the majority of observed values being of greater value than the median - and that the action potential distribution is negatively-skewed, but fairly close to a Poisson (random) distribution.

Fig.23b was obtained by observing the muscle membrane response at a fixed locus to many successive stimuli, which were applied to the nerve at a rate of about twelve per minute. The resulting range of action potential sizes was plotted as a frequency-distribution histogram (Fig.23b)

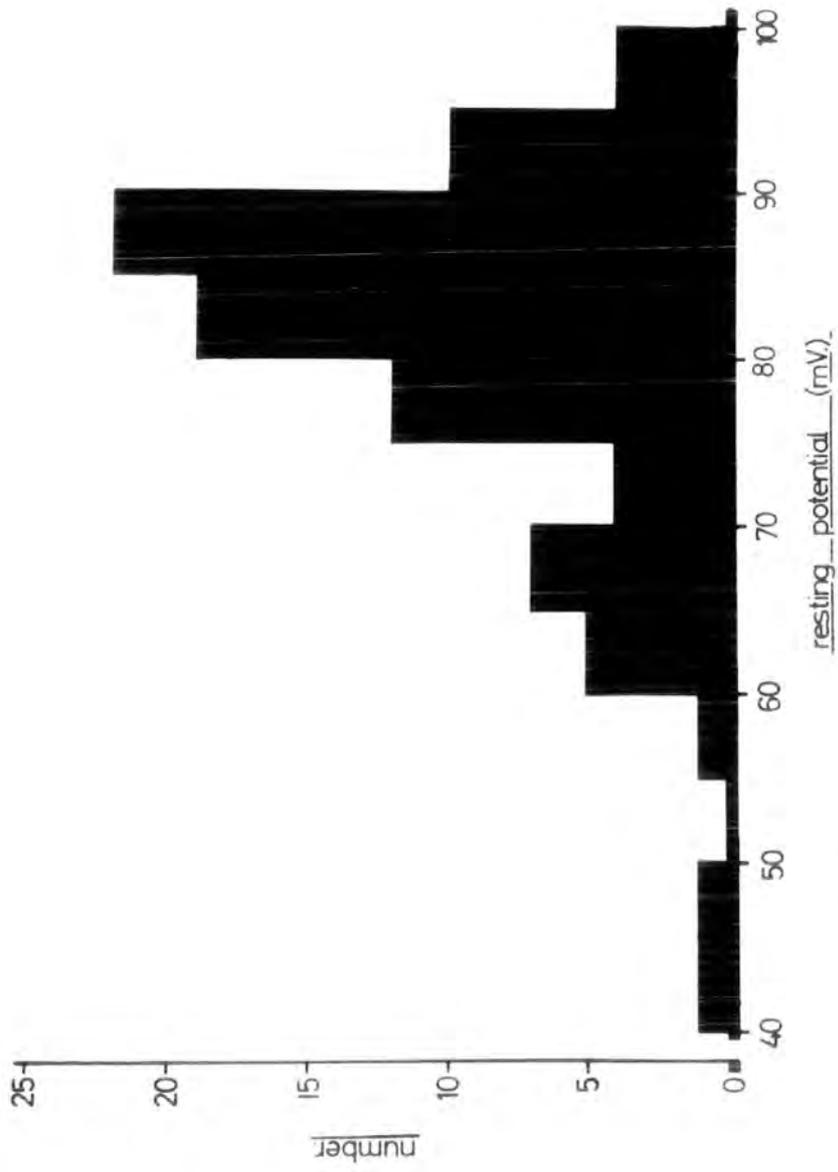
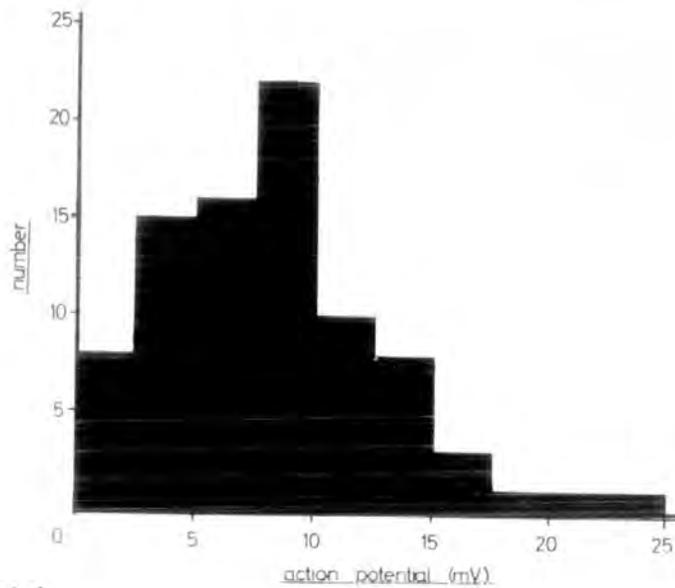
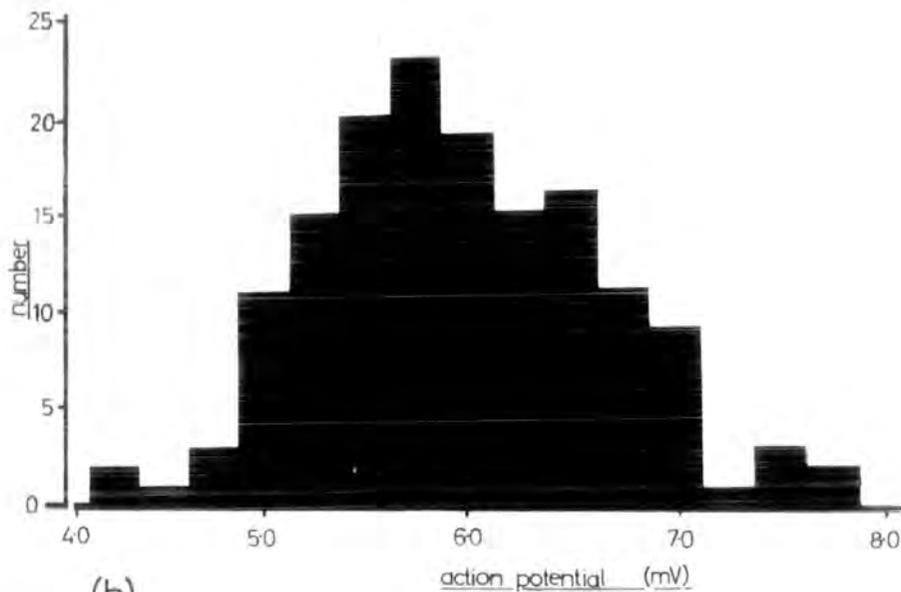


Figure 22.

Frequency Distribution of the Resting Potentials
Occuring Within a Single Muscle Block
 Mean value 81.6 mV. \pm 1.15.



(a)



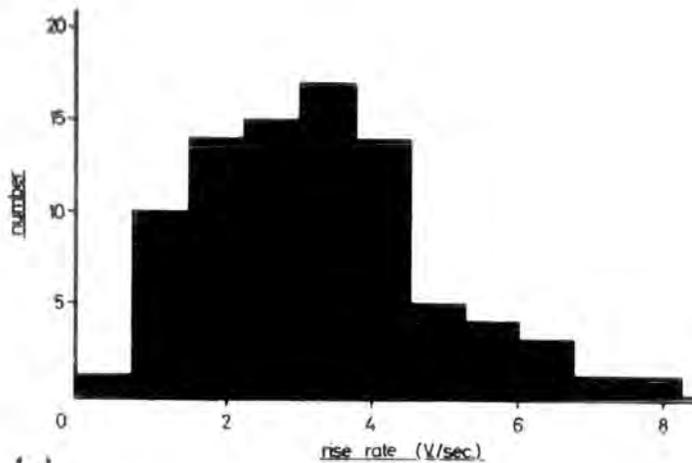
(b)

- (a) The frequency distribution of the slow action potential sizes occurring within a single muscle block.
- (b) The variation in size of slow action potentials successively recorded from the same locus. Mean value 6.00 ± 0.05 se., standard deviation 0.65

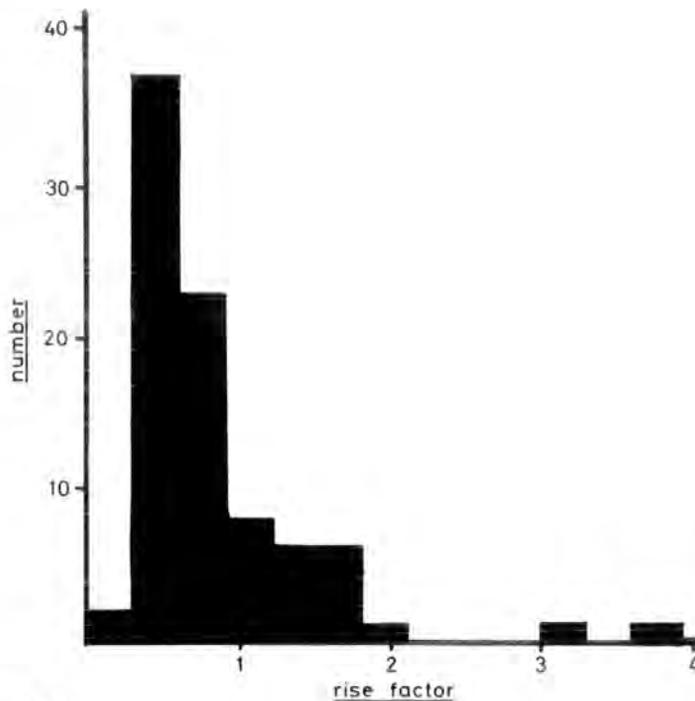
which is thus a representation of the variability of each one of the events, the mean of which appears in Fig.23a. The variation of the action potentials at each locus can clearly be seen to be normally distributed; the very slight negative skew is almost certainly a consequence of fatigue of the response as the observation run progressed. A normal distribution represents a random fluctuation of an event about a theoretically-expected mean. That Fig.23b is a true normal distribution is confirmed by calculation of the statistical standard deviation; the majority of the curve lies within \pm two standard deviations of the mean, as do more than 90% of the individual observed events.

Values obtained for resting and action potentials of individual loci were compared to see if there was any relationship; a correlation coefficient (see Appendix C) was calculated for the two sets of data, and showed that there was no correlation of any significance; there was, in fact, no significant correlation between resting potential and any other measured function.

The distribution histogram of the measured rise rate (Fig.24a) can be seen to have the same general shape as that of the action potential size (Fig.23a). A plot of the two variables (Fig.25a) shows them to be linearly related to each other; the correlation coefficient of the two



(a)



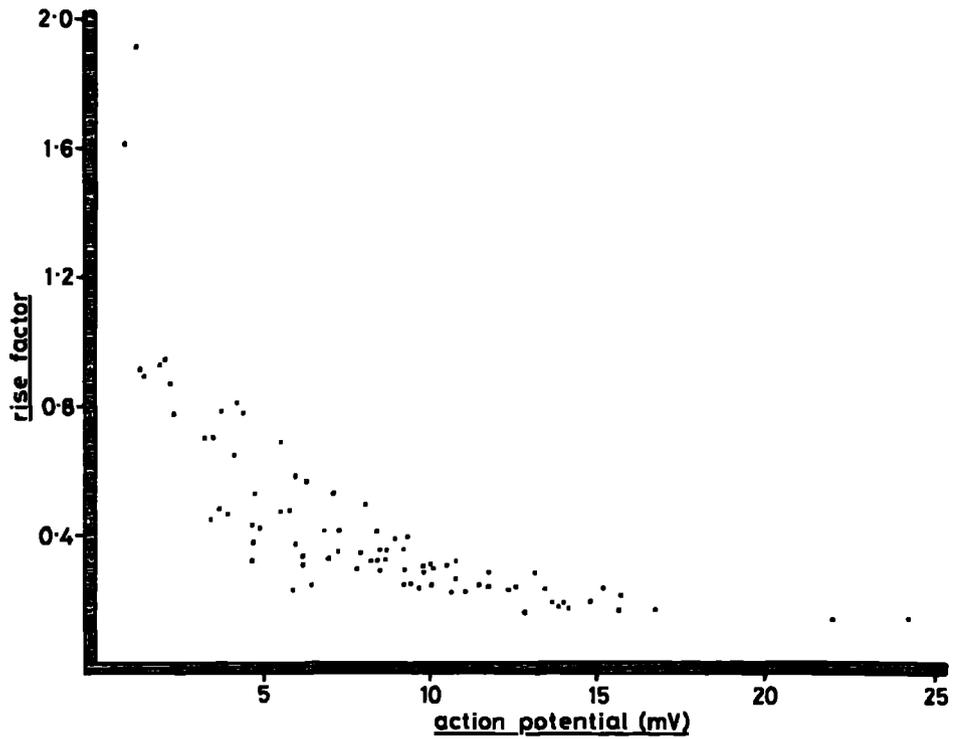
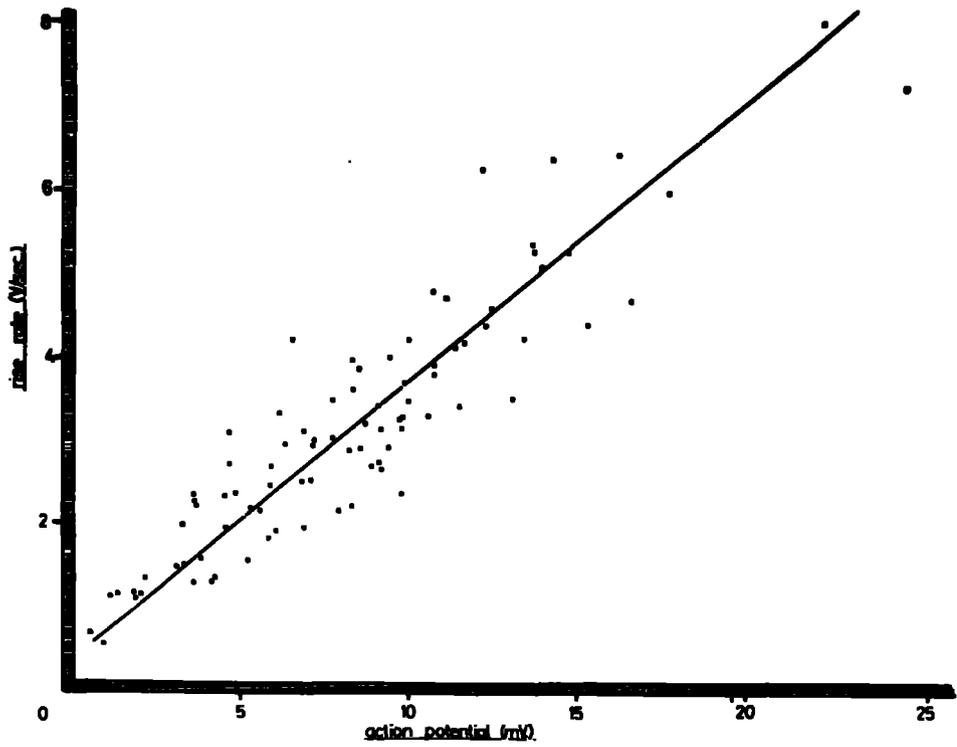
(b)

The Variation of the Rising Phase of Slow Action Potentials Within One Muscle Block

Frequency distribution of the rise rate

Frequency distribution of the rise factor:

$$\text{rise factor} = \frac{\text{rise time}}{\text{action potential size}}$$



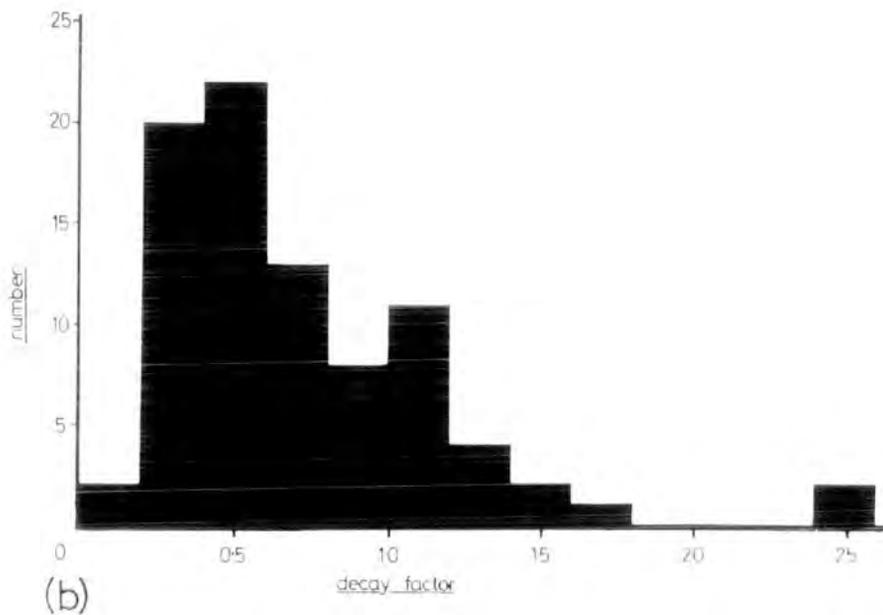
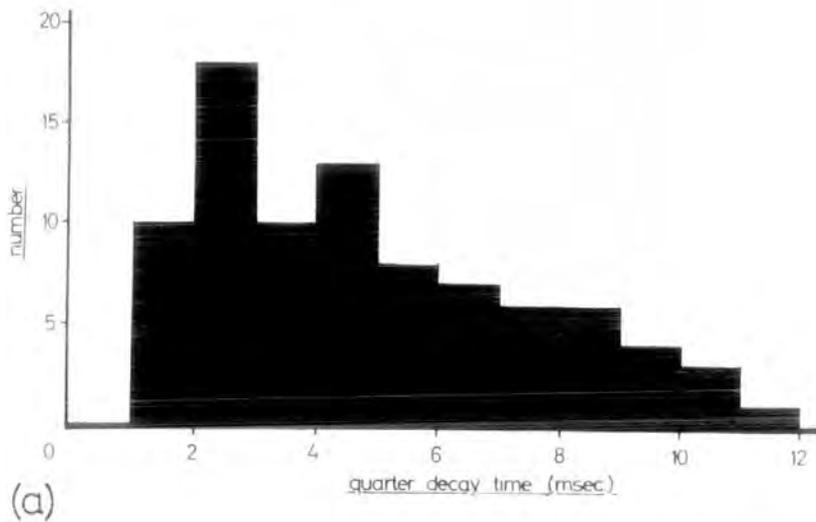
The Relationship Between the Action Potential and its Rising Phase

Figure 2b

variables, 0.91, indicates that there is a negligible chance ($p < 0.001$) that this configuration arose by chance. The two are therefore related to a very significant extent. As discussed above, this is to be expected, since as the rise time of most of the observed action potentials is of the order of 2msec (see Table 6, Appendix A), the rise rates of the largest potentials are bound to be the fastest.

It was to eliminate this numerical interference that the rise factors were calculated. The distribution of these is extremely skewed; it is illustrated in Fig.24b, and it can be seen that the great majority of values fall between 0.20 and 0.45sec/V. Although the numerical influence of the action potential size has theoretically been removed, a plot of size against rise factor (Fig.25b) clearly shows that an exponential relationship exists between them. It can be seen in this graph that the largest rise factors are associated with the smallest action potentials. The correlation coefficient of the rise factor and log. action potential size is 0.78; the relationship has a probability of less than 0.01 of having occurred randomly. There is thus a very definite indication that the smallest action potentials are the only ones associated with the largest rise factors.

A similar study of the decay time and the decay factor distributions (Fig.26) show that the decay rate is



The Variation of the Decay Phase of Slow Action Potentials Within One Muscle Block

- (a) Frequency distribution of the quarter decay time
- (b) Frequency distribution of the decay factor

$$\text{decay factor} = \frac{\text{quarter decay time}}{\text{action potential size}}$$

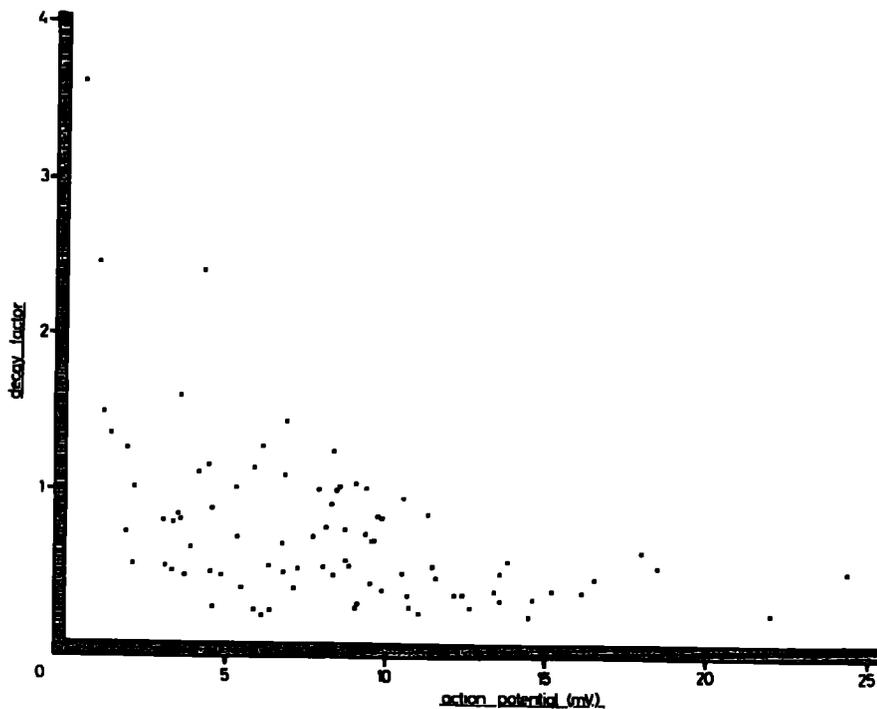
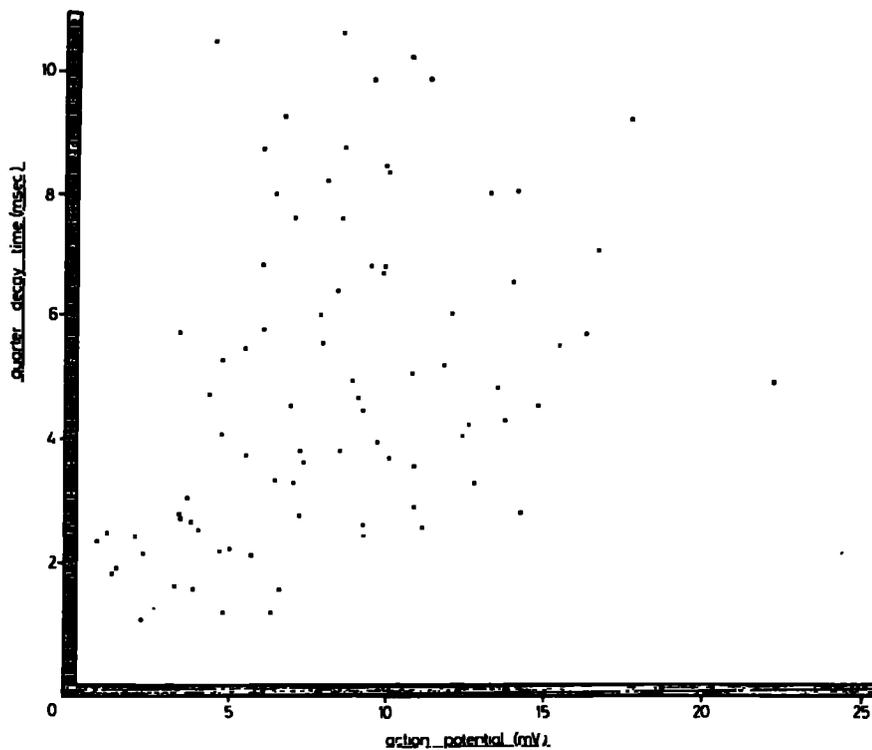
Figure 26.

much more variable than the rise rate (Fig.24a); the decay rate does, however, have a statistically significant ($p = 0.05$) relationship with the action potential (Fig.27a) even though there is more scatter than in the corresponding relationship of the rise rate (Fig.25a). In the relationship between decay factor and action potential size (Fig.27b) there is considerably more scatter than in the corresponding relationship of the rise factor, but a significant exponential correlation ($p = 0.01$) exists.

Because of the great variability of decay rates in particular, there is no significant correlation between rise rate and quarter decay time (Fig.28a). Possibly a much larger sample would reveal some correlation. There is, however, a very highly significant ($p < 0.001$) relationship between rise and decay factors; this can be seen in the plot of the two (Fig.28b). It is clear, therefore, that high rise factors are associated with high decay factors in the same responses; at lower values of the factors there is considerably more scatter in the relationship.

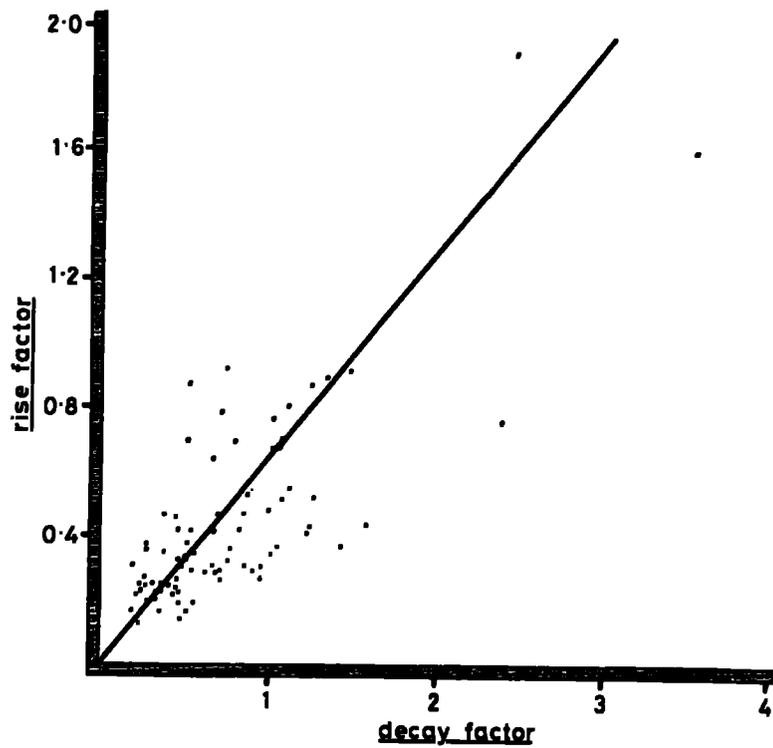
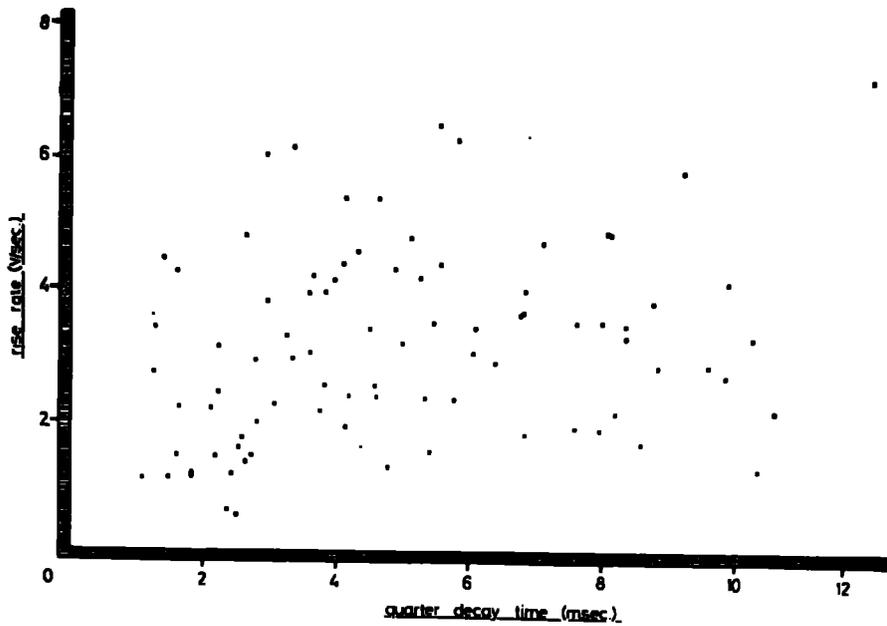
DISCUSSION

The results of the survey set out above are chiefly remarkable for the range of apparent variation in the parameters of the active membrane. If they are examined carefully while bearing in mind that the size of the action potential varies



The Relationship Between the Action Potential and its Decay Phase

Figure 27.



The Relationship Between the Rise and Decay Phases
of the Action Potential

Figure 28.

considerably along the length of the fibre, it is possible to obtain an indication of the likely way in which the underlying mechanism works.

As discussed above, the likely error of the results presented as means is not in excess of $\pm 10\%$. The great variation observed both along each fibre and between fibres is not, therefore, a product of the normal variation of the action potential at any one locus such as is illustrated in Fig.23b. In any case, the variation of rise and decay factors, which have been determined quantitatively in a way specifically designed to exclude the effects of potential size, is far too great to be explained on a basis of random variation at any one locus.

Investigation of the membrane parameters (Chapter 3) showed that the muscle fibre membrane of Aeschna has an extremely short length constant, of the order of 0.25mm. According to the theory of Hodgkin & Rushton (1946), describing the passive spread of potential, an applied voltage decays to about 0.35 of its value in travelling a distance equal to its length constant, to about 0.12 in two length constants, and to 0.05 in three. In the membrane of Aeschna, therefore, an action potential spreading out from an end-plate will appear to have decreased to 0.1 of its original size within about 500 μ . The actual amount will vary with the length constant from fibre to fibre. No

figures are available for spatial distribution of end-plates on these muscle fibres, but it may be significant that the circumference of the fibres is of the order of 100 - 200 μ , observed diameters being in the range 30 - 60 μ .

If, therefore, the assumption is made that some regions of the muscle fibre membrane are of the order of 300 - 400 μ from the nearest end-plate, and that the average inter-endplate distance approaches the magnitude of the length constant (250 μ), the observed variation of action potentials showed by random sampling is relatively easily explained. The probability of sampling a fibre near to one of its end-plate regions is evidently low, and the majority of samples must have been obtained at some distance from the nearest end-plate. Insufficient samples were taken to obtain many high values in the survey. It may be that a mechanism such as this is responsible for the variety of responses obtainable by action of a single axon in some crustaceans. It seems to be general that where such variation occurs only a very few observed potentials reached the upper limit of observed range, as has been found in Aeschna (e.g. Hoyle & Wiersma, 1958a).

Explanation of the variation of rise and decay rates is somewhat more difficult, however. The chief clue is provided by study of the propagation of applied square-wave current pulses, as described in Chapter 3. The square

pulse, initially almost perfect in shape, degrades as it is conducted electronically away from its point of origin. Both the rising and falling edges, originally sharply-rising, gradually decrease in gradient, and follow an exponential rise and fall of increasing period. The lack of sharpness of the rising and falling edges of a square-wave even at a very short distance from point of application can be seen in Fig.15. The electrode will, of course, have some effect on the rising and falling phases (see Fig.12b); this effect is much less than that due to degradation, and is in any case constant. The degradation is in fact predicted by the theory of Hodgkin & Rushton (1946).

An action potential, whether it has a component of active membrane response or not, does not have an instantaneous rise rate, and the decay is exponential even at point of origin of the potential (i.e. the end-plate). There seems no reason to doubt that in processes of passive propagation the rising and falling phases of an action potential will become degraded in the same way as a square-wave. The effect of this will be to lengthen the rise and decay times relative to the size of the action potential.

The underlying significance of the rise and decay factors can now be seen. A high factor (in units of seconds of rise or decay time per volt of action potential size) implies a longer time of rise relative to size of action

potential. Figs. 25b & 27b show clearly that the high factors, both rise and decay, are found in association with the smallest potentials. These potentials are thought to represent the electrical disturbances furthest from an end-plate; the high factors, it is now suggested, indicate those potentials which have undergone most degradation in shape - giving reductions in rate of change and consequently increased factors - by travelling the greatest distance from the point of origin. That both arguments lead to the conclusion that the smallest potentials are those furthest from the end-plate only serves to reinforce the theory.

The scatter of points in the relationships between rise and decay factors and action potential size, and also between the two factors (Figs. 25b, 27b & 28b) is a product of two features; these are the natural variation of living systems (especially living excitable membranes), and the smallness of the numbers of samples averaged at each locus.

The value of the maximum size of potential occurring on each fibre - at the end-plate - is quite impossible to estimate from these graphs, except very roughly. A value of between 20 and 30mV is a likely estimate, and the highest value of action potential size seen in any experiment so far is 25mV. It is quite likely that the maximum size in different fibres varies to a considerable extent, and such

variation would, in fact, tend to produce scatter of results such as has been observed. A close examination of a fibre by the two-electrode method would be necessary to determine the maximum size, but to be certain of having sampled one locus showing a value significantly near the maximum the fibre would have to be sampled at intervals which are small compared to the length constant - probably at intervals of less than 25μ . To take such samples along a 3 - 4mm fibre without dislodging the current-passing electrode which "signposts" the fibre would be technically almost impossible.

Statistical techniques such as those described above are probably the best means of obtaining an idea of the range of actual action potential sizes occurring within a group of muscle fibres, and will show whether there is true variation in size between fibres or whether, as here, an apparent variation is simply a product of random sampling.

In the light of the proposed model it is hardly surprising that there is no correlation of any kind between resting potentials of fibres and the size or any other aspect of the action potentials observed; any fibre, presumably having the same resting potential along its length, can show a whole range of sizes and shapes of action potential.

One object of the survey - to discover a basis of variation - is therefore achieved. The other - to form a basis for prediction of action potential size at any locus -

is theoretically possible, but would involve erection of a complex theory. It would be possible, by calculation of rise or decay factor, to show by how much they have changed from those occurring in the potential at its origin. This change will have been made in a way independent of initial or final potential size, being a function of the membrane cable properties. From this change of factor, and by measurement of the length constant of the particular fibre under experiment, the distance travelled by the potential from the end-plate could be calculated. The known size of the action potential could then be extrapolated back to discover what it was at the end-plate.

This procedure, besides being far too involved to make it worthwhile, would be dependent on so many approximations and assumptions (such as assuming a point-source end-plate, and having to ignore the variability of the potential at one locus) that it would not produce a very accurate answer. The information obtained in this part of the study is relevant to other parts in one important respect, however. When any experiment involves changes in size of action potential by such experimental means as changing ionic balance in the bathing saline, the changes must be observed through one micro-electrode which is left inserted in the same fibre throughout the experiment. To

make quite certain that any changes in action potential size reflect changes at the neuromuscular junction, and not changes in the fibre membrane, it is then only necessary to measure the length constant.

CHAPTER 6

THE FATIGUE OF THE SLOW RESPONSE.

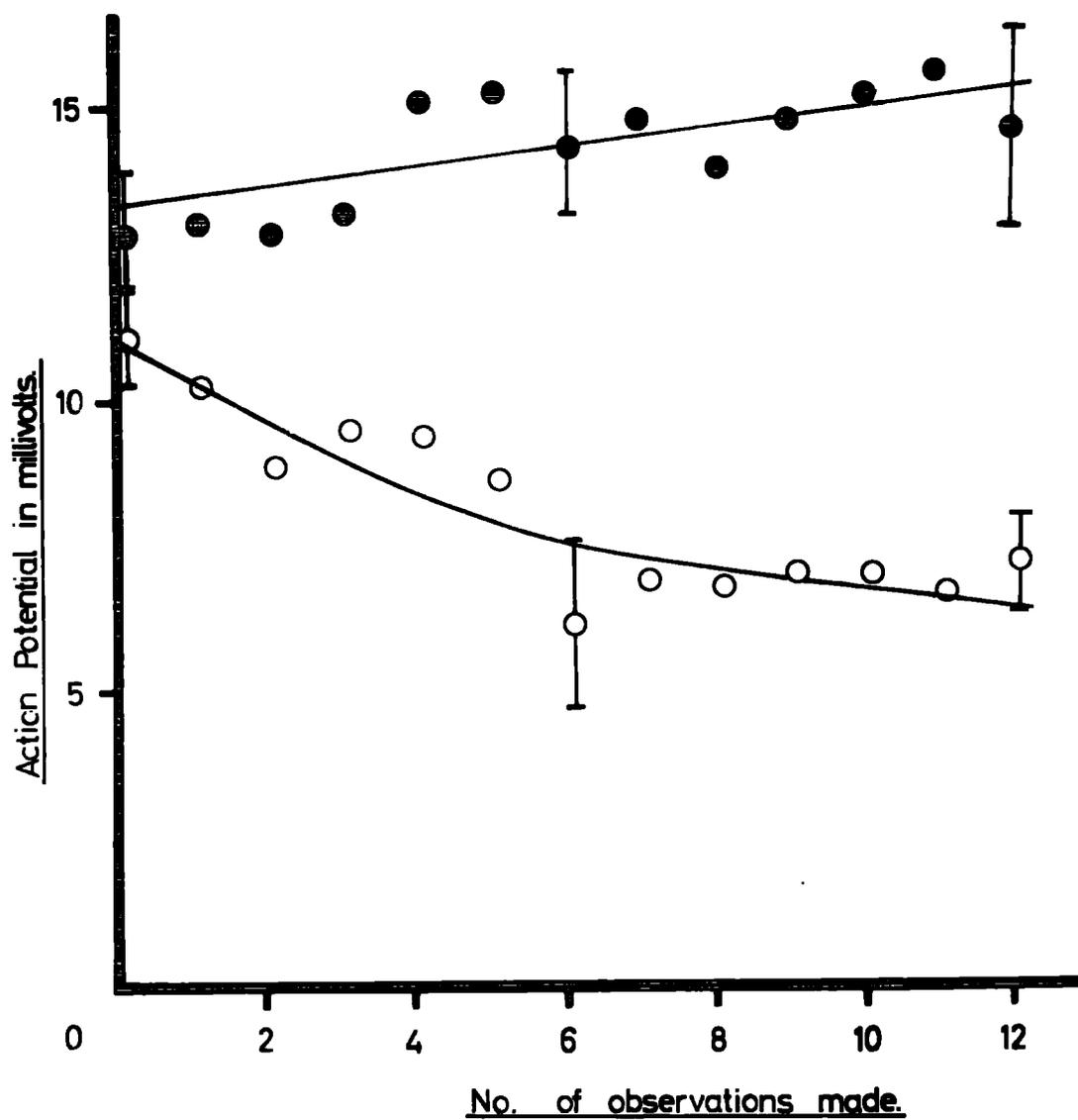
CHAPTER 6

THE FATIGUE OF THE SLOW RESPONSE.

INTRODUCTION

While other experiments were being carried out it became apparent that both electrical and mechanical muscle responses were subject to processes of fatigue. A preliminary study of the responses of the muscle fibre to paired neural stimulation led to the discovery that a fatigued preparation showed considerably more electrical facilitation than was to be found in a fresh one.

Examination of the detailed results obtained in several sets of experiments, involving various work loads on the preparation in unit time, seemed to indicate the presence of two types of response with differing fatigue patterns. In Fig.29 the progress of size of action potential is traced through two typical experiments from the series described in the next chapter and illustrated in Fig.43. Confidence limits indicated in Fig.29 are two standard errors ($p = 0.05$ for significance of difference). Each point describes the control size of action potential in each of 13 sets of observations in which considerable additional activity was induced. The points of the graph of Fig.29 are roughly equally separated both in time and in amount of activity elapsed.



THE FATIGUE OF EXPERIMENTAL PREPARATIONS

Figure 29.

It can be seen that one preparation markedly and significantly fatigued, while the other showed no significant effect. Over all the experiments which have been carried out in this whole study in which the size of the action potential to normal single stimulation was determined at intervals to give some indication of the progress of fatigue, there was a roughly equal number of each of the types illustrated in Fig. 29. This 1:1 ratio has been obtained entirely from coxal muscle preparations; no results are available for the flexor tibialis. Since the coxal muscle is innervated by only one (slow) axon, differences in fatigue must reflect differing properties either in the end-plates or in the fibres themselves.

Varying muscle fibre properties, giving different responses to the same innervating fibre, have been reported in a number of arthropods. The slow fibre innervating locust extensor tibialis, designated S1 by Hoyle (1955c), produces one type of response in two-thirds of the fibres it innervates, but a completely different response in the other third. Workers such as Kennedy & Takeda (1965a & b), Dorai Raj (1964) and Hoyle & Wiersma (1958) have demonstrated many neuromuscular systems of crustaceans in which one axon produces varied responses in muscle fibres that are all innervated by it.

An investigation into the processes of fatigue was carried out in order to see whether more than one muscle cell type could be identified; knowledge of the time-course of fatigue was also required to assist in interpretation of other experiments, especially those in which any processes of facilitation were suspected to be important.

It became apparent that a long series of experiments was required, however, so the results reported in this chapter are preliminary, and some conclusions are very tentative.

METHODS

The changes in size of action potential and mechanical response were followed by stimulating the preparation at a constant rate and sampling the evoked responses at intervals.

Coxal preparations, linked to the mechano-electronic transducer and covered with normal saline, were used with intact central nerve connections, a loop of the crural nerve being drawn into a large-tipped suction electrode.

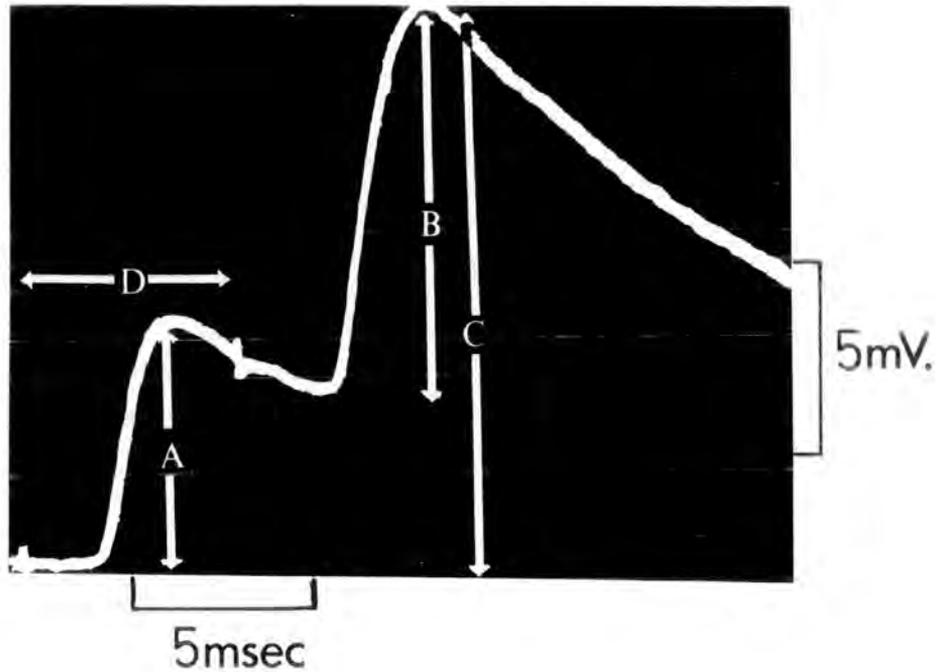
In the majority of experiments the switchbox was used in conjunction with two micro-electrodes; the facility for switching the "stimulating" electrode into the display circuit (and the "recording" one out) could be used to display

responses successively from the two electrodes, each of which was used as a recording electrode and left inserted into a fibre throughout the experiment. In this way a single set of display equipment could be used to obtain results from two different fibres of a single preparation which, if not obtained simultaneously, were evoked under the same conditions at all times, innervated by the same axon, and being stimulated the same number of times.

Experimental procedure was as follows :-

The preparation was isolated and set up, and briefly stimulated to make sure that the nerve had not been damaged, mechanical responses being observed. The preparation was left for a short time, and then the two micro-electrodes were successively inserted into fibres, the resting potential and response to single stimulation being used as indicators of satisfactory penetration. The preparation was then left for at least half an hour so as to be as non-fatigued as possible at the start of the experiment.

To fatigue the preparation at a steady rate the nerve was stimulated at a rate of 24/min; this rate was set accurately so that a time-clock could be used to count the number of stimuli applied; counting accuracy by this method was probably an error of two per thousand. The stimulus applied consisted of pairs of shocks of varying gap (interval). The response elicited by this stimulation appeared as in Fig.30,



A – Size of First Potential.

B – Size of Second Potential.

C – Overall Size.

D – Stimulus Gap.

Parameters for Measurement of
Dual Stimulation Data

which defines the terms used in this and subsequent chapters to describe electrical responses. This type of stimulation allowed three parameters to be followed during the course of progress of fatigue :-

- (i) The size of the normal action potential. The interval between applied pairs of stimuli ($2\frac{1}{2}$ sec) was sufficiently long to ensure that the first potential (Fig.30) was effectively a response to single stimulation.
- (ii) The amount of facilitation. This parameter is expressed as the ratio of the size of the second action potential to that of the first (see Appendix D). In different experiments, the stimulus gap between the pairs was varied, but not the rate of application of the pairs, which remained constant at $2\frac{1}{2}$ sec. The facilitation at various stimulus gaps was established both in non-fatigued and fatigued conditions.
- (iii) The mechanical response. A paired stimulus applied to the slow axon elicits a significant mechanical response, most or all of which corresponds to the second stimulus. Single stimulation would evoke virtually no mechanical response.

After the preparation had been allowed to rest for at least half an hour after its last stimulated activity (when the electrodes were inserted) twenty pairs of stimuli

were applied to the preparation, and the evoked responses recorded from one of the two micro-electrodes. The preparation was then allowed to rest for another quarter of an hour. The process of experimentally-applied fatigue was then started, by stimulating at a steady rate; the first twenty responses were recorded through the other micro-electrode. Standard responses from two fibres in non-fatigued condition had now been obtained. As fatigue progressed, samples of responses were recorded at intervals from each electrode in turn. Twenty responses were recorded in each sample.

Recorded data was measured, transferred to punched paper tape, and mean values for the size of each potential, the amount of facilitation, and the size of the mechanical response, were calculated by computer (see Appendix E).

RESULTS.

In all experiments, not only of this type but also of any other type involving calculation of facilitation, no preparation or fibre of a preparation has been found in which significant facilitation was demonstrable in the fresh condition. The only exceptions to this were those cases in which the stimulus gap was so small that the rising phases of the action potentials interacted, presumably reflecting the effects of a release of transmitter arriving at the

post-synaptic membrane before the effects of a previous unit of transmitter had worn off. It can safely be concluded that the slow response of the coxal muscle of dragonflies exhibits no facilitation in the fresh condition.

Table 3 is a collection of results from fresh preparations used in a variety of other experiments, and it can be seen that in no case is there significant ($p = 0.05$) facilitation; in addition, in no case is there a decrease in the relative mean size of the second potential, significant or not.

Fig.31 illustrates results typical of those obtained in other experiments. The two fatigue curves shown were recorded by two micro-electrodes from the same preparation during the same activity run; they may illustrate a difference between the two types of fatigue pattern described in the introduction to this chapter. The stimulus gap for the experiment of Fig.31 was 5msec, and the detailed results from which the curves were constructed appear as Table 7, Appendix A.

The preparation of Fig.31a was the only one of seven which definitely showed this fatigue pattern, in which considerable activity can be elicited before the size of the first action potential falls significantly (Fig.31a). In five of the other six experiments the pattern followed was that of Fig.31b, a more-or-less

exponential decrease with fatigue, significant within 100 applied stimuli. In the other experiment the decrease came rather later, and was an intermediate case.

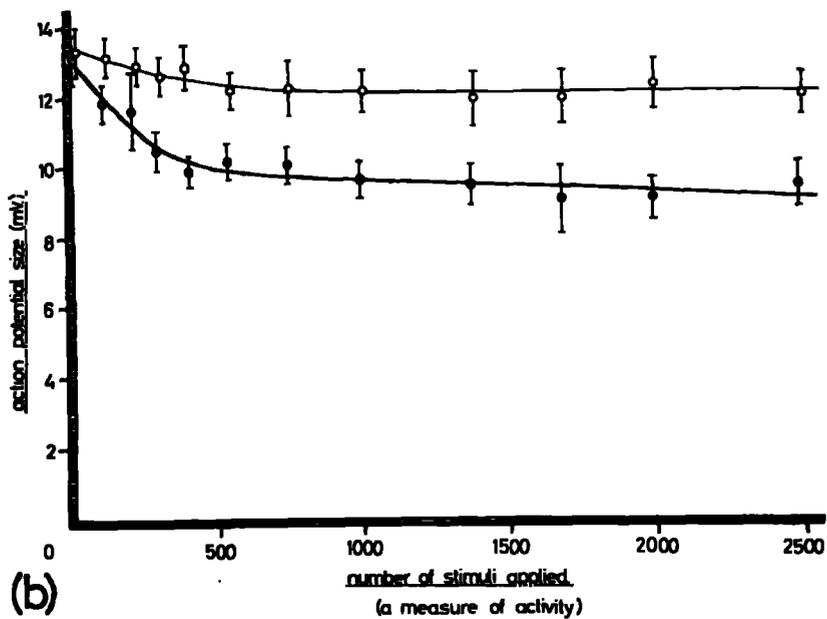
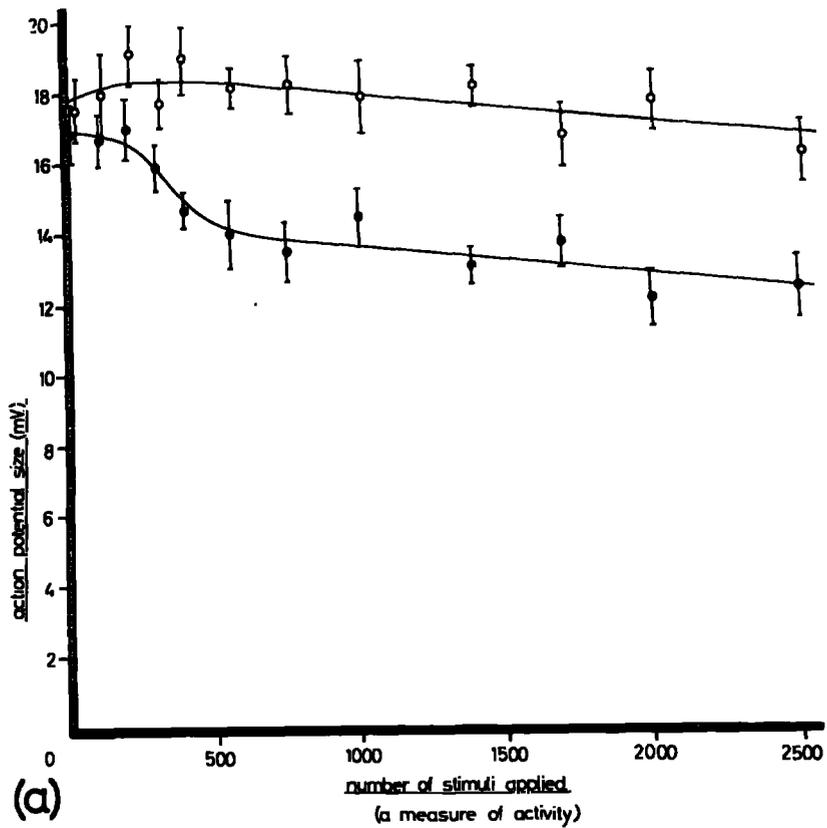
The progress of facilitation with advancing fatigue is illustrated in Fig.32, in which curves 32a and 32b correspond with curves 31a & 31b, i.e. from the same fibres; the results are detailed in Table 8, Appendix A. It can be seen that there is a rapid development of significant facilitation, the initially rapid increase slowing until it reaches an approximately plateau level. Comparison of Fig.32b with Fig.33, the facilitation curve of an exactly similar experiment with another fibre, but at a different stimulus gap, suggests that the value of the plateau level of facilitation might be a function of the stimulus gap to some extent. The rate of increase, and the amount of activity necessary to bring the facilitation level to a plateau, can be seen to be considerably greater in the preparation of Fig.31a & 32a. than it is in the fibre of Figs.31b & 32b. This is probably related to the correspondingly slower decrease in size of the first action potential with activity.

In Fig.34 the results of an experiment are given in which, after an observation of the fatigue of the preparation by stimuli of 100msec gap, it was allowed to recover for two hours, after which time the process was repeated.

TABLE 3

The Responses of Fresh Coxal Muscle Preparations to
Paired Stimulation collected from Control Observations
of various Experiments

STIMULUS GAP msec	SIZE OF FIRST POTENTIAL (mV) \pm s.e.	SIZE OF SECOND POTENTIAL (mV) \pm s.e.	FACILITATION \pm s.e.
5	13.10 \pm 0.35	13.30 \pm 0.35	1.03 \pm 0.04
5	13.45 \pm 0.45	13.55 \pm 0.65	1.06 \pm 0.08
5	16.85 \pm 0.40	17.50 \pm 0.45	1.06 \pm 0.05
10	13.85 \pm 0.25	14.05 \pm 0.35	1.02 \pm 0.03
20	7.55 \pm 0.35	8.25 \pm 0.25	1.14 \pm 0.07
20	7.70 \pm 0.30	7.95 \pm 0.30	1.09 \pm 0.08
25	15.35 \pm 0.50	15.50 \pm 0.55	1.03 \pm 0.06
25	9.30 \pm 0.15	9.35 \pm 0.15	1.01 \pm 0.03
25	11.00 \pm 0.25	11.00 \pm 0.25	1.01 \pm 0.01
100	14.95 \pm 0.35	15.00 \pm 0.25	1.05 \pm 0.03

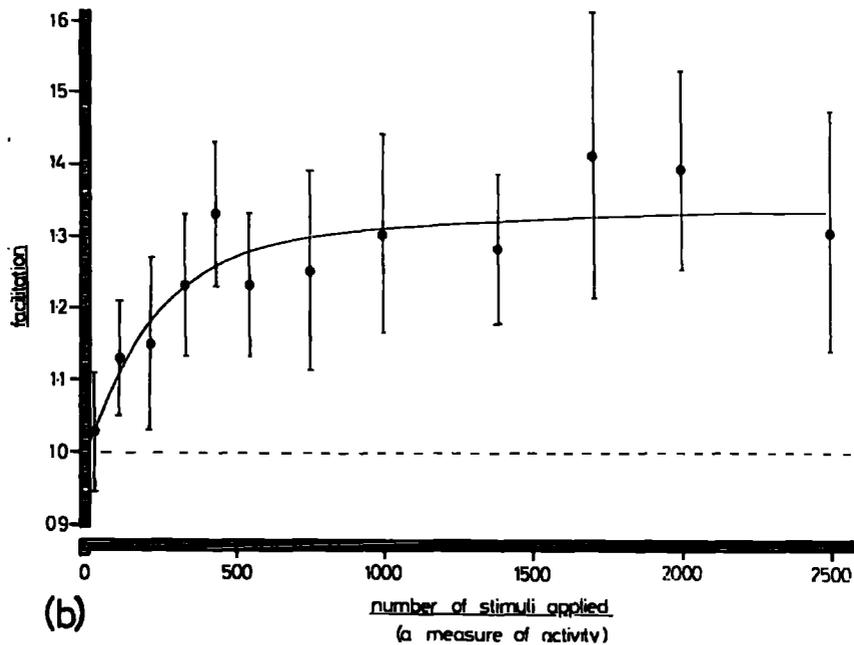
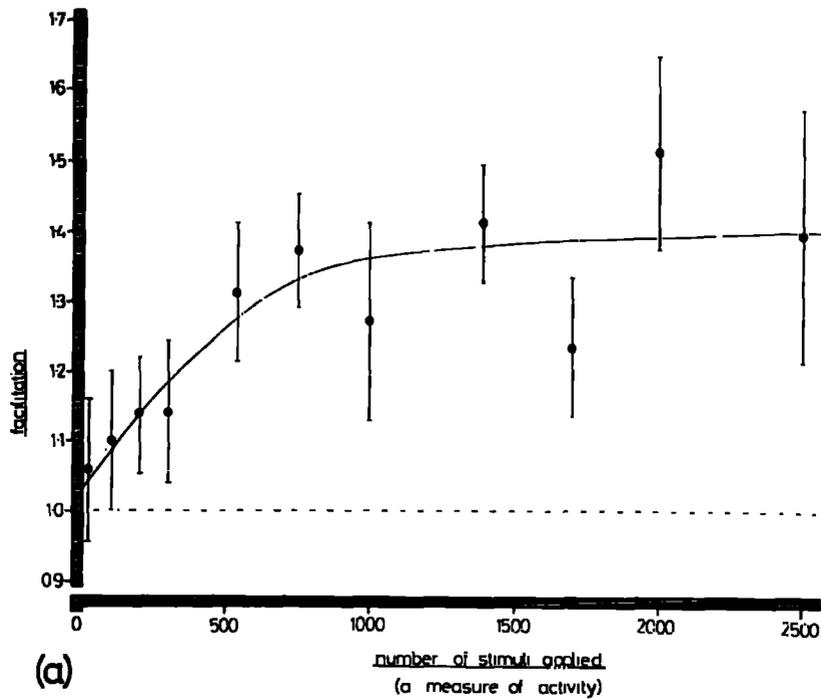


The Effect of Fatigue on the Slow Action Potential

The effect of paired stimuli, 5 msec. gap, delivered at 2 pairs/min.

The two preparations were recorded simultaneously

Figure 31.



The Effect of Fatigue on the Facilitation of the Slow Action Potential

The effect of repeated paired stimulation at 24/min. on the ratio of the second potential to the first. The two preparations were recorded simultaneously. Stimulus gap 5 msec.

Figure 32.

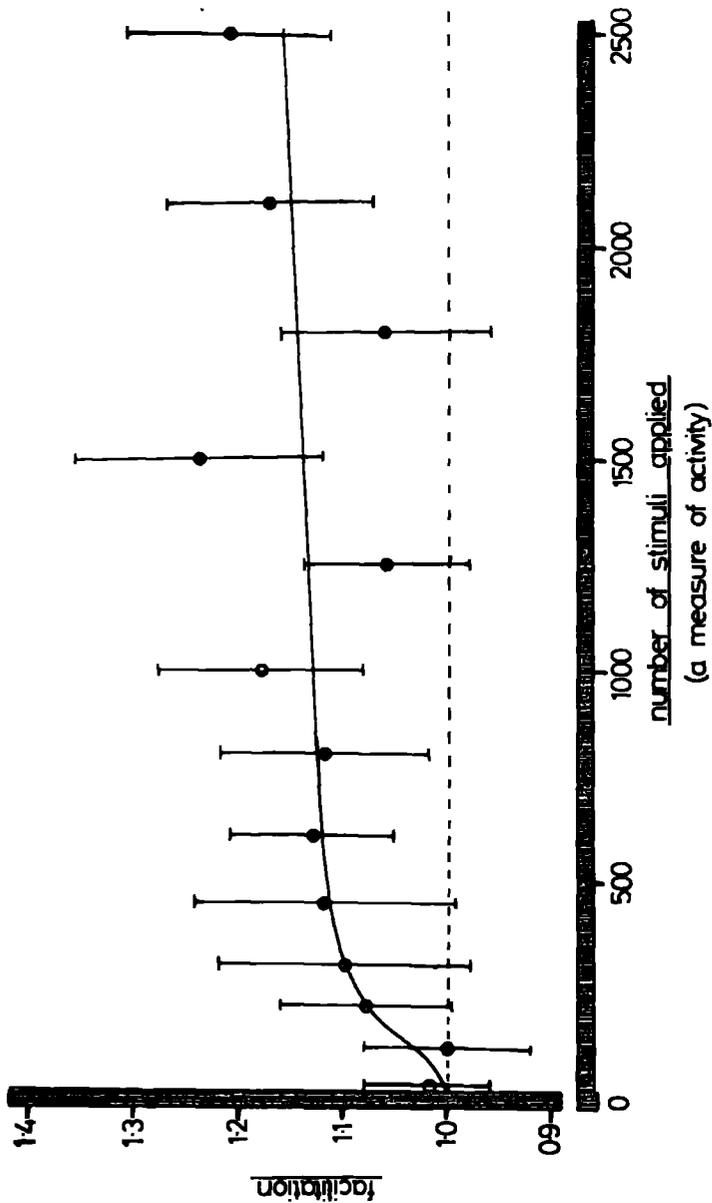
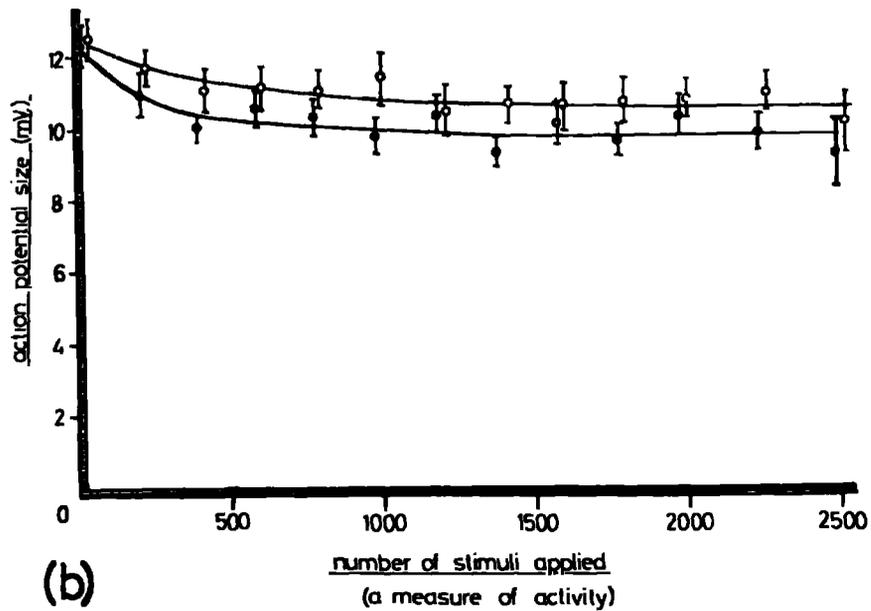
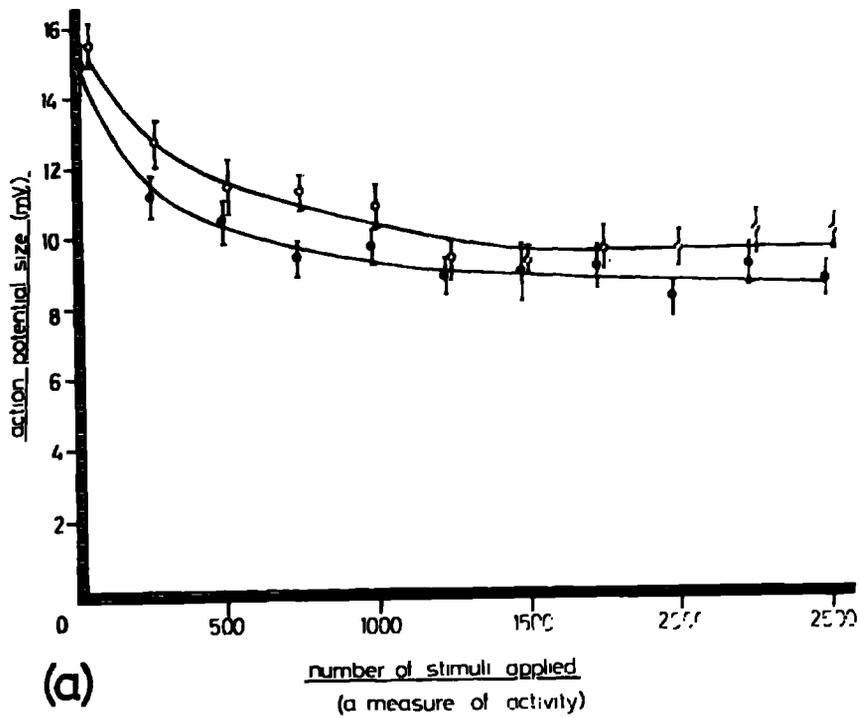


Figure 30.

The Effect of Fatigue on the Facilitation of the

Slow Action Potential

The effect of repeated paired stimulation at 24/min. on the ratio of the second potential to the first. Stimulus gap 10msec.



The Effect of Fatigue on the Slow Action Potential

The effect of paired stimuli, 100msec. gap, delivered at 24pairs/min.

(a) Fresh preparation.

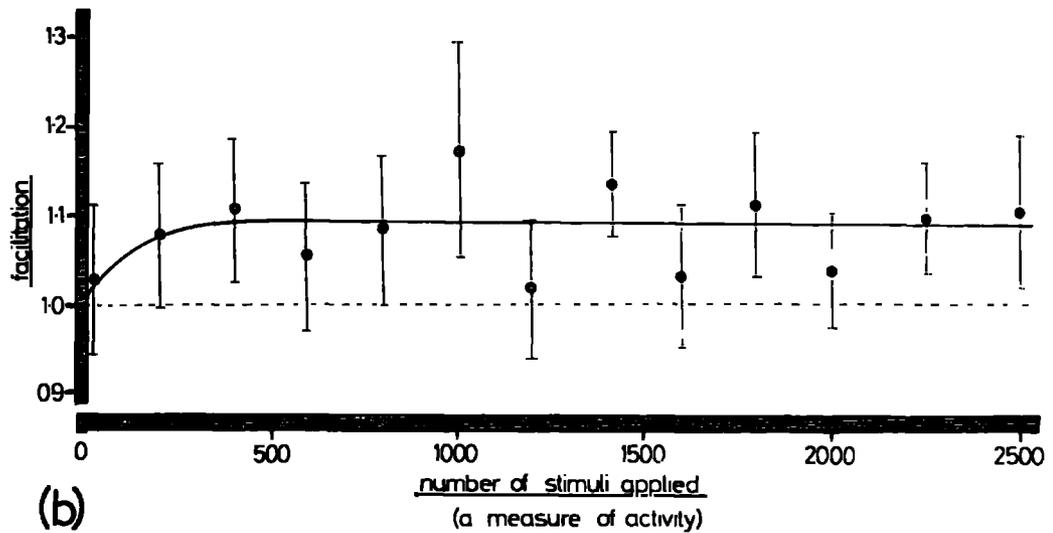
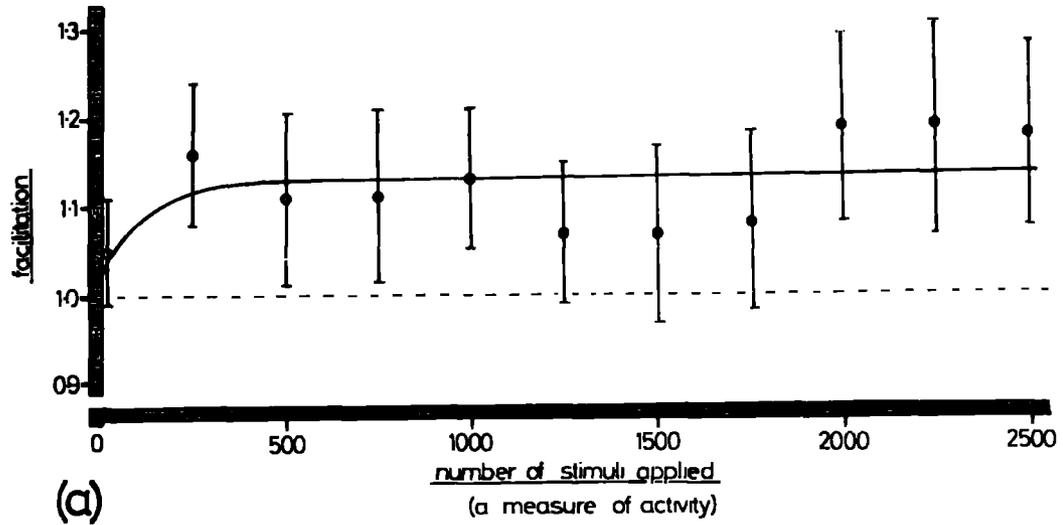
(b) The same preparation after two hours rest.

Figure 34.

Corresponding changes in facilitation are illustrated in Fig.35. It can be seen from the results of this experiment that the processes of fatigue caused by steady activity are reversible, but the recovery of the action potential to its former size is a long process, only partly completed in two hours; return of facilitation processes to zero is much more rapid. This would seem to indicate some distinction at the neuromuscular junction or in the muscle fibre membrane between the two.

Fig.35a demonstrates that there is very little more facilitation in the fatigued preparation at 100msec gap than there is at 10msec gap. Conclusions concerning the magnitude of facilitation cannot be drawn, however, because of the small number of sets of results available. Data from experiments carried out for other purposes suggests, however, that there is considerable variation.

Experiments on the fatigue of the mechanical response - necessarily of the whole coxal muscle - show what appears to be an exponential decrease in magnitude, suggesting a simple process of metabolic fatigue as a consequence of depletion of energy store, build-up of metabolic wastes etc. Results from two typical experiments are illustrated in Fig.36.



The Effect of Fatigue on the Facilitation of the Slow Action Potential

The effect of repeated paired stimulation at 24/min. on the ratio of the second potential to the first. (a) Fresh preparation. (b) The same, after two hour's rest. Stimulus gap 100 msec.

Figure 35.

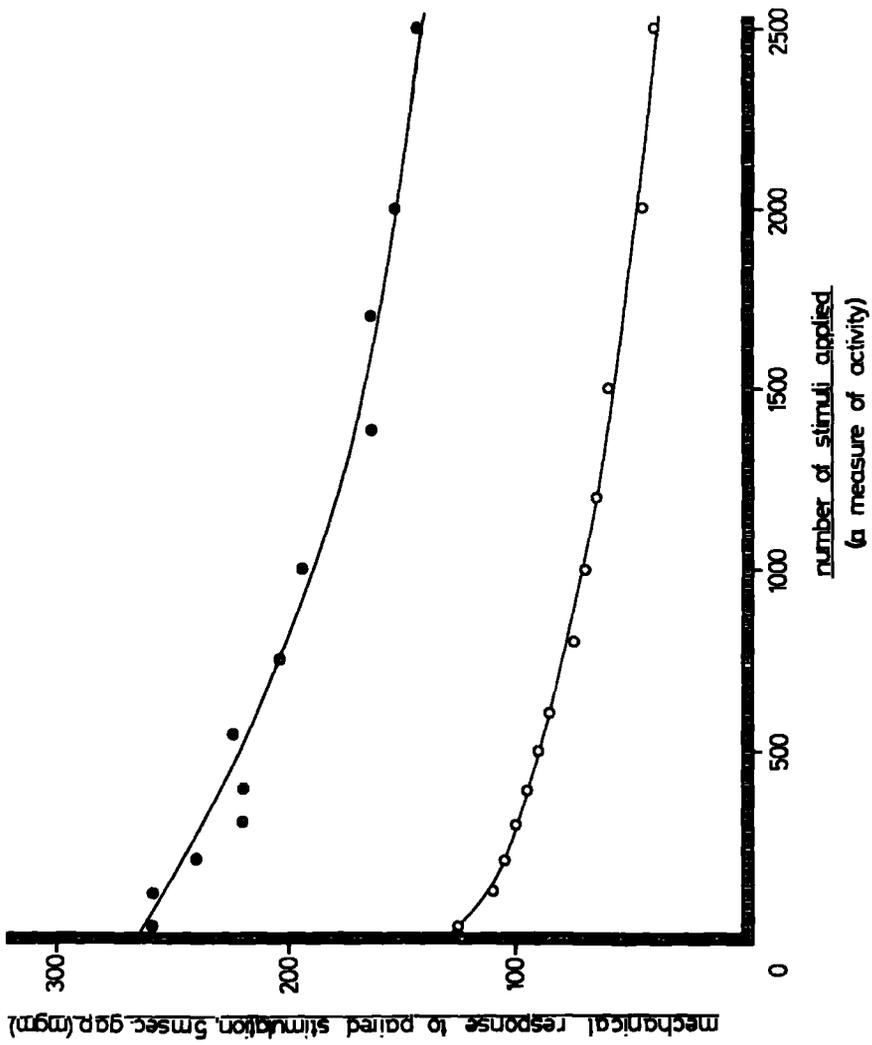


Figure 36.

The Effect of Fatigue on the Slow Mechanical Response

The response of two separate preparations to paired stimuli, 5msec gap.

DISCUSSION

The few experiments reported in this chapter offer only an indication of the nature of fatigue processes occurring in the coxal muscle fibre neuromuscular junction and excitation system. The only definite conclusion that can be drawn is that any observed facilitation of the slow action potential can be ascribed solely to the processes of fatigue.

The absence of facilitation in fresh preparations is a property which sets the dragonflies somewhat apart from other known arthropods, in which facilitations of both fast and slow action potentials have been reported. Facilitation of the slow response in other insects is often considerable (e.g. Hoyle, 1957). The process is much more gradual in both fast and slow responses of crustacea (e.g. Hoyle & Wiersma, 1958; Wiersma, 1961) and it would be interesting to carry out a study on various of the decapod muscles used by other workers to see if any part of the facilitation processes known to occur there can be ascribed to fatigue.

Dudel & Kuffler (1961b) have studied the processes of facilitation in crayfish, and concluded that there is no change in sensitivity of the post-synaptic membrane to the action of the transmitter, nor an increase in penetration of the impulse to more distant terminals of the axon. They believed that facilitation is pre-synaptic, a greater

number of quanta being released per impulse. Some observations made by them in the same study indicate a process of fatiguing of the action potential, and this they ascribe to a reduction in recruitment of quanta per impulse.

It would therefore seem likely that the observations reported in this chapter can be ascribed to changes not in the amount of transmitter per quantum, but in the numbers of quanta released per nerve impulse. The number released would seem to be nearly maximal in the fresh preparation and to reduce with fatigue. If the different fatigue patterns of Fig.31 are in fact representative of the definite differences illustrated in Fig.29, it is possible that the single innervating axon may have two different types of ending, in one of which quantal release in the fresh condition is maximal, and in the other it is sub-maximal. The former type will tend to fatigue more quickly, and to show less facilitation in the fatigued state. There may be graduations between the two.

All such speculation requires much more experimental evidence for substantiation, however. The only conclusion to be drawn - the lack of facilitation in fresh preparations - must be taken into account whenever any other than single stimulation is applied to a preparation for experimental purposes.



CHAPTER 7

THE MECHANICAL SLOW RESPONSE AND ITS RELATIONSHIP TO

ELECTRICAL EVENTS.

CHAPTER 7

THE MECHANICAL SLOW RESPONSE AND ITS RELATIONSHIP TO

ELECTRICAL EVENTS

INTRODUCTION

The variability of the responses, both electrical and mechanical, of arthropod muscle fibres has caused great difficulty in attempting to relate electrical and mechanical events to each other. In vertebrate striated muscle fibres it is generally accepted that electrical activity at the membrane is a pre-requisite of, and initiates, contractile processes; the majority of workers consider that contraction is a direct result of reduction of membrane potential (Kuffler, 1946; Katz, 1950; Sten-Knudsen, 1954, 1960; Watanabe, 1958), but some consider longitudinal internal current flow to be more important (Csapo & Suzuki, 1958). Invertebrate muscle fibre activity is much more difficult to explain satisfactorily in this way, for two reasons. Firstly, there is considerable variation in the amplitude of electrical activity observable in different fibres of the same muscle; secondly, many fibres are innervated by more than one axon, and the axon which produces the greater electrical activity does not necessarily produce the larger mechanical response (Wiersma & van Harreveld, 1938; Hoyle & Wiersma, 1958; Atwood & Hoyle, 1965).

The mechanical responses of the coxal muscles of Aeschna, as outlined in Chapter 4, are particularly interesting since a process of mechanical facilitation in which individual mechanical responses increase in size as well as summate seems to occur in the absence of any comparable electrical phenomenon at the membrane. This problem is explored in detail in this chapter, and the relevance to the problem of existing theories of linkage between excitation and contraction in other animals is discussed.

METHODS

Coxal muscle preparations, immersed in normal saline, were linked to the mechano-electronic transducer, and the electrical activity of randomly-selected fibres was observed with single micro-electrodes. Stimuli were applied to the crural nerve at carefully-measured frequencies via a suction electrode. The nerve was left intact in some preparations, but cut from the ganglion in others. Where a limited number of responses were to be measured, the remotely-connected shorting keys of the stimulator were used and the minimum of excess stimuli were applied in order to reduce fatigue effects as far as possible.

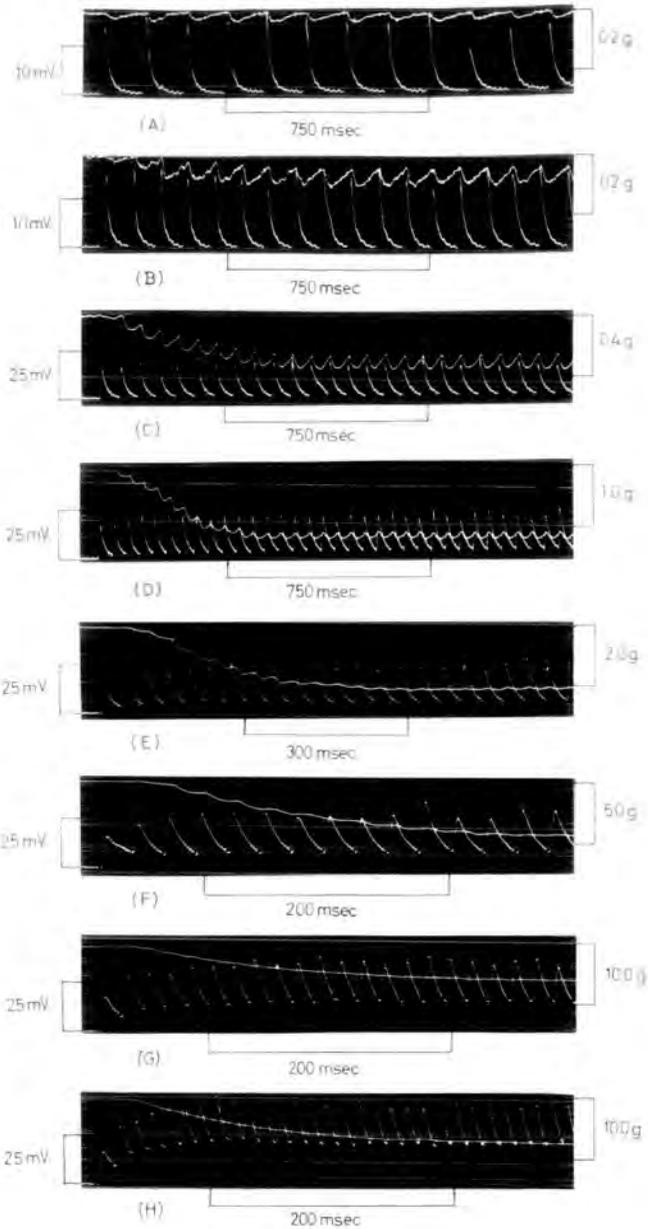
RESULTS

Application of trains of stimuli to the preparation produced responses similar in appearance to those of Fig.37. In this figure the upper trace follows the tension of the whole muscle and the lower trace shows simultaneous electrical activity in one of the fibres of the muscle. Each pair of traces shows the activity of the preparation immediately after initiation of stimulation, i.e. the first few responses of the train. In each case it can be seen that mechanical responses gradually increase and summate until a plateau is reached, and that the plateau is reached more rapidly as stimulus frequency is increased; the magnitude of the response is also increased.

There are two alternative ways of measuring the rate of application of stimuli. One is simply to measure the frequency, the other is to express the rate as the time interval between two successive stimuli - the stimulus gap. Each is equally valid, and in fact the stimulus gap is the reciprocal of the frequency. Data from the experiment of Fig.37 is plotted in each way in Fig.38; in Fig.38a the stimulus frequency is used as the X-axis, and in Fig.38b the stimulus gap is used. In each of these graphs two variables are plotted on the Y-axis - the mean size of the action potential and the base depolarisation.

FIGURE 37

The electrical and mechanical responses of coxal muscle
to repetitive trains of neural stimuli.



The Electrical and Mechanical Response to Repetitive Stimulation (Coxal Muscle)

	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)
STIMULUS GAP(msec)	150	100	70	50	40	30	20	15
FREQUENCY (stimuli/sec)	6.5	10	14.5	20	25	33	50	67

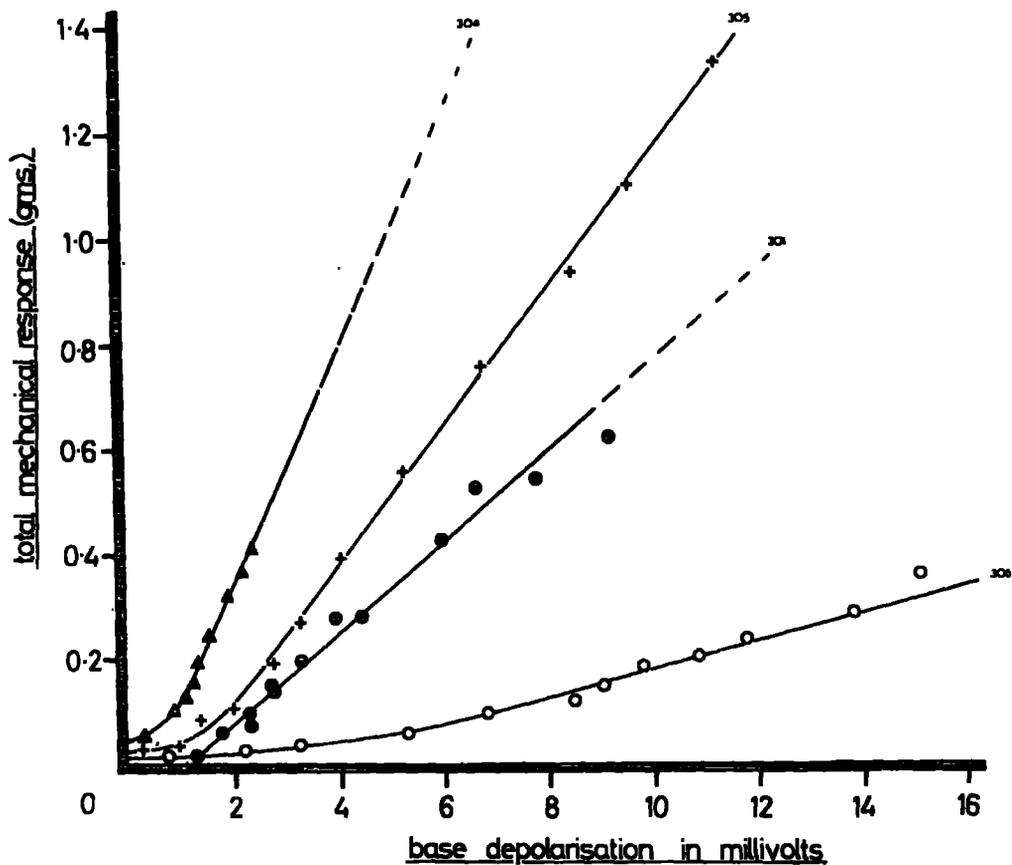
Appendix A. The mechanical response indicated is the maximum plateau level of tension developed. The experiment was carried out in the order of traces given in Fig.37, applying the lowest frequencies (largest stimulus gaps) first. The increase in size of the action potential with decreasing stimulus gap, shown in Fig.39b, which occurs in all experiments carried out, would seem to indicate a gradually increasing frequency-dependent facilitation, but this effect is caused by fatigue of the electrical response, and is a consequence of the progressive fatigue of the preparation (see Chapter 6). It is also probable that facilitation in a fatigued preparation is frequency-dependent. The decrease in size of action potential at the highest frequencies is probably a consequence of the large overall depolarisation of the membrane, since at these levels the potentials are superimposed upon a large base depolarisation; total membrane excursion is consequently very large.

These explanations of the variation of the action potential size clearly preclude any relationship between this variation and the amount of tension developed in the muscle. However, the amount of base depolarisation at each value of stimulus gap does seem to have an approximate numerical relationship with the tension developed, though there is

no causal relationship immediately evident. In Fig.40 the values of the plateau levels of tension plotted against base depolarisation for four experiments of this type; the four selected cover the range of results obtained from all experiments, and the small numbers labelling each curve identify the set of experimental results, which were calculated from the observed responses by computer (Appendix E). It can be seen from this figure that there is a general relationship between mechanical response and base depolarisation.

Base depolarisation varies greatly from fibre to fibre in a muscle block; it does not seem to be related at all closely to the size of the action potential in a fibre, and is probably a function of the rate of decay of the potential rather than of its maximum size. It is probably this variation which is responsible for the different gradients obtained in plots such as those of Fig.40, where mechanical events are those of the whole muscle, but electrical events are from representative fibres.

The lack of linearity of the relationships shown in Fig.40 at the lower values on each axis (produced at low stimulation frequencies) is thought to be a consequence of the fact that there is a little mechanical activity at low frequency even though the stimulus gap is too large to allow any summation of individual responses. Consequently there



THE RELATIONSHIP BETWEEN BASE DEPOLARISATION AND MECHANICAL SLOW RESPONSES

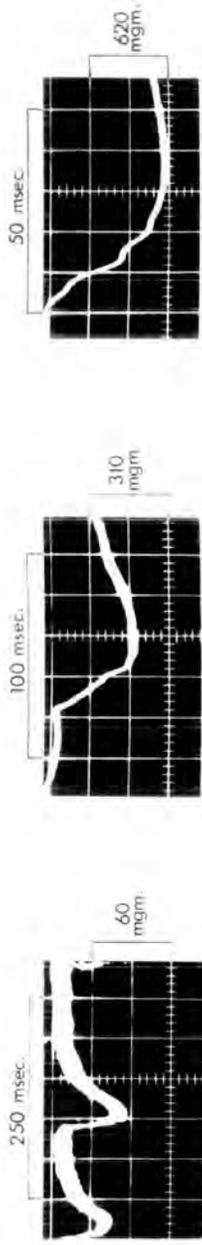
Figure 40.

is significant mechanical response in the absence of significant electrical base depolarisation. Mechanical and electrical fatigue must also have some effect, producing variation of responses in a non-linear way to some extent. Further experiments were therefore devised to try and offset these effects.

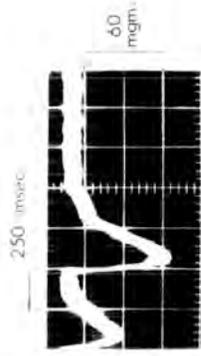
Mechanical responses to pairs of stimuli were observed and measured at different intervals between the two stimuli. Representative traces appear in Fig.41. The size of the response to the second stimulus was expressed as a ratio to that of the first; in those events where recording sensitivity was too low to measure response to the first stimulus, or where it was masked by response to the second, a mean value from other measurements was used. Detailed results of this experiment appear as Table 10, Appendix A, and are plotted in Fig.42. It is quite clear from this graph that the size of the response to the second of a pair of stimuli is related directly to the interval between the two, and that the relationship is an exponential one. Whatever factor of the active fibre phase is responsible for reinforcement of the effect of a subsequent active phase clearly dies away exponentially, as does the decay phase of an action potential. Only a minority of action potentials take 150msec to decay fully, however.

FIGURE 41.

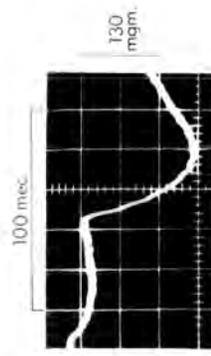
The mechanical responses of a whole coxal muscle to pairs of neural stimuli.



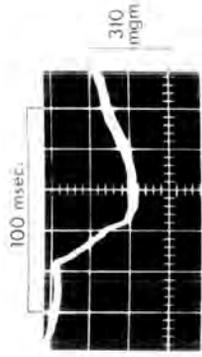
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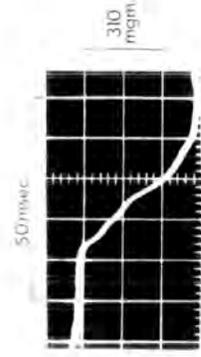
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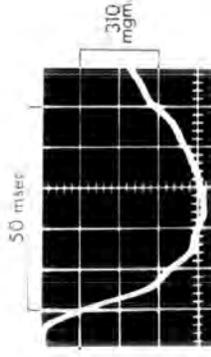
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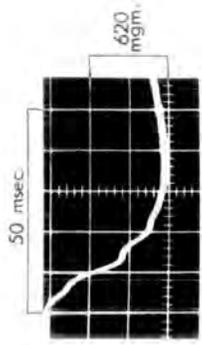
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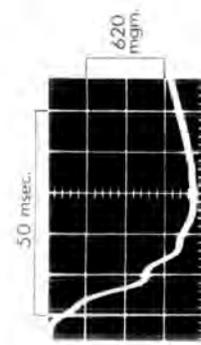
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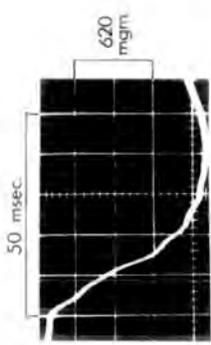
F



G



H



I

The Mechanical Response of Coxal Muscle to

Paired Stimulation

Stimulus Gap (msec.) A-150 B-100 C-50 D-40 E-25 F-14 G-6 H-3.5 I-2

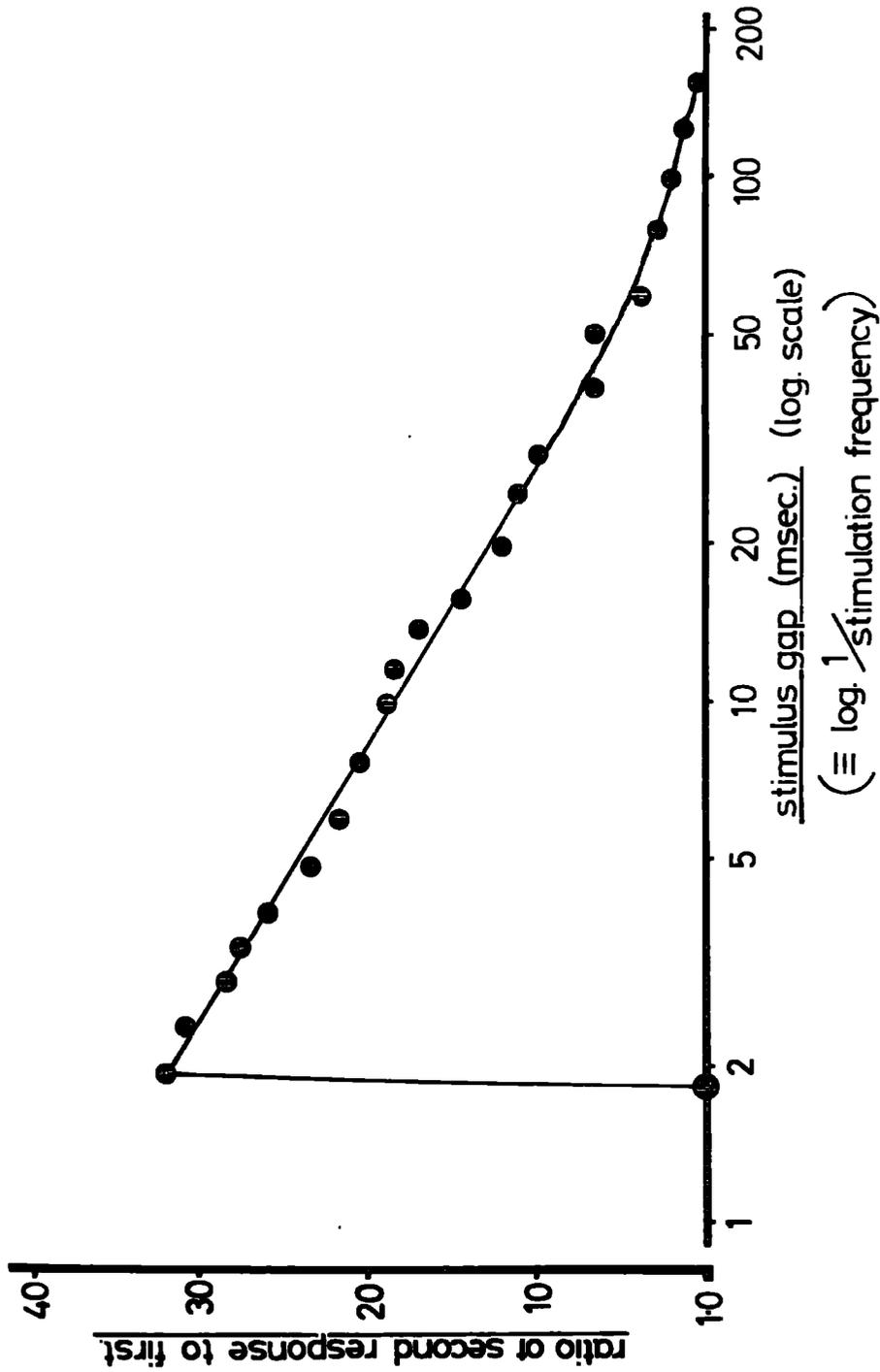


Figure 42.

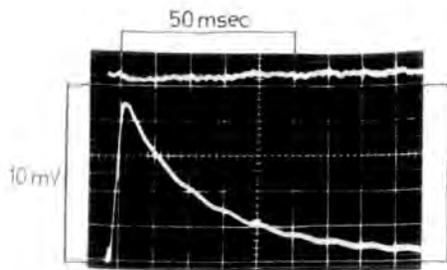
THE MECHANICAL RESPONSE OF COXAL MUSCLE
TO THE SECOND OF A PAIR OF STIMULI

A series of experiments was carried out in which the sizes and base depolarisations of the first few responses of a train were observed. Typical traces of this type of experiment appear in Fig.43. Several sets of responses were taken and averaged for each stimulus gap value, measurements being made of the size of each action potential in the train of responses, and of each mechanical response. Each of these mean values was then referred to the mean value of the first electrical or mechanical response and expressed as a ratio. In this way figures were obtained largely independent of the effects of fatigue, the first mechanical and electrical response acting as a control. Additional to these means and ratios, base depolarisation of the last (usually the fifth) potential to be observed was measured, and so was the overall mechanical response (summed) after the same number of responses. The long calculations were carried out by computer.

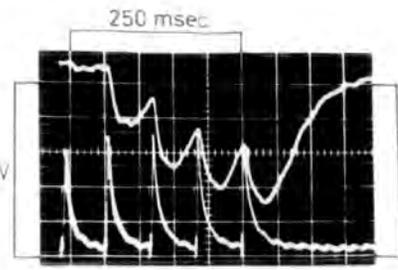
In Fig.44 total mechanical response after the final potential observed is plotted against base depolarisation at initiation of that potential from three representative experiments. These results are clearly very comparable to those obtained in the experiments of Fig.40 and indicate that the same general relationships apply between sub-maximal responses as between maximal ones, such as are illustrated.

FIGURE 43

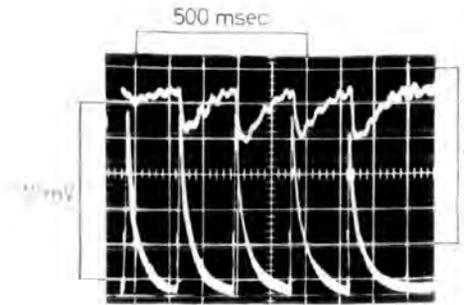
The electrical and mechanical responses of coxal muscle
to the application of short trains of neural stimuli.



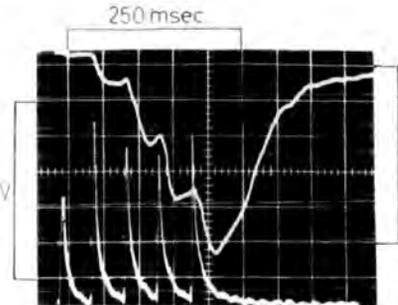
1000+ msec gap



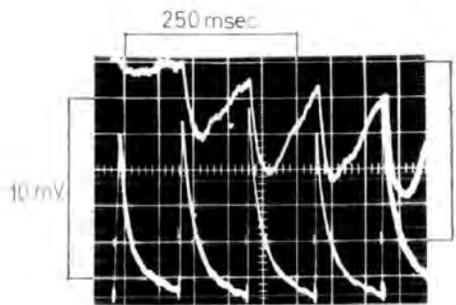
60 msec gap



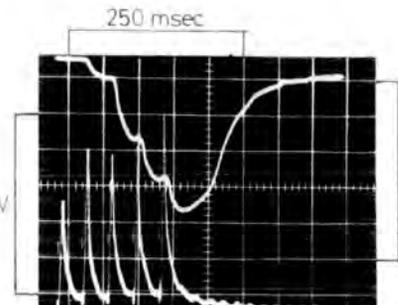
100 msec gap



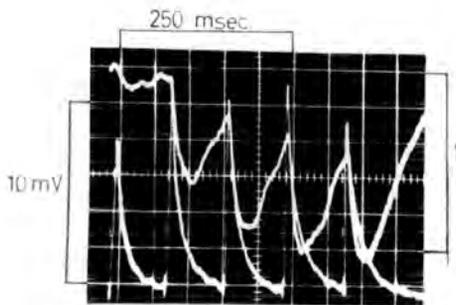
40 msec gap



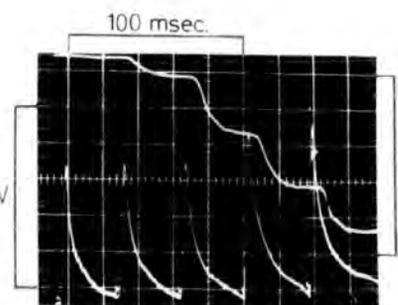
90 msec gap



30 msec gap



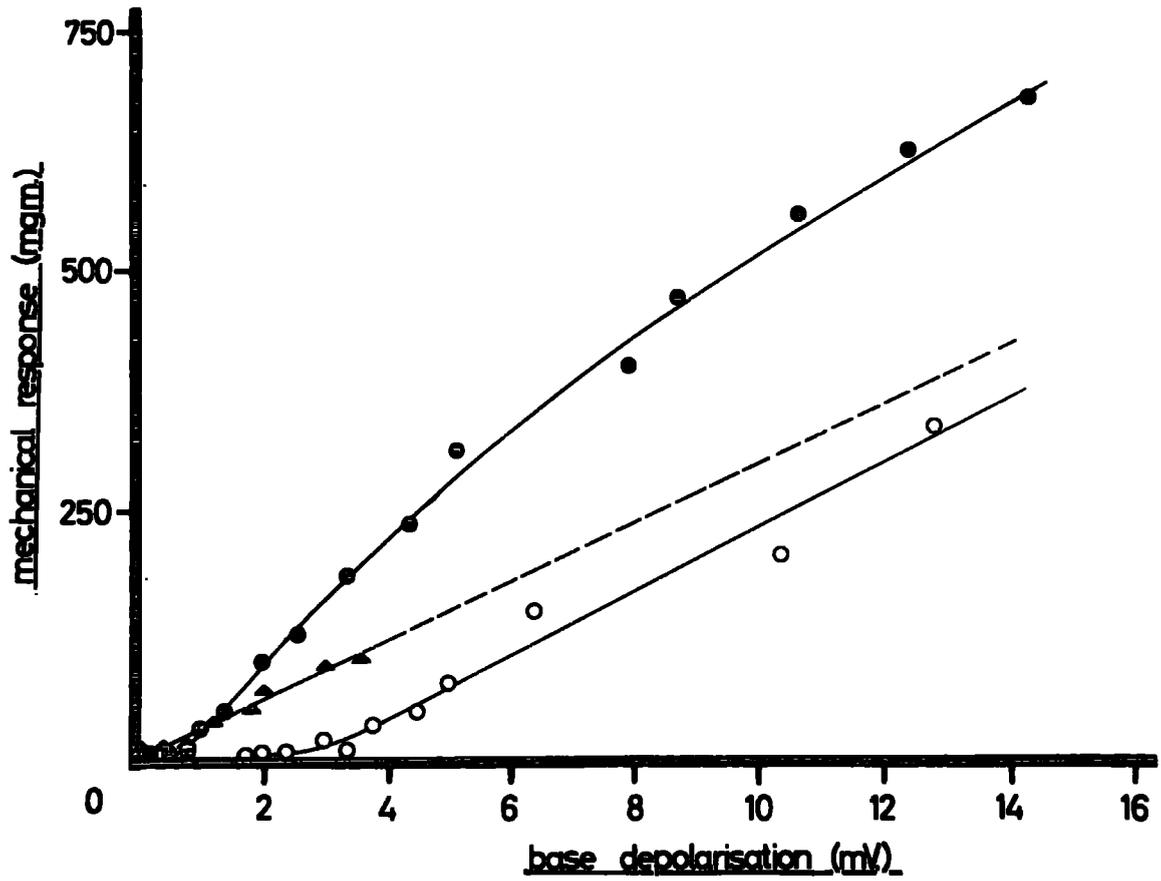
80 msec gap



25 msec gap

The Electrical and Mechanical Response to Multiple Stimulation.

(Coxal Muscle)

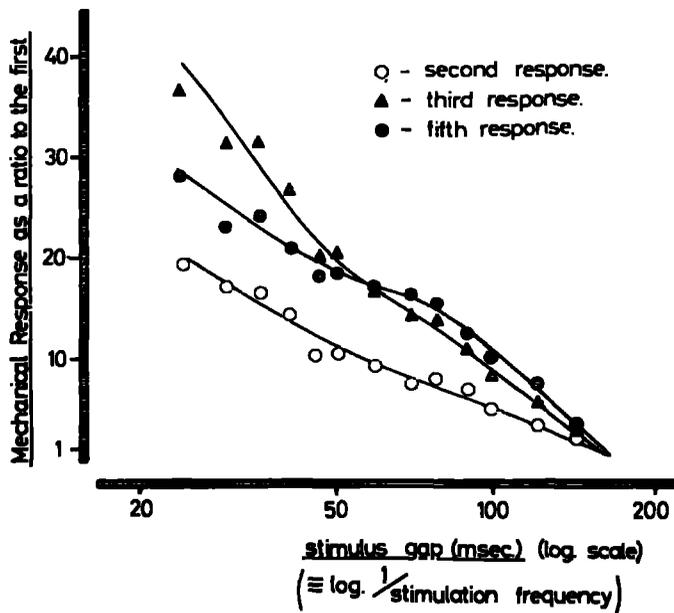
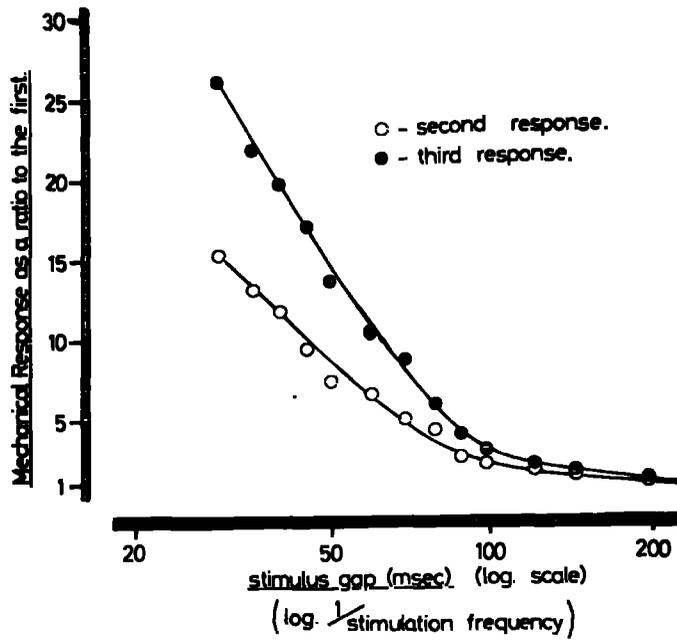


THE RELATIONSHIP BETWEEN BASE DEPOLARISATION AND
TOTAL MECHANICAL RESPONSE

Figure 44.

in Fig.40. In fact it is likely that the base depolarisation at initiation of the fourth or fifth responses is equal to the final plateau level, since experiments of the type of Fig.37 indicate that it is rare for equilibrium not to be reached within five responses.

In Fig.45 some of the results obtained from individual measurements of responses are plotted. Both types are expressed as ratios, as previously described, to eliminate fatigue effects. Size of mechanical responses to second, third, fourth and fifth stimuli were used to draw these graphs; in Fig.45a the line for the fourth and fifth response curves followed that of the third; in Fig.45b the fourth response curve followed that of the third. In the majority of experiments the response patterns were those of Fig.45a over the first six or seven responses, and subsequent responses followed the line of the fifth response of Fig.45b, where reduction in size seems to have occurred early in the response train. In other experiments the ratio of the size of the third response to that of the first at a stimulus gap of 30msec varied from about 10:1 to 40:1. In spite of this great variation in relative size, or degree of mechanical facilitation, the pattern of response remained the same. The actual value of the ratio was presumably dependent to some extent on the condition of the preparation.



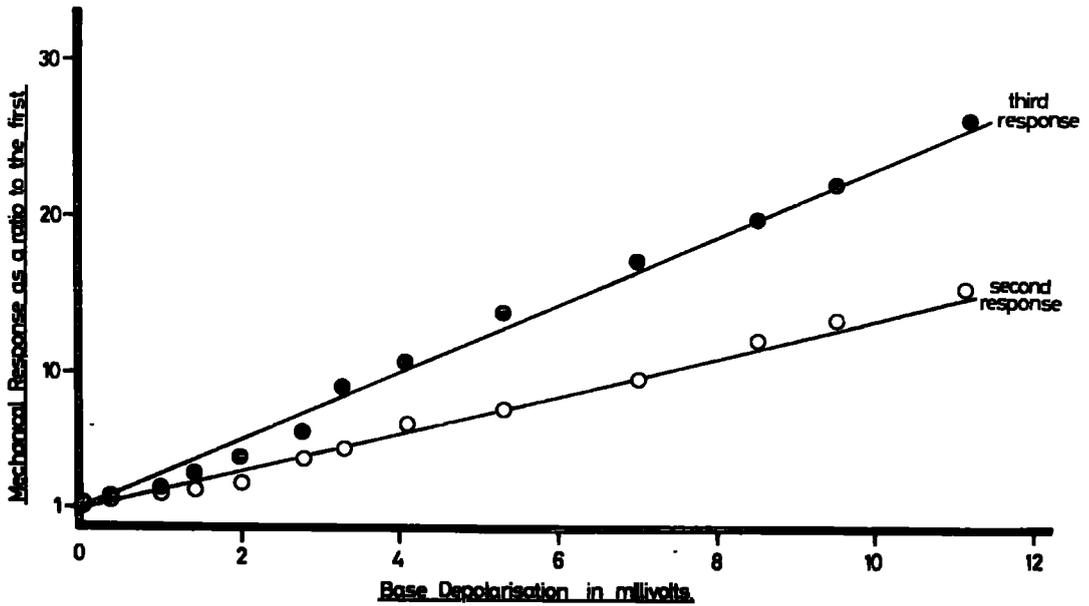
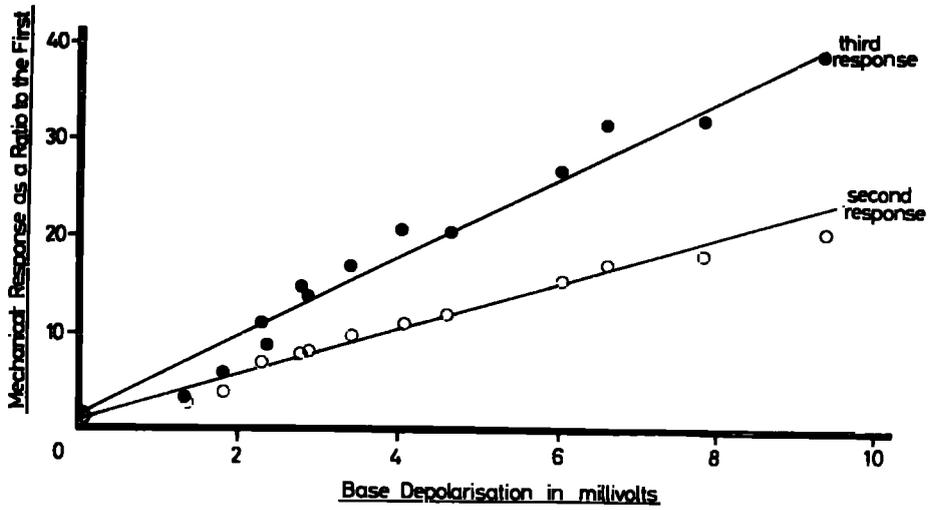
THE INDIVIDUAL MECHANICAL RESPONSE TO REPEATED SLOW STIMULATION

Figure 45.

When the sizes of individual responses such as these are plotted against base depolarisation, graphs are produced which are linear to a very high degree of significance. Fig.46a is a typical plot, and the graph appearing in Fig.46b is the best one obtained. The data from which Figs.45 & 46 were plotted is reproduced in Tables 11 & 12, Appendix A. The graphs of Fig.46, which are plotted to exclude effects of fatigue and non-linearities produced by the significant mechanical response to single stimulation indicate quite definitely the relationship between the base depolarisation and the size of the mechanical response.

DISCUSSION

It is the pattern of electrical and mechanical response to repetitive stimulation in which dragonfly muscle fibres most resemble those of crustaceans. The only major difference in appearance between the electrical responses illustrated in Fig.35 and responses similarly obtained by Kennedy & Takeda (1965a & b) in crayfish abdominal muscles, or by Hoyle & Wiersma (1958a & b) in Panulirus stretcher muscle is the absence of facilitation in dragonfly fibres in fresh condition. Repetitive stimulation of the slow nerve innervating insect muscles has not been shown to give large discrete e.p.p.s superimposed on a relatively small



**THE RELATIONSHIP BETWEEN BASE
DEPOLARISATION AND MECHANICAL
SLOW RESPONSES**

Figure 46.

plateau depolarisation in any species; instead large plateau depolarisations with little or no variation due to individual e.p.p.s are produced (Wood, 1958; Huddart, 1966.). The difference, of course, is only one of degree, the insect slow response to repetitive stimulation showing much more summation, often much more facilitation, and a greater total membrane potential excursion, than do crustaceans (Hoyle, 1957).

It is this relatively small excursion of the base level of polarisation, combined with the small size of the action potential, which produces the great difficulty in attempting to relate contraction, or development of tension, causally to membrane potential. In the case of dragonfly muscle fibres, difficulties are accentuated by the fact that the amount of depolarisation in response to neural activity varies considerably along the individual fibres (see Chapter 5) as well as by the inter-fibre variations which are found in other arthropods.

The tentative suggestion that tension development is initiated when the membrane potential is decreased to a threshold level, and is proportional to depolarisation thereafter, was made originally by Fatt & Katz (1953). Studies by Orkand (1962) in which he observed the tension developed by individual fibres when the membrane was depolarised directly have suggested that this might indeed be

the case, no tension being developed until a threshold potential, termed E_c , was reached. The problem of the "paradox phenomenon" of Panulirus closer muscle (Wiersma & van Harreveld, 1938), in which an apparently large mechanical response to small electrical activity evoked by slow-nerve stimulation could be observed alongside very little mechanical response and large electrical activity from fast-nerve stimulation, has been explained by Atwood & Hoyle (1965) in terms of the inter-fibre variability of E_c . They have found a few fibres in the muscle in which the depolarisation produced by slow-nerve activity is much greater than that evoked by the fast nerve, and suggest that these few fibres alone produce the mechanical activity observed in the whole muscle.

Atwood, Hoyle & Smyth (1965) also observed the mechanical response of single fibres to neural stimulation, and state that in no fibre could they observe tension development until neurally-evoked activity caused depolarisation in excess of E_c .

This angle of theoretical approach has been found to be tenable in several decapods as well as in other crustacea (Edwards, Chichibu & Hagiwara, 1965; Atwood, 1963a & b, 1965a & b) and any apparent "paradox" observable in the contraction of the whole muscle is usually attributable

to functional diversity of the fibres of that muscle into "tonic" and "phasic" types (Atwood & Dorai Raj, 1964). As yet no attempt has been made to apply these theories to muscle fibres such as those of locust or cockroach, and some difficulty can be expected here, in view of the large depolarisations produced by both fast and slow activity (Hoyle, 1957; Wood, 1958) and of the great differences in tension elicited by fast and slow stimulation (Usherwood, 1962b).

Hoyle & Wiersma (1958b) considered the mechanical response to repetitive stimulation was a function of the plateau depolarisation attained (i.e. the base depolarisation). This has been clearly shown to be the case in dragonfly muscle fibres, in experiments such as that of Fig.46. Attempts to apply the theories of membrane potential/tension linkage set out above are likely to be accurate only when experiments have been carried out in which the tension of single fibres is recorded. It is likely, however, that the effect of the base depolarisation is to lower the membrane potential sufficiently for individual action potentials to exceed E_c for that fibre. The conclusion of Chapter 5 that large action potentials occur on all fibres and that small observed potentials represent the product of decrementally-propagated large ones could be applied here to suggest that as base depolarisation increases with stimulation frequency more

of the membrane of each fibre reaches E_c , and more of the contractile mechanism is incorporated into the response. Conversely, less of the membrane is beyond the range in which the electrical response, as it spreads out, falls below the level necessary to exceed E_c .

The variability of observed base depolarisations may reflect different membrane properties such as time and length constants, rather than the size of potential or properties of the decay phase. There would thus be inter-fibre differences in base depolarisation, and consequently different fibres may have different proportions of their membrane in excess of E_c at the same stimulation frequency. Thus with increasing frequency greater areas of membrane of more fibres become depolarised sufficiently, and tension of the whole muscle increases accordingly.

The process of mechanical facilitation can also be explained in terms of the different E_c values of different fibres, since the process has been observed in the responses of whole muscle blocks. As base depolarisations in the various fibres advance in the early stages of response to repetitive stimulation, each fibre unit contracts more to each stimulus, since the membrane potential exceeds the depolarisation required to reach E_c by more in each response; also the fibres which show the greatest base depolarisation will contract the most during initial responses, only being

equalled by other fibres with lower base depolarisations when these have built up sufficiently.

Experiments of the type illustrated in Fig.43, carried out on a very few fibres or, better still, single ones will, it is hoped, give some idea of how nearly the above speculations are likely to be the truth. If they are found to be untenable it may become necessary to fall back on the idea of Hoyle & Wiersma (1958b) that tension is dependent on changes in membrane permeability to ions which do not greatly affect membrane potentials, e.g. to calcium ions, while electrical responses are due to movement of ions, monovalent cations for example, which are irrelevant to production of tension.

At the same time some cause must be sought, perhaps by the methods of Chapter 5, to explain differences between fibres in levels of base depolarisation. Further studies still, perhaps using drugs or changes in external ionic concentrations, will be needed to elucidate mechanisms by which membrane potentials control tension development.

CHAPTER 8

THE EFFECT OF VARIATION OF DIVALENT CATIONS ON THE

SLOW MUSCLE RESPONSE

CHAPTER 8

THE EFFECT OF VARIATION OF DIVALENT CATIONS ON THE

SLOW MUSCLE RESPONSE

INTRODUCTION

The ions which occur in greatest concentrations in association with excitable membranes are sodium and chloride (in the haemolymph) and potassium (in the myoplasm or axoplasm) (see, e.g., Florey, 1966). Many studies have been carried out in the nerve and muscle tissues of a great variety of animals to investigate the relations of these ions. Experiments involving the divalent cations, particularly calcium and magnesium, have shown increasingly in recent years that these ions, although present in living systems in concentrations small relative to those of sodium or potassium ions, play vital roles in the excitation and osmotic relations of membranes as well as in the initiation of contractions.

Calcium ions have a general role in the maintenance of the integrity of membrane systems. In the absence of calcium the membranes of both nerve and muscle undergo changes in permeability to potassium and sodium ions which render them inexcitable; potassium conductance is increased, as is the inactivation of sodium (Narahashi, 1964, 1965;

Werman & Grundfest, 1961; Werman, McCann & Grundfest, 1961; Wright & Tomita, 1965). Frankenhauser & Meves⁽¹⁹⁵⁸⁾ have demonstrated that magnesium has a qualitatively similar effect on nerve fibres at least, that it can replace calcium, but that it has about two-fifths the activity of calcium.

Techniques which circumvent the problems of excitation of muscle in abnormal calcium concentrations, using potassium contracture, have confirmed that calcium must be present in muscle fibres before they will contract (Frank, 1960, 1964; Neidergerke, 1956; Lorkovic, 1967), and the observation of Hoyle (1961) that contraction occurs in calcium-free conditions in the spiracular muscle of locust has been criticised (Aidley, 1965). It is now generally assumed that calcium ions form an essential part of the excitation-contraction link in muscle (Aidley, 1965); influxes of calcium have been observed in muscle during action potentials (Bianchi & Shanes, 1959), though a similar influx has been observed in nerve (Hodgkin & Keynes, 1957). Gilbert & Fenn (1957) have described a calcium pump in frog muscle which extrudes the ion from the resting cell.

The most obvious effect of calcium and magnesium levels on vertebrate neuromuscular preparations is a result of the close relationship between transmitter release and the concentrations of these ions. An increase in the quantum content of the acetylcholine released by a nerve

impulse at the pre-synaptic membrane of vertebrate twitch neuromuscular junctions is brought about by an increase in external calcium concentration, or a decrease in magnesium concentration; conversely a decrease is produced by calcium deficiency or magnesium excess. The amount of acetylcholine contained in each quantum is unaffected (del Castillo & Stark, 1952; del Castillo & Katz, 1954a. & b; Katz & Miledi, 1965, 1967). Further, an excess of one of the two ions can counteract the effect of a deficiency of the other (del Castillo & Engbaek, 1954). Magnesium ion also has an effect on the post-synaptic membrane, producing a decrease in the sensitivity of the membrane to directly-applied acetylcholine (del Castillo & Engbaek, 1954).

It is clear that a similar dependence of transmission mechanisms upon calcium and magnesium ions is present in the junctions of arthropods (Fatt & Ginsborg, 1958; Hoyle, 1955a; Dudel & Orkand, 1960; Dudel & Kuffler, 1961a; Rathmeyer, 1965), although in some cases adaptation to abnormal ionic levels, particularly of magnesium, has been necessary (Wood, 1957).

A knowledge of the quantitative dependence of the responses of Aeschna upon magnesium and calcium levels would be a useful tool in investigations of the spread of electrical activity along the muscle fibre membrane, and studies of the excitation-contraction link involving

alteration of ionic levels of either calcium or magnesium will clearly need to be interpreted in the light of other known effects of those ions. Consequently an investigation of the effects produced by changes in external concentrations of the two ions was undertaken, and some preliminary results are reported in this chapter.

METHODS

Coxal muscle preparations, with ganglia removed to avoid spontaneous activity of neurons, were used in all experiments. In order to follow the effects of changes of ions on both electrical and mechanical responses, paired stimuli were applied to the preparation via a suction electrode, evoking two end-plate potentials and a significant mechanical response to the second of the stimuli. By this means facilitation effects, if any appeared, could also be followed.

Preparations were set up and bathed initially in normal saline. Experimental salines were introduced into the experimental chamber and unwanted saline drained away in the manner described in Chapter 2. Experimental salines were made up with varying amounts of calcium or magnesium. It was not thought necessary to substitute any other cation in cases of deficiency, or to remove any other constituent in cases of excess, since in no case did the change in

overall molarity of the saline exceed 11mM, which is about 4% of the total.

Experimental salines were made up in quantities of 100mM, which was about four times the capacity of the experimental chamber. The whole 100ml was added to the chamber within 10 seconds during a change, excess saline draining away equally rapidly, and it is likely that the relevant ionic changes were 90% complete within two seconds, since there was considerable turbulence within the chamber during the change.

Because of the variability in response of fibres, micro-electrodes were left in the same fibres throughout each experiment. In most experiments only a single micro-electrode was used. After initial recordings of electrical and mechanical activity under normal saline conditions the saline was changed as rapidly as possible without dislodging the electrode.

THE EFFECT OF CHANGES IN CALCIUM

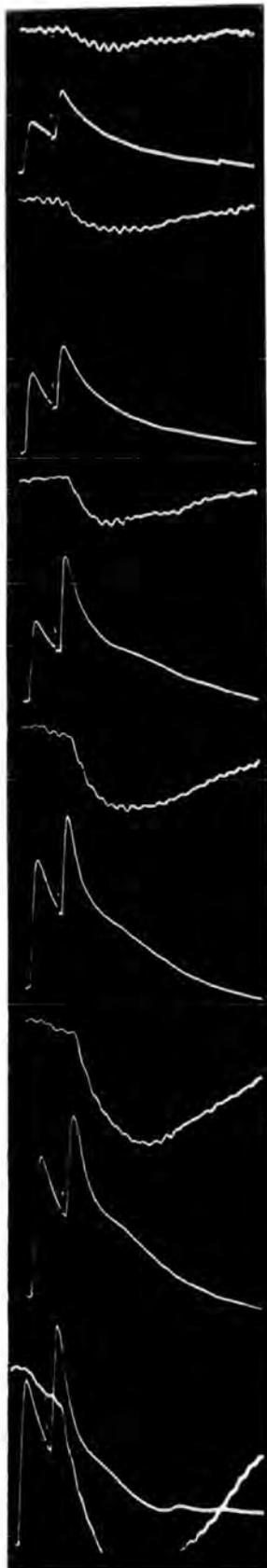
Within a few seconds of a change in the calcium level of the saline, changes were apparent on both electrical and mechanical responses of the muscle to neural stimulation. All changes were found to be fully and completely reversible when normal calcium levels were restored. No changes in either calcium or magnesium levels have been observed to have

any significant effect on the resting potential.

In order to obtain an impression of the time-course of the changes in response produced by changes in calcium level, paired stimuli were applied to the preparation at a steady rate of 15 per minute (one pair every four seconds). This rate was chosen to reduce fatigue effects such as those described in Chapter 6 to a minimum. At least twenty pairs of stimuli were applied to the preparation at this rate while it was bathed in normal saline; continuous recordings were then made during and subsequent to the changing of salines.

Figs.47 & 48 illustrate the changes in mechanical and electrical responses brought about by altering the saline; in Fig.47 the experimental saline contained twice the normal level of calcium (20mM instead of 10mM/l) and in Fig.46 half the normal level. In spite of the usual fluctuations of the electrical response it is evident from these traces that changes in response occur almost as quickly as the saline is changed; the effect is so quick that any lag must be due simply to uneven changes of saline locally within the muscle, a delayed washing-out or penetration.

The recordings of a series of such experiments were measured, and in the graphs which form Figs.49 - 51 the sizes of individual responses are plotted against time.



Time - 0

50msec

Time - 12 seconds

100 mgm (upper trace)

25 mV (lower trace)

Time - 16 seconds

Lower Trace

The electrical response to paired neural stimulation, 20msec gap

Upper Trace

Time - 24 seconds

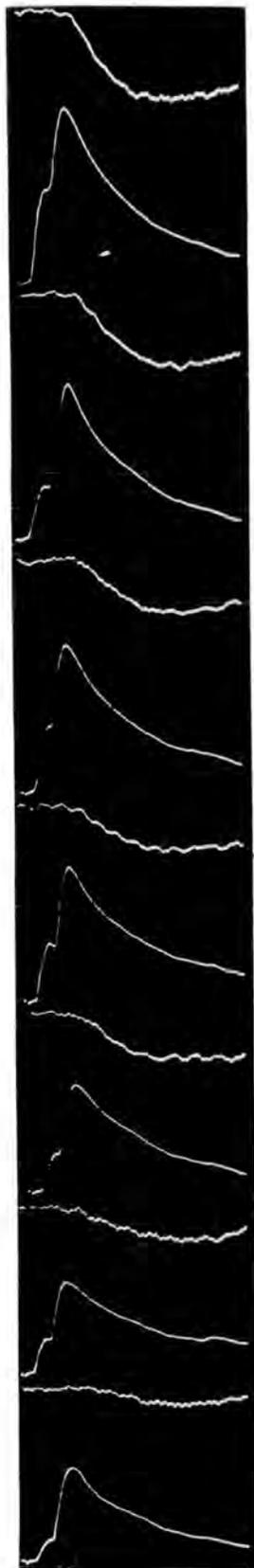
The simultaneously-recorded mechanical response of the whole muscle

Time - 30 seconds

THE EFFECT OF DOUBLED
SALINE CALCIUM ON THE
SLOW RESPONSE

10mM/l. - 20mM/l.

Time - 1 minute



Time - 0

25msec

Time - 12 seconds

100 mgm. (upper trace)

10 mV (lower trace)

Time - 24 seconds

Lower Trace

The electrical response to paired neural stimulation, stimulus gap 5msec.

Time - 27 seconds

Upper Trace

The simultaneous mechanical response of the whole muscle

Time - 36 seconds

Time - 48 seconds

THE EFFECT OF REDUCED SALINE CALCIUM ON THE SLOW RESPONSE

Time - 1 minute

10mM/l - 3mM/l

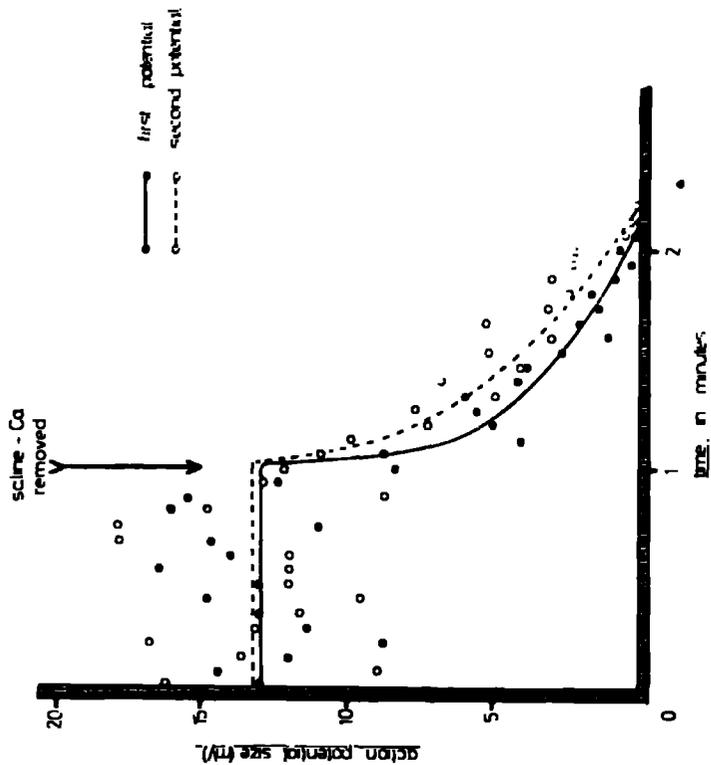
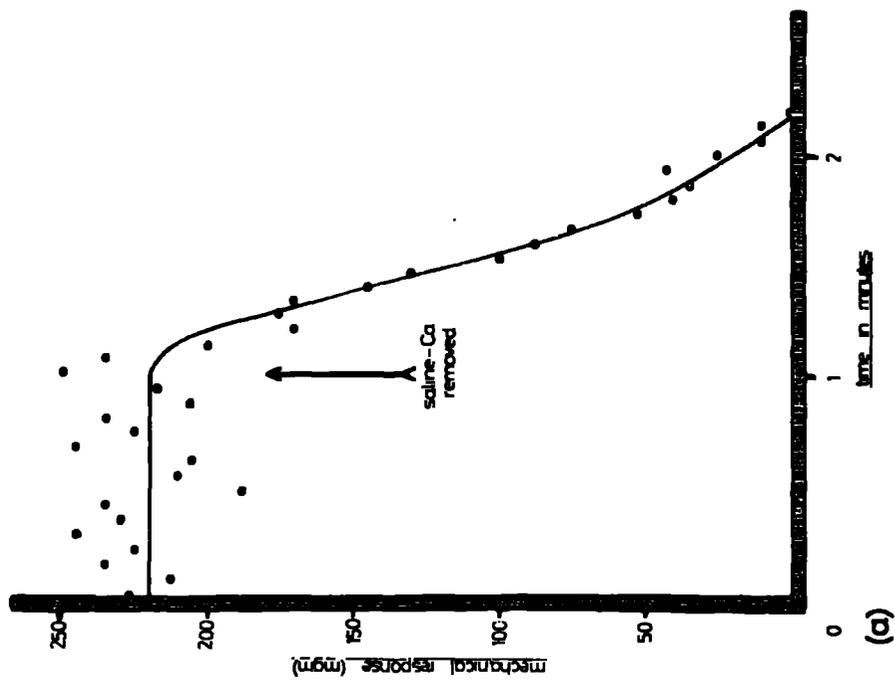
Figure 48.

from initiation of saline change. Each point represents a single response, there being one every four seconds; consequently there is some scatter of the points due to the normal variation of the electrical responses.

In the experiment of Fig.49 calcium-free saline was introduced into the experimental bath. Both electrical and mechanical responses were abolished within one minute, the process being completely reversible.

In doubled saline concentration (Fig.50) the size of the response to single stimulation (upper line of Fig.50a) approximately doubles within one minute; in this very short time the change was from $15.35\text{mV} \pm 0.50$ (s.e.) to $32.05\text{mV} \pm 0.55$ (s.e.). These values are averaged from the twenty responses recorded before the saline was changed, and twenty responses recorded consecutively commencing one minute after the change. Results obtained in exactly the same way from an experiment in which saline calcium was reduced from 10 to 3mM/l show the change to be from $11.10\text{mV} \pm 0.30$ to $4.60\text{mV} \pm 0.30$. In Fig.51 individual responses from this experiment are plotted.

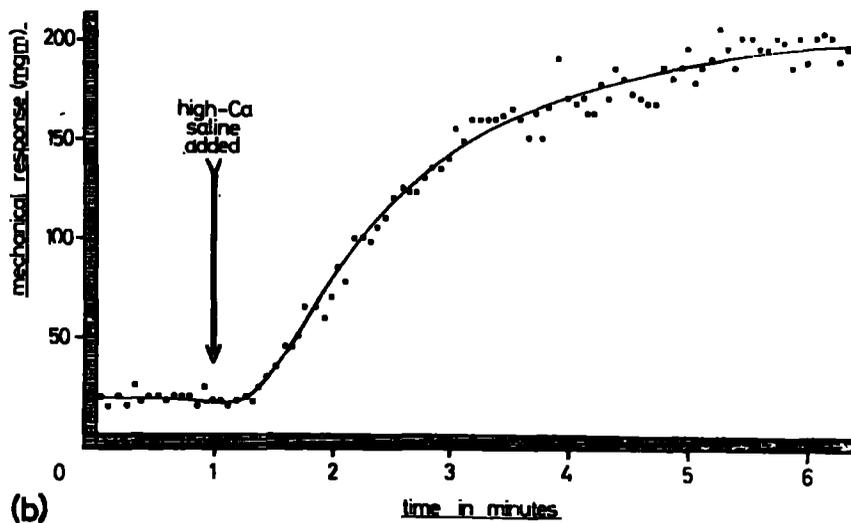
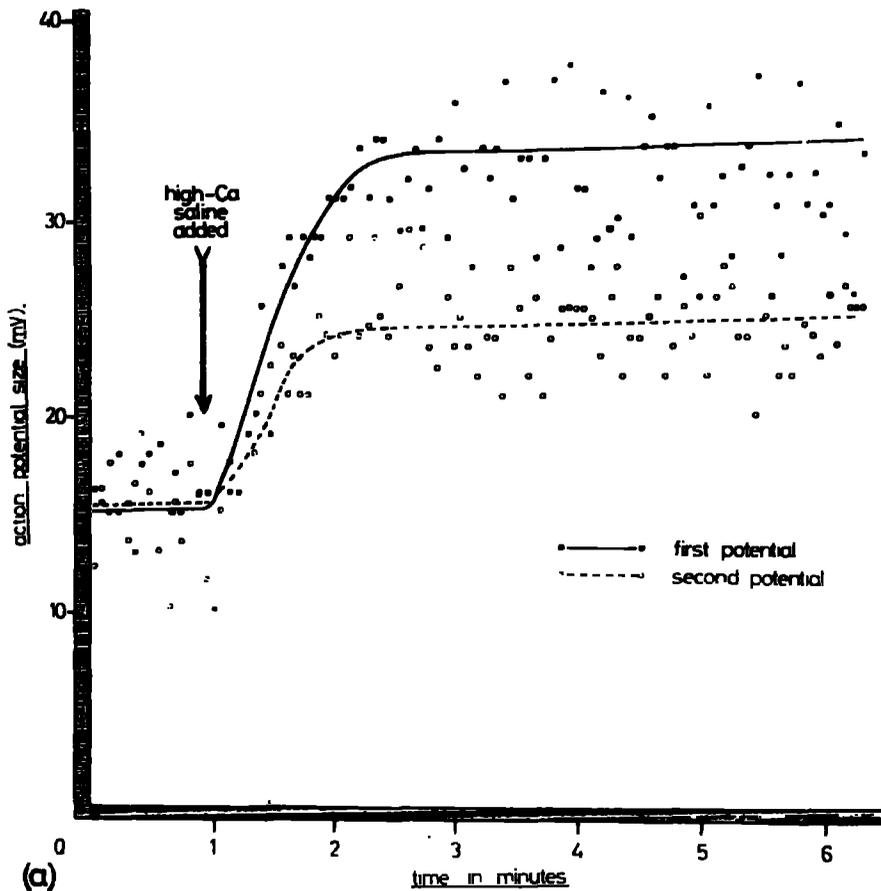
It will be seen from Figs.50b & 51b that the mechanical response to the second stimulus of the applied pair increases or decreases in a way parallel to changes in electrical response. In fact in increased calcium a mechanical response to the first (effectively single)



THE EFFECT OF CALCIUM-FREE SALINE ON THE SLOW RESPONSE

- (a) The mechanical response of the whole muscle to the second of a pair of stimuli, 5msec gap.
- (b) The simultaneous electrical activity of a superficial fibre.

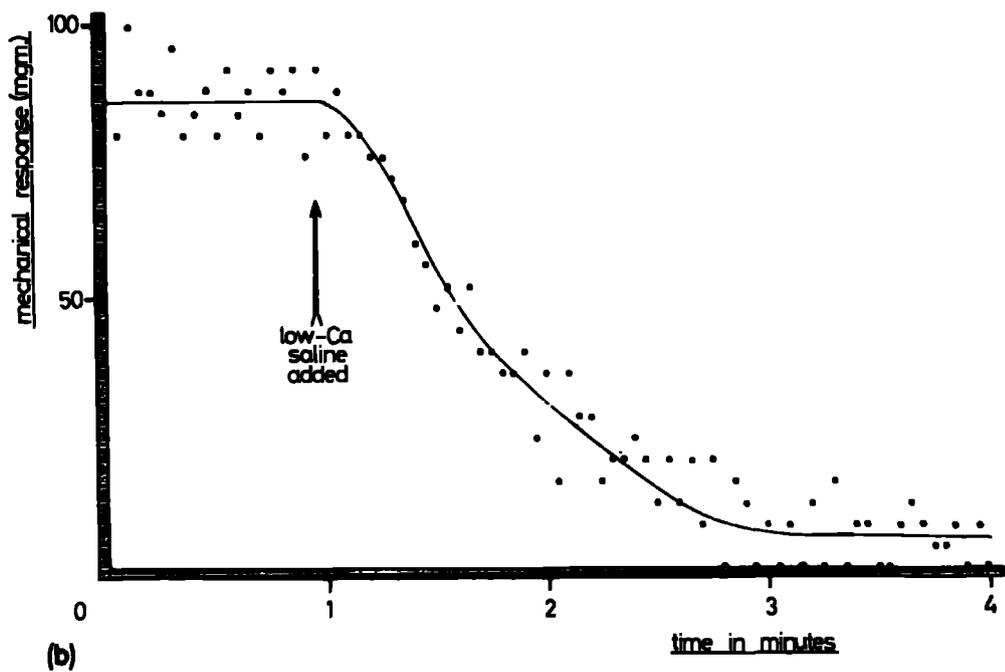
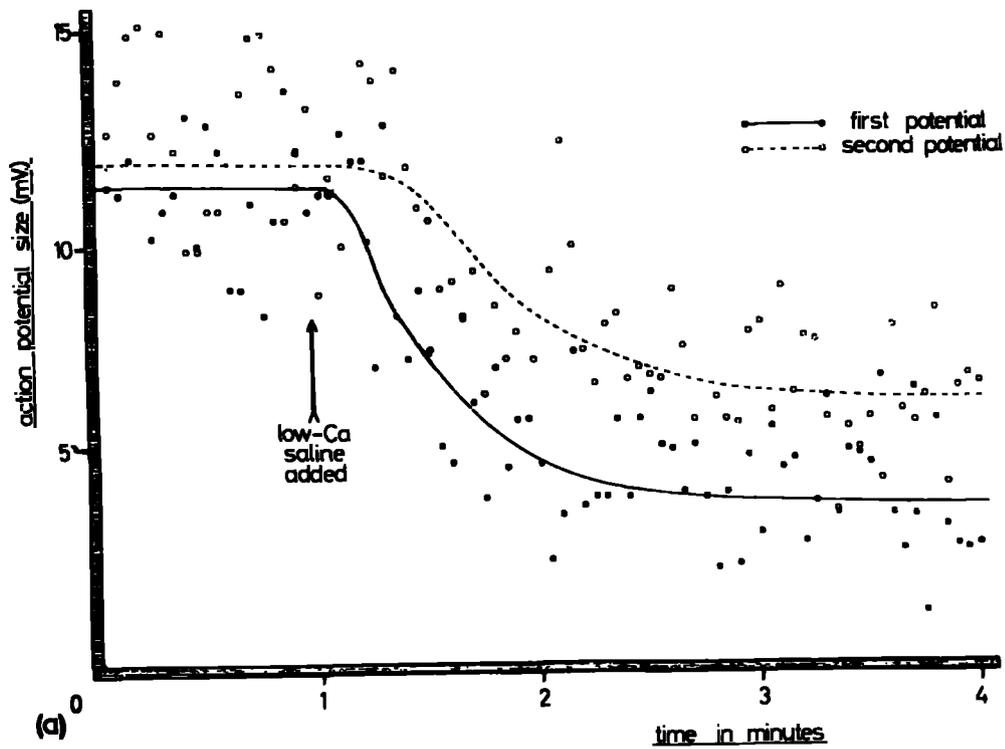
Figure 49.



The Effect of Doubled Saline Calcium (10mM/L-20 mM/L) on the Slow Response

- (a) The effect on the responses of a superficial fibre to paired neural stimulation, stimulus gap 20msec.
 (b) The simultaneous effect on the mechanical response of the whole muscle to the second stimulus.

Figure 50.



The Effect of Calcium Deficiency on the Slow Muscle Response

- (a) The effect of 3mM. Calcium - Saline on the electrical response to paired neural stimulation, stimulus gap 5msec.
- (b) The simultaneous mechanical response of the whole muscle to the second stimulus.

Figure 51.

stimulus rapidly develops, in addition to the increase in size of the response to the second stimulus. This can be seen in the traces of Fig.47.

Observations of the responses evoked by paired stimuli show that the effect of changed ionic conditions have the greatest effect upon the first response; the second response is much nearer in size to responses evoked under normal ionic conditions. Thus in reduced saline calcium (Fig.51) there is an apparent facilitation of the response, and in increased saline calcium the second response is smaller. Changes in the sizes of both responses, and in the facilitation of the second response by the first, are summarised in Table 4. As a general rule the change in size produced in the second response is about half that produced in the first.

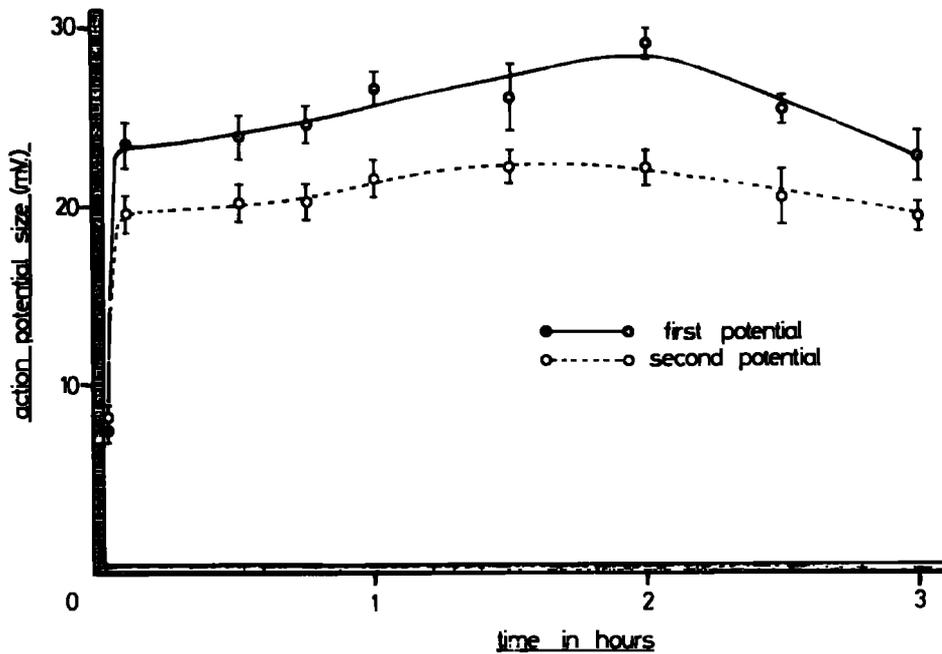
When trains of responses are evoked by repetitive stimulation responses subsequent to the second decline (or increase) still further, though the original size is not reached.

In order to discover whether these rapid changes were in any way transitory, a preparation was left for some time in an experimental saline containing 20mM/l calcium - double the normal amount. The response to paired stimulation in the graphs of Fig.52 were obtained by sampling at intervals; detailed results appear in Table 13, Appendix A.

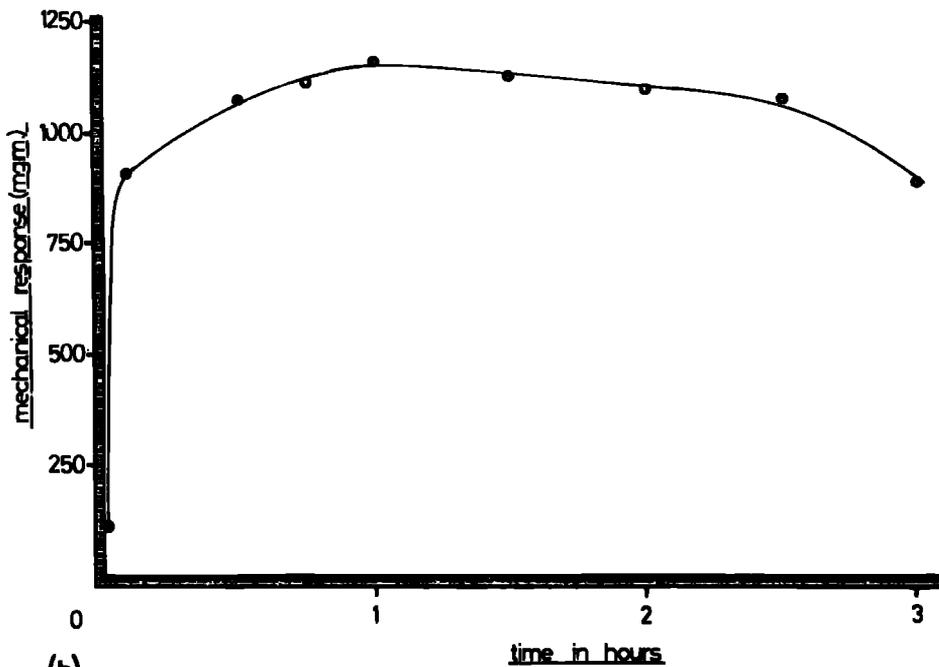
TABLE 4

The Immediate Effect of Changes in External Calcium Ion Concentration upon the Electrical Slow Response to Paired Stimuli

	RESPONSE TO FIRST STIMULUS (mV) ± s.e.	RESPONSE TO SECOND STIMULUS (mV) ± s.e.	FACILITATION ± s.e.
Initial (10mM/l)	15.35 ± 0.50	15.50 ± 0.55	1.0 ± 0.06
1 min after change to 20mM/l	32.05 ± 0.55	24.75 ± 0.70	0.78 ± 0.02
Initial (10mM/l)	11.10 ± 0.30	12.55 ± 0.45	1.13 ± 0.07
1 min after change to 3mM/l	4.60 ± 0.30	7.40 ± 0.40	1.75 ± 0.15



(a)



(b)

The Effect of Doubled Saline Calcium on the Slow Response

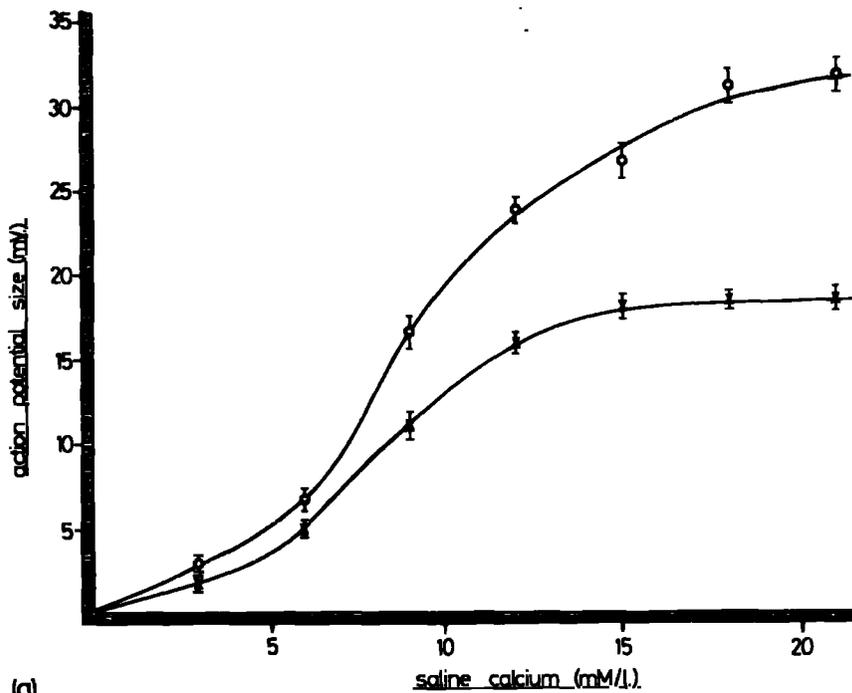
- (a) The effect of 20mM. Calcium - Saline on the electrical response to paired neural stimulation, stimulus gap 5msec.
- (b) The simultaneous mechanical response of the whole muscle to the second stimulus.

Figure 52

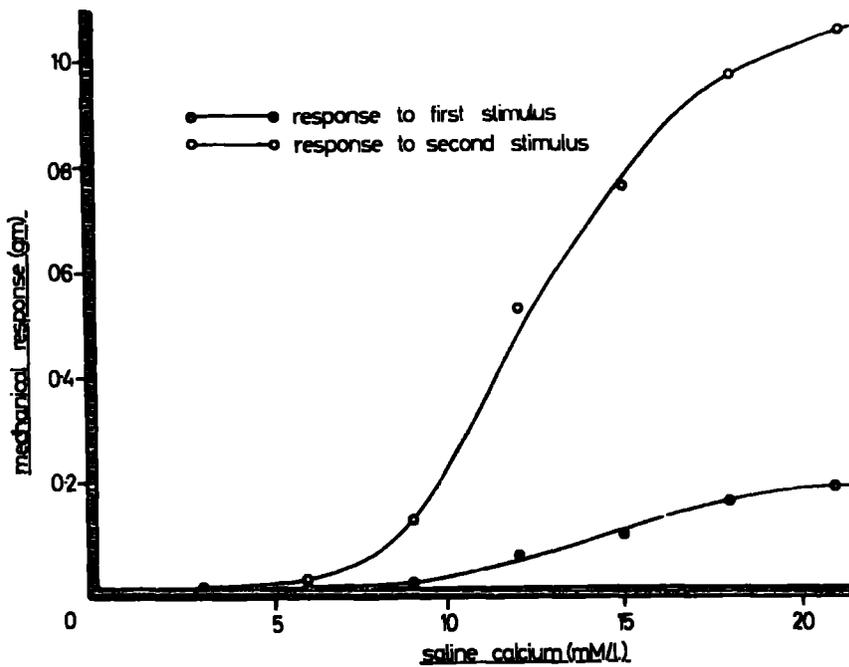
These results show that there is a second phase of increase in size of both mechanical and electrical response after the initial rapid change. There is a gradual change-over the first hour of exposure to doubled calcium concentration.

The decline in size subsequent to the first hour seen in the experiment of Fig.52 is attributed to the effects of fatigue. The train of 25 responses evoked, and averaged to obtain the mean values plotted as points on the graph, showed a steady progressive decline in size. In the period of rest between each sampling time there was some recovery of size. In view of the fatigue observed in the much smaller normal responses it is very likely that the process is accentuated under these conditions. The minor differences in apparent time-courses of mechanical and electrical effects are not significant; errors introduced by observing single-fibre electrical responses and whole-muscle mechanical responses are entirely adequate to explain this diversity.

In the experiment of Fig.53 (detailed results - Table 14, Appendix A), two fibres were impaled with separate micro-electrodes and the responses of these fibres observed consecutively as described in Chapter 6. Paired stimuli were applied, though only the responses of each fibre to the first stimulus are plotted in Fig.53a. In Fig.53b mechanical response of the whole muscle to each of the two stimuli are plotted.



(a)



(b)

The Relationship Between the Slow Muscle Response and the Level of the Saline Calcium

- (a) The effect of variation of saline Ca on the size of action potential evoked by neural stimulation in two fibres simultaneously.
- (b) The corresponding mechanical response of the whole muscle to the same stimulus, and to a second, 25 msec. gap.

Figure 53.

The preparation, after being set up in normal saline, was immersed in 3mM/l calcium saline and allowed to equilibrate for 15 minutes. Fifteen responses to paired stimuli (25msec gap) were then recorded; the experimental saline was changed to one containing 6mM/l calcium, and the same process repeated. Further recordings were made in the same way at various calcium levels, the amount being increased at each change by 3mM/l. In this way any problems caused by retention of calcium ions, either intracellularly or in extracellular spaces within the muscle block, were evaded.

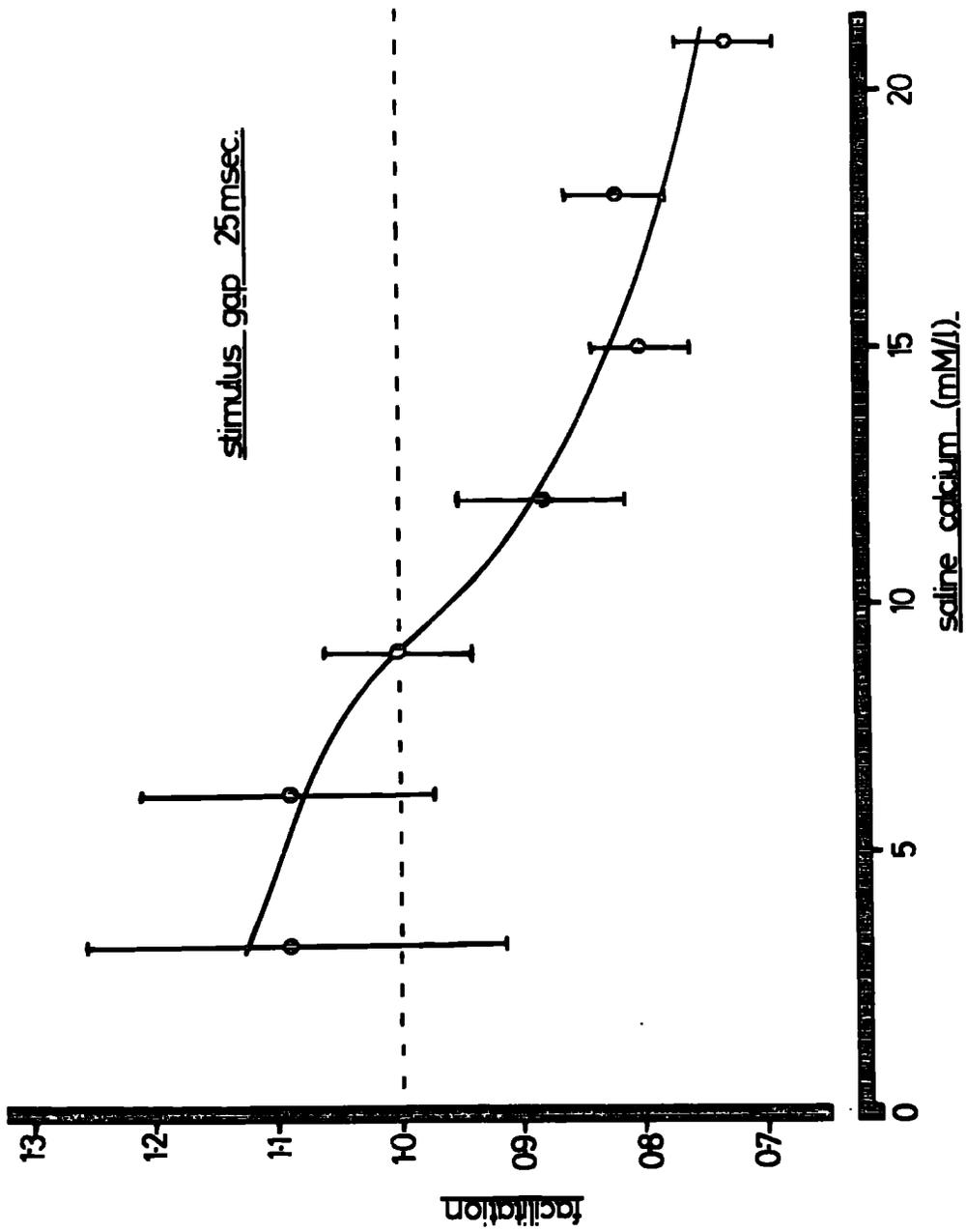
As can be seen in Fig.53a there is an S-shaped relationship between potential size and calcium level in the saline. Increase or decrease of size with changed ionic conditions does not change the relative sizes of potentials which exist under normal conditions - the largest potentials increase in size proportionally as much as do the smaller ones. However, there are indications from this and other experiments that the smallest potentials reach maximum size at lower levels of calcium than do larger ones, i.e. the relating curve levels off into a plateau earlier (see Fig.53a).

Increases in size of mechanical responses with increase in calcium seem to follow a relatively similar shaped curve to that of electrical responses; however, that part of the curve which represents lower levels of calcium does not appear because there is no mechanical

response to single or even to paired stimuli below certain levels of calcium. This may be related to threshold levels of membrane polarisation such as were referred to on the previous Chapter. At all levels of calcium at which electrical activity can be detected, mechanical responses can be evoked by repetitive stimulation, but as many as ten stimuli may be required in succession before any tension is developed.

Fig.54 is drawn from some of the same data as was used to plot the graphs of Fig.53, and illustrates the tendency of potentials subsequent to the first in a pair or train to vary from level to level of saline calcium to an extent rather less than does the first. At levels of calcium less than normal second responses are facilitated by the first, but at higher calcium levels they are considerably depressed (ratio of less than unity). Detailed results of this experiment appear in Table 15, Appendix A.

It is clear from these experiments that the size of the end-plate potential response is closely dependent upon external calcium levels. The relationship between mechanical responses and external calcium, while evidently close, is not so easy to discover from these experiments because of the differences in size between individual responses of a train. Mechanical responses to single stimuli, just detectable at normal calcium levels, are absent at any lower level, but may be of considerable magnitude at



THE EFFECT OF VARIATION OF SALINE CALCIUM ON THE FACILITATION OF THE SLOW ACTION POTENTIAL

Figure 54.

higher concentrations. This can be seen in Fig.53b. In an effort to discover the basic pattern behind these changes an experiment was conducted following the same procedure, but recording the mechanical responses to each stimulus of a train. The results are plotted in Fig.55.

The general pattern of changes in the development of tension can clearly be seen. As external calcium is increased, tension is developed by earlier responses of a train. A level is reached at which the very first response produces tension. With increasing external calcium the earlier responses make the greatest contribution to total tension.

THE EFFECT OF CHANGES IN MAGNESIUM

Only a small number of experiments have so far been carried out in which the levels of magnesium have been altered. The results which follow must therefore be considered to be preliminary, and not fully substantiated.

All experiments have been of the type described above in which rapid changes in response to paired stimuli have been followed by means of steady stimulation; in these experiments pairs of stimuli were applied at 20/min (one per three seconds). Results obtained from changes of external magnesium from the normal level of 6mM/l to 12mM, to 3mM and to zero are given in Figs.56, 57 & 58 respectively. As was

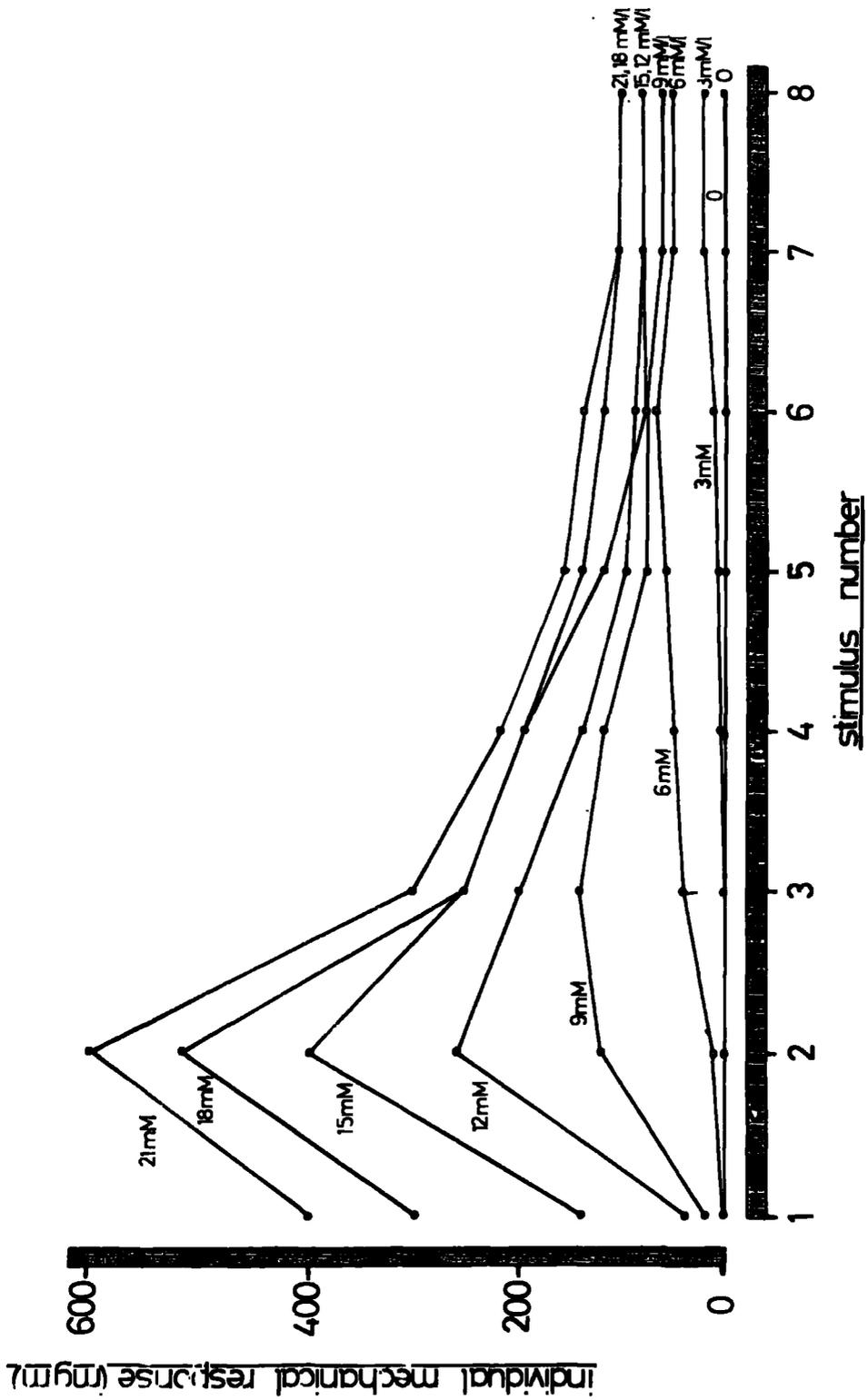


Figure 55.

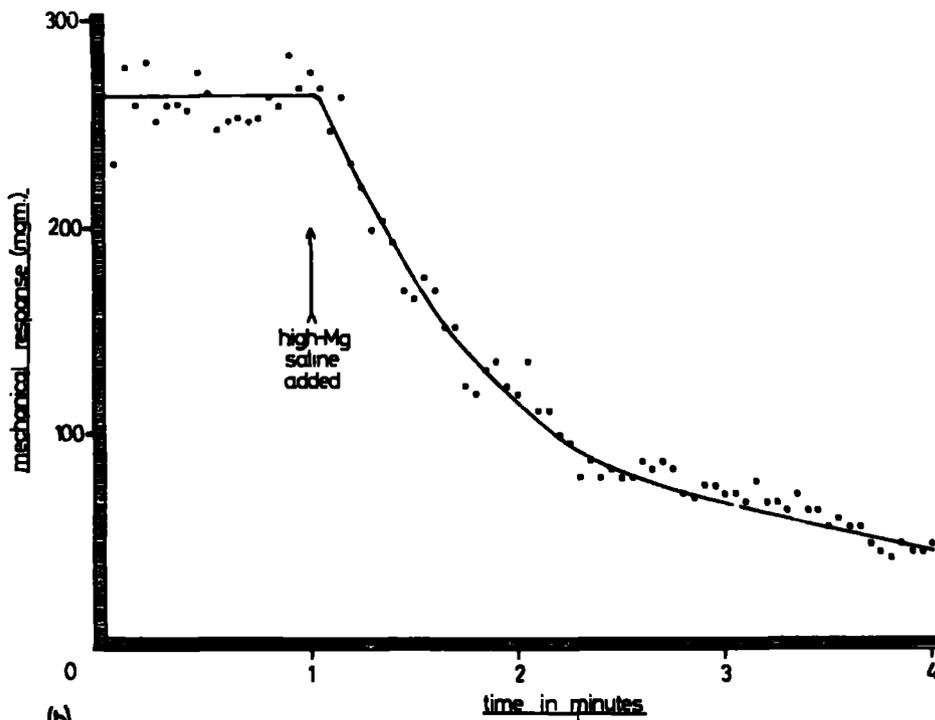
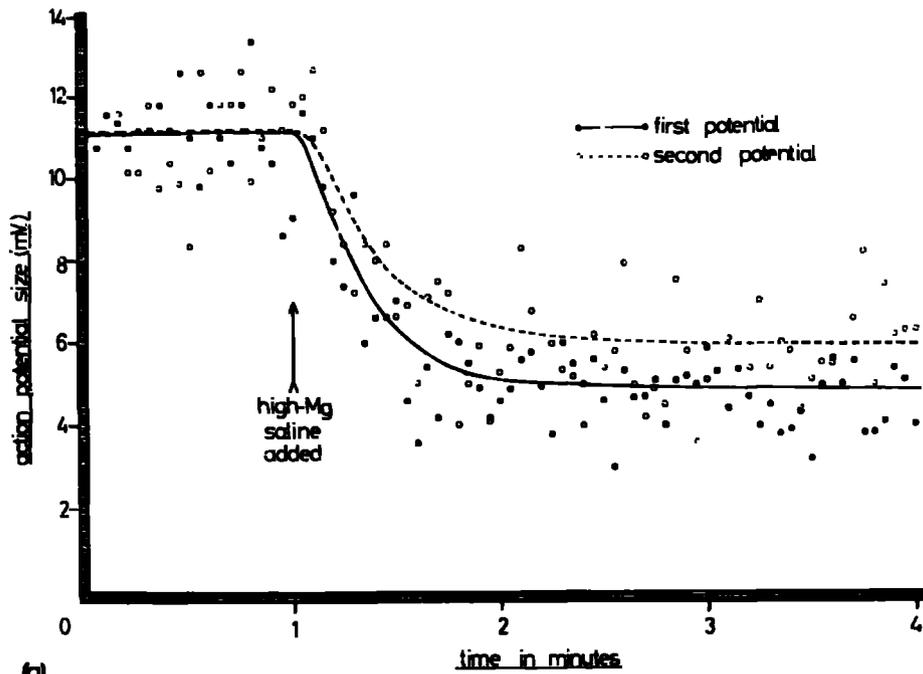
THE EFFECT OF VARIATION OF SALINE CALCIUM ON THE INDIVIDUAL MECHANICAL RESPONSES TO STIMULUS TRAINS, 40msec. GAP

the case in changes of calcium level, all effects are completely reversible.

The effect of doubled magnesium is exactly as one would predict by analogy with vertebrate responses, being the opposite to the effect produced by increased calcium. As can be seen in Fig.56, both mechanical and electrical responses are greatly reduced by doubled external magnesium. The change within one minute in this experiment was from $11.00\text{mV} \pm 0.25$ (s.e.) to $4.95\text{mV} \pm 0.15$. Differentials between first and second potentials of a pair are rather less marked than those produced by changes in calcium, the difference between sizes of first and second potentials in this experiment being only just significant ($p = 0.05$); the facilitation is in fact 1.15 ± 0.07 (s.e.). Mechanical responses are correspondingly reduced (Fig.56b).

In reduced saline magnesium (Fig.57) the effect on the electrical response is again predictable, there being a significant increase in size - $13.90\text{mV} \pm 0.30$ (s.e.) to $21.55\text{mV} \pm 0.30$ in this case. Reduced magnesium also brings about a significant depression of responses subsequent to the first, the ratio of the second response to the first in this experiment being 0.90 ± 0.02 .

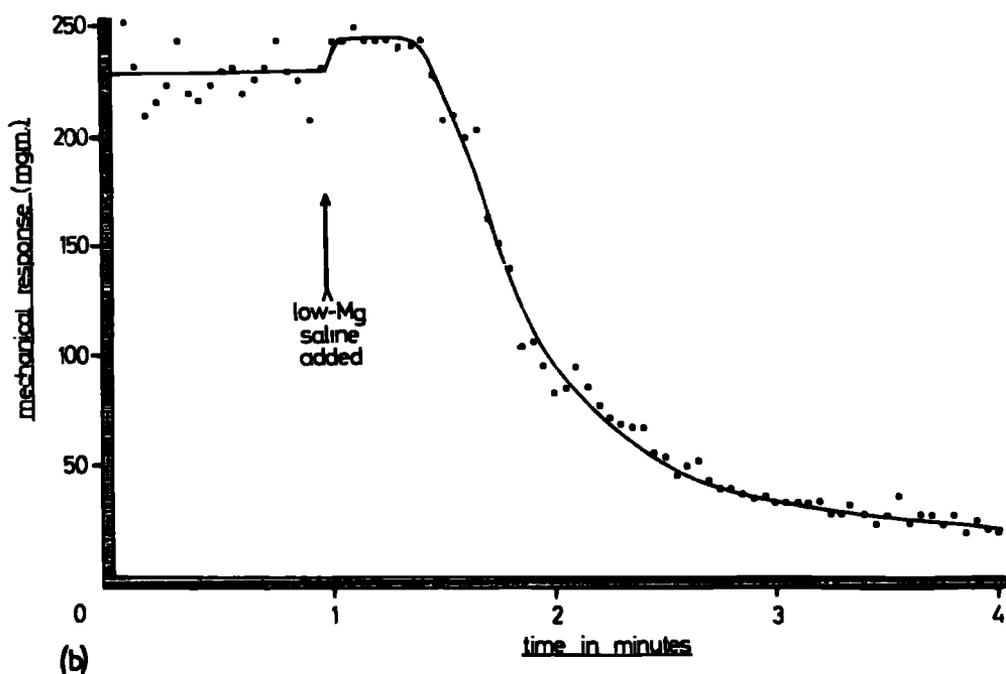
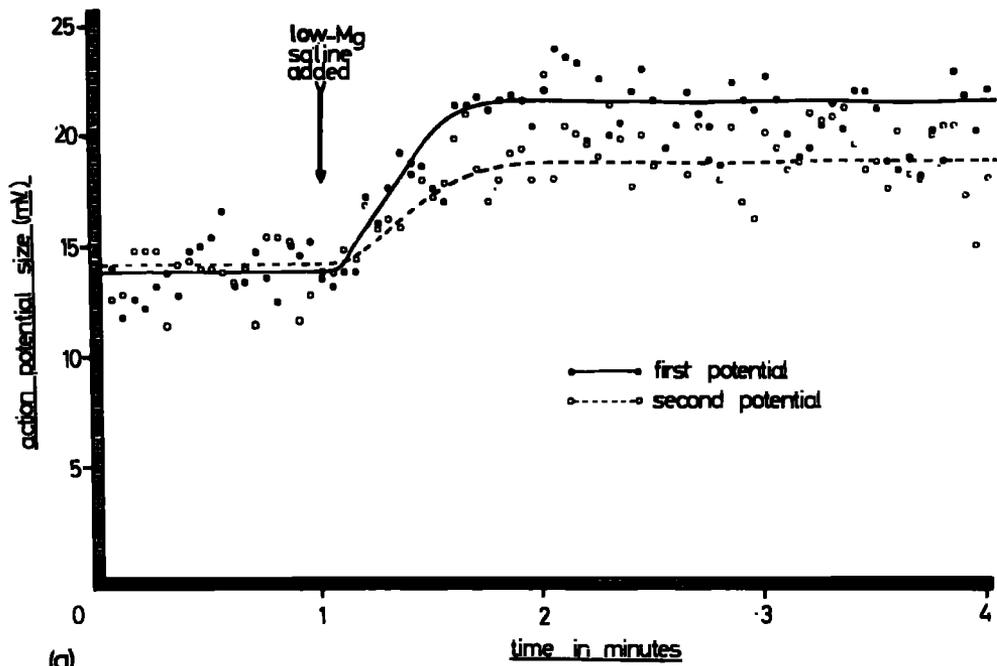
The mechanical responses evoked in decreased magnesium are very unexpected, however. After a very small



THE EFFECT OF DOUBLED SALINE MAGNESIUM (6-12mM/L)
ON THE SLOW RESPONSE

- (a) The effect of 12mM. saline Magnesium on the electrical response to paired neural stimulation, stimulus gap 5msec.
- (b) The simultaneous mechanical response of the whole muscle to the second stimulus.

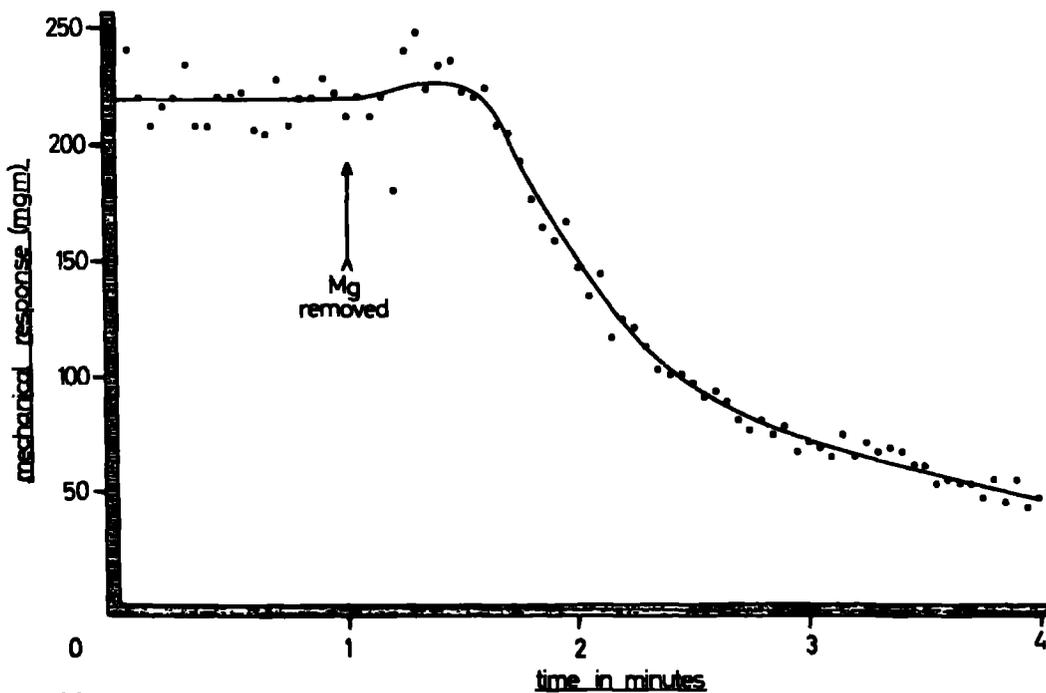
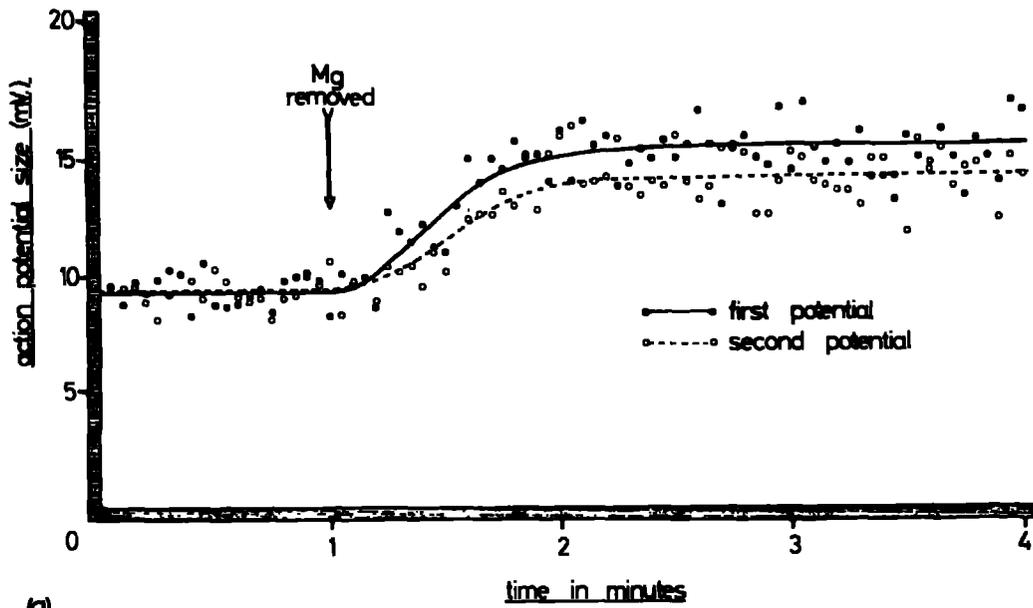
Figure 56.



THE EFFECT OF REDUCED SALINE MAGNESIUM (6-3mM/l.)
ON THE SLOW RESPONSE

- (a) The effect of 3mM. saline Magnesium on the electrical response to paired neural stimulation, stimulus gap 5msec.
- (b) The simultaneous mechanical response of the whole muscle to the second stimulus.

Figure 57.



THE EFFECT OF MAGNESIUM-FREE SALINE ON THE SLOW RESPONSE

- (a) The effect of zero saline Magnesium on the electrical response to paired neural stimulation, stimulus gap 5msec.
- (b) The simultaneous mechanical response of the whole muscle to the second stimulus.

Figure 58.

initial apparent increase in mechanical response (Fig.57b) there is a steady, rapid decline, until a very low level is reached. It is not known whether a total blockage of tension development is eventually produced.

In magnesium-free conditions a very similar state of affairs is found (Fig.58). An initial small increase in mechanical response is soon succeeded by a rapid decline. In these conditions the increase in electrical response, while significant, is actually somewhat less than that brought about by reduction of magnesium to a low level. The depression of the second response by the first (0.95 ± 0.03) is not significant ($p = 0.05$).

Much elaboration of these investigations using experiments such as those carried out with calcium levels, will be required to explain these discrepancies between electrical response and tension development.

DISCUSSION

The general picture which has emerged from this investigation so far is that external calcium ions affect neuromuscular transmission in two ways, one of which ensues immediately upon a change in the concentration, the other becomes fully operative only after some time has elapsed. The effects are operative both upon electrical and mechanical responses. Changes in external magnesium ions affect

electrical responses in a way opposite to calcium in the short term, and it is possible that the longer-term effect produced by calcium is also operative, but in reverse.

The mechanical responses in abnormal magnesium concentrations follow electrical effects only in higher magnesium environments; in deficient salines they seem to increase initially along with electrical responses, but a different effect of magnesium-ion deficiency becomes rapidly apparent, and tension development becomes blocked, apparently in the presence of normal transmission of electrical events.

Antagonism between the two ions has not been investigated; since the effects of the two upon excitable cells are generally opposite, it is likely that increased magnesium concentrations will nullify the effect of increased calcium concentrations.

The rapid time-course of generation of the effects of changes in concentrations of these ions can be explained satisfactorily only in terms of changes in the conditions at the end-plate synapse. It seems most unlikely that internal conditions, either in the muscle fibre, or in the pre-synaptic nerve fibre, could be so rapidly altered by relatively slight adjustment of the external environment (see Gilbert & Fenn, 1957).

It is well known that both calcium and magnesium ions greatly affect the production of neuromuscular transmitter

by vertebrate motor end-plates (e.g. del Castillo & Stark, 1952; del Castillo & Katz, 1954b). Recent work by Katz & Miledi (1965b, 1967) has demonstrated that external calcium and magnesium ions are utilised by the pre-synaptic membrane in transmitter release only at the time of release; ionophoretic local application of either ion was fully effective if made only 20msec before transmitter was released. Since a dependency of transmission on calcium and magnesium ions very similar to that of vertebrate neuromuscular junctions has been demonstrated in a great variety of invertebrates (e.g. Hoyle, 1955a; Wood, 1957; Eatt & Ginsborg, 1958) it is probable that these ions are used from external sources in a way similar to that demonstrated by Katz & Miledi (1965b, 1967) and within time intervals of similar order.

It is very probable that the rapid effects of changes in calcium and magnesium ion concentrations are due to changes in the local environment of the sites of transmitter release at the neuromuscular junction. This conclusion is likely to be correct in spite of the fact that the transmitter substance is almost certainly not acetylcholine, no experiments have been carried out on the transmission system of Aeschna concerning possible transmitter substances, but acetylcholine has no effect on those arthropod neuromuscular junctions so far studied with only one possible exception

(McCann & Reece, 1967); they are indications that analogues of glutamic acid may be functional as transmitters (see Usherwood & Malachi, 1966). If the conclusions concerning action of calcium and magnesium can be confirmed, it will suggest that transmitter release mechanisms work in basically similar ways even if different transmitters are involved.

The extreme rapidity of the effect of changes in ionic concentrations are presumably a reflection of the accessibility of the end-plate region to external fluids. This confirms physiologically the electron-microscope observation of Smith (1961) that the lemnoblast which ensheaths the peripheral axon is absent at the myoneural junction. The end-plate region is clearly more accessible to externally-applied agents than are the end-plates of some insects (see, e.g., Wood, 1957). In the great majority of experiments carried out with invertebrate materials effects of changes in applied ionic environment are equilibrated within half an hour, and the period is frequently shorter.

The second phase of adaptation to changes in external calcium shown by the muscle fibres of Aeschna is presumably due to an equilibration of calcium concentrations inside and outside the fibre. Since these changes affect the electrical response as well as the mechanical, the effect cannot be upon the excitation-contraction link. It seems likely, therefore, that internal calcium ions affect the

sensitivity of the post-synaptic membrane in some way. The effect could be upon either the transmitter receptor sites, or upon permeabilities of the post-symaptic membrane. Since changes in calcium ion have little effect on the resting potential, any direct membrane effects must concern permeabilities in the active, excited state. Such changes have been reported in the excitable membranes of other arthropods (Narahashi, 1966; Werman & Grundfest, 1961). Attempts to confirm that active membrane potentialities are affected will be complicated by the fact that if the muscle fibre membrane is affected, so must be the pre-synaptic membrane.

It was suggested in Chapter 6 that process of facilitation and fatigue are likely to be dependent on the probabilities of quantal release by pre-synaptic impulses, facilitation occurring when a greater number of quanta are released and fatigue being a consequence of decreased release. Increased fatigue effects have been observed in high-calcium environments (Fig.52), and facilitation or depression of repetitive responses have been seen in abnormal ionic conditions. It would seem likely, therefore, that calcium ions may govern the number of quanta released by a pre-synaptic impulse, rather than the amount of transmitter in each quantum. Katz & Miledi (1965b) have demonstrated this to be the case in the action of calcium ions on acetyl-

choline release in the neuromuscular junctions of frog's sartosius.

Wakabayashi & Iwasaki (1964) have reported that reduction or increase in size of vertebrate end-plate potentials by changes in calcium or magnesium concentration is greatest in singly-evoked responses. When end-plate potentials are repetitively evoked, responses subsequent to the first show facilitation when the size of the first has been depressed, and depression when the size of the first has been enhanced by ionic effects. Wakabayashi & Iwasaki (1964) established that this effect was pre-synaptic. This pattern of response is very similar to that observed in Aeschna, and increases the likelihood that preliminary conclusions linking calcium ion concentration with probability of transmitter release are correct. The probability of release of quanta is decreased in low calcium concentrations, but repetitive stimulation somewhat increases the probability in responses subsequent to the first by action similar to that seen in normal processes of facilitation.

The observation that potentials of different sizes retain their size differential in different calcium concentrations is to be expected in the light of the conclusions of Chapter 5. This observation also suggests that calcium has little effect on the rate of decrease of size of potentials as they spread out electrotonically from

points of origin; the length constant of the membrane is thus unaffected.

The correlation between electrical activity and the development of tension will require close examination, since the increase in mechanical response to individual stimuli is much greater than increase in depolarisation in single responses, though to later stimuli of a repetitive train there is relatively much less change. It is likely that tension development is related not only to membrane potential but also to existing tension within the muscle fibre.

The most likely explanation of the results obtained when magnesium concentrations were changed is that the action of magnesium on the pre-synaptic membrane is opposite to that of calcium, as is the case in other known instances (e.g. del Castillo & Engbaek, 1954). There is in addition an action of magnesium upon the post-synaptic membrane; in the absence of magnesium ions neuromuscular transmission is not blocked, but the link between excitation and contraction is partly or wholly decoupled. If the small initial upward excursion of the curves of Figs. 57b & 58b are significant, it would seem that magnesium ions diffuse somewhat more slowly from the site of excitation-contraction linkage than from the site of activation of quantum release.

The observations that there is more increase in size of the response in magnesium-free conditions than is seen in magnesium-free conditions, and that there is apparently less depression of repetitive responses in high magnesium than occurs in low-calcium environment, are not explained by this hypothesis of dual effect of magnesium ions upon the pre-synaptic release mechanism and the excitation-contraction link. Much further work is required.

It is clear from these preliminary observations that ionic control of neuromuscular transmission in Aeschna has many features in common with previously-elucidated mechanisms, but that there are some unusual effects, mostly involving magnesium ions. These effects may not be a part of neuromuscular transmission, but may concern subsequent stages in the development of tension by muscle fibres in response to the pre-synaptic neural activity.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 9GENERAL DISCUSSION AND CONCLUSIONS

The results which have been presented and discussed in this thesis have demonstrated fairly clearly that the organisation of the neuromuscular physiology of dragonflies is, by comparison with other insects and with many crustaceans, somewhat primitive. While many of these investigations have necessarily been preliminary, it has become clear that these animals will prove to be of value in the investigations of general problems of neuromuscular physiology because of their primitive nature. Primitive organisations have often proved to be of use in the investigation of advanced problems because of the reduced number of variables which need to be allowed for in drawing conclusions; for example, inter-neuronal interactions such as occur in vertebrate central nervous systems can be interpreted in the light of experiments conducted on the ganglia of molluscs, where model systems involving only a few neurons can be investigated. In the same sort of way the responses of the flexor tibialis muscle of Aeschna may well be of use in an investigation to determine why different mechanical responses are evoked by the slow and fast innervations of insect muscles; one variable which is absent in this muscle is the active membrane response present in the

fast response of higher insects. It is hoped that such an investigation may be carried out in the future as one of the possible expansions of the results of this thesis.

When experiments are carried out in isolated experiments, as have most of the experiments reported here, one tends to lose sight of the significance of the results to the physiology of the whole animal. It is intended, therefore, in this discussion to consider the implications for the animal under natural conditions of the conclusions reached in each of the individual discussions which have for the most part been considered in isolation.

Innervation and Normal Response

As detailed in Chapters 2 & 4, only one of the two major power segments of the leg of dragonfly nymphs is innervated by a fast axon; the basal segment of the leg - the coxa - which together with the muscles situated inside the thorax but inserted in the proximal regions of the coxa controls the overall position of the leg, has no fast innervation.

While innervation and responses of the corresponding muscles of the adult dragonfly are unknown, there is no reason to suppose that they are any different, for several reasons. Dragonflies, being exopterygote insects, do not exhibit dramatic morphological changes from one form to another

(such as metamorphosis from pupa to adult); all changes are gradual and step-wise, changing a little at each of the many ecdyses. The only major morphological changes at maturation and final adult emergence are the development of wings and maturation of sex apparatus. Even the development of wings is in no way sudden, wing buds having been present from very early stages together with the operating musculature in a very reduced form. It is unlikely, therefore, that any of the skeletal muscles not associated with flight should suddenly develop additional innervations to existing functional muscles at a late stage in development. In addition to there being no precedent for mobilisation of a hitherto quiescent innervation, it is likely that the stimulating techniques used to evoke experimental responses would stimulate all available systems, whether they were used in vivo or not. For these reasons alone, it is unlikely that innervation of the leg muscles in adults is in any way different from that of nymphs; whether a fully-functional system is ever used in vivo is, of course, a different matter, but for the purposes of this discussion is irrelevant.

No consideration of the significance of observed patterns would be valid without consideration of the natural history of the insect. There is a general tendency to suppose that the most important habitat in which an animal

lives (if it occupies more than one in its life-time) is that one in which it breeds; all other evolutionary considerations will have been subordinated to this. It is very clear, however, that this is not the case in the life of dragonflies, or for that matter of the closely related mayflies (Ephemeroptera). Dragonflies spend a minimum of two years as larval forms, the period being controlled to some extent by food availability; they emerge from the water only to breed, and this completed, they die, being very susceptible to the hostile environment. Practically every advanced feature of dragonflies has clearly evolved to promote survival in the aquatic environment - feeding mechanisms, specialisation of the rectum into an actively irrigated gill and into a jet-like escape mechanism, for example. Interpretation of physiological data should therefore be considered against this background, treating dragonflies primarily as aquatic animals.

It is not really surprising, therefore, that fast innervation has not evolved to any great extent in the leg muscles of these basically floating animals since the only apparent function of the legs is "walking" along pond beds and along the stems of weeds and reeds, and anchoring the animal to the latter when at rest. Escape mechanisms, with which fast innervations are usually associated, involve only positioning of the legs along the body in a stream-

lined manner; no great expenditure of energy is required.

Disuse of an available neuromuscular mechanism could conceivably lead to its atrophy and loss. Dragonflies, being an ancient form of insect pre-dating most present-day forms may quite easily have lost a fast mechanism in their evolution. This is most unlikely, however, firstly because it would imply that fast mechanisms were present in the most primitive forms of insect, and secondly because it is unlikely that all traces of a fast innervation would be lost from some muscles; it is at least probable that a dual innervation would have remained, even if the fast-contracting capability had been lost.

Mechanical Responses and Action Potential Variability

The preliminary results which have been obtained suggest that the evolution of muscle fibres of dragonflies has not been in the direction of large excursions of membrane potential - there is no facilitation, no great summation, and no active membrane response. Instead, it seems that mechanical responses are controlled with relatively small potential excursions, but none-the-less have a great capability of fine control. This conclusion was reached in the discussion of Chapter 7. Apart from giving an impression in conjunction with evidence from experiments on crustaceans that small potential excursions represent a primitive state,

this evidence would seem to have little evolutionary importance. There is no apparent environmental pressure in favour of either method of control of movement, nor for that matter in favour of the vertebrate method involving regenerative action potentials, and it is evident that all variants on the basic primitive system have been equally successful within the requirements of each individual group of animals.

Since the majority of the fine movements of the leg of dragonfly nymphs is likely to originate in the coxa from where the position of the whole leg is controlled, it is likely that the animals have utilised the potential for fine movement innate in their neuromuscular organisation to a great extent. Traces such as those illustrated in Fig.17 have shown that natural activity in the crural nerve tends to be in bursts of two or more spikes; as has been seen in Chapter 7, large contractions, almost as rapid as fast twitches, though with less force, can be elicited by very short bursts of neural activity.

The coxal muscles must also serve postural functions, especially when the animal is at rest. Slow responses are those almost invariably used in the postural activity of invertebrates, and even of some lower vertebrates because of their ability to exert steady tension for considerable periods. Fast responses are much more prone to fatigue.

The fact that the coxa must bear the brunt of posture maintenance, especially when the animal is at rest on a reed stem, is presumably one environmental pressure which has led to the retention of slow innervation to every muscle fibre.

For fine control of tension of whole muscles two mechanisms are available; innervation by several axons of one or more types (including inhibitory), or great variation in threshold of fibres innervated by a single axon. The evolution of dragonflies has clearly led to the latter method; the combination of varied base depolarisations (presumably reflecting varied membrane properties) and the vastly different depolarisations which are induced at the same time over the surface of the membrane of each fibre produces a system in which fibres with lowest thresholds may well be fully contracting long before those with the highest thresholds have begun to contribute any tension to the tetanus at all. It is doubtful, in fact, if the majority of the tension-developing capability is ever utilised, since trains of nerve impulses of sufficient frequency and length are unlikely to be produced in the normal environment.

Fatigue Processes

Without an exhaustive series of experiments in which various patterns of activity other than the regular type used in the experiments of Chapter 6 are evoked, it is very difficult to perceive the relevance of the properties of the neuro-muscular system discovered in Chapter 6 to the natural functioning of the animal.

Although it is known that fatigue processes are reversible, it is difficult to know by just how much fatigue processes proceed in normal activity. A second difficulty is that it is not certain that such fatigue processes do in fact occur in the whole animal, though it is most unlikely that the observed pattern is partly or wholly a consequence of the experimental conditions.

It would seem likely that the animal is permanently in a condition of very mild fatigue, particularly in muscles of such size and importance as those of the leg. If the two types of fibre which are suggested by Fig.29 do exist - and experimental evidence so far obtained is by no means decisive - then the type of muscle fibre which exhibits no fatigue during experiments in which periodic, rather than steady, activity is evoked is probably not affected by fatigue processes in the normal animal.

The other type of fibre illustrated in Fig.29 would be affected, and by very little activity. It is

tempting to suppose that the processes of facilitation which are very much involved in and connected with processes of fatigue are an evolutionary development to counteract the effects of fatigue. It seems reasonable to suppose that fatigue such as was created in the experiments of Figs. 31 & 34 is much more extreme than would occur in the normal animal; it is likely that facilitation comes into action before fatigue processes have reduced the size of the response to any great extent, and that the results are rather like those illustrated in Fig. 31a, responses subsequent to the first being as large as, or larger than, single responses of unfatigued muscle. The amount of fatigue involved would not, of course, be as much as was applied in the experiment of Fig. 31a, which is thought to represent the other type of muscle fibre.

It is important in this context to bear in mind that in the normal animal the effect on the animal of the first action potential of a burst is virtually nil - there is no mechanical response. It is to subsequent impulses that movements are made, and the mechanisms suggested above would, of course, involve these responses. The first response, which is ineffective and acts only as a conditioning response, does not need to be corrected for effects of fatigue.

In the living animal, then, most of the experimental observations of this thesis have some relevance. Actions of changed ionic conditions have no such relevance, of course, but they form the basis of experiments in which the basic mechanisms may be elucidated. In particular, the unexpected action of magnesium ions on the mechanical response (Chapter 8) form at least one basis for experiments combining the techniques of Chapters 3 & 7, observing mechanical responses of fibres to directly-applied depolarisation; the excitation-contraction link could be studied directly by this means to see if contraction does directly follow membrane polarisation or if it does not.

APPENDIX A.

ADDITIONAL TABLES

TABLE 5

The Effect of Various Sucrose Concentrations in Normal Saline on the Resting Potential

SUCROSE. gm/l	T I M E I N H O U R S					
	0	1	2	3	4	5
40	58.4 ± 2.5	62.2 ± 2.7	66.5 ± 1.4	66.7 ± 2.7	57.5 ± 2.7	50.0 ± 2.3
45	69.0 ± 1.4	61.7 ± 2.7	46.1 ± 4.3	49.7 ± 4.7	50.3 ± 4.2	43.1 ± 3.9
55	63.1 ± 1.8	61.3 ± 2.4	61.1 ± 2.0	61.3 ± 1.7	60.5 ± 2.7	58.4 ± 2.9
60	58.3 ± 4.0	63.6 ± 2.2	65.5 ± 2.2	64.0 ± 2.5	60.0 ± 4.2	65.6 ± 4.0
70	60.7 ± 2.3	67.0 ± 3.2	67.4 ± 2.5	63.0 ± 3.2	58.1 ± 3.2	56.5 ± 4.0
75	57.1 ± 2.1	65.9 ± 2.0	61.5 ± 2.7	46.9 ± 2.7	45.0 ± 2.9	31.7 ± 9.7

Figures are resting potentials in millivolts ± one standard error.

This table relates to Fig. 4.

TABLE 6

Copy of Computer Presentation of Results obtained from the
Survey of Action Potential Relationships

resting potential mV	mean action potential mV	mean rise rate V/sec	rise factor	qtr.decay time msec	decay factor
89.00	10.62	3.32	0.30	10.18	0.96
90.00	11.36	4.18	0.24	9.80	0.86
90.00	6.88	3.13	0.32	3.26	0.47
80.00	13.60	5.40	0.18	6.54	0.48
86.00	17.56	5.71	0.17	9.12	0.52
67.00	11.62	4.21	0.24	5.18	0.45
86.00	16.20	6.28	0.16	5.70	0.35
85.00	22.08	8.06	0.13	4.90	0.22
85.00	6.18	3.36	0.30	1.22	0.20
73.00	3.18	1.47	0.69	1.64	0.52
83.00	0.12	2.75	0.37	9.80	1.07
98.00	7.22	3.03	0.33	3.64	0.50
87.00	24.44	7.27	0.14	11.92	0.49
65.00	9.16	2.73	0.36	2.62	0.29
89.00	8.72	3.23	0.37	4.92	0.56
66.00	12.24	4.43	0.23	4.02	0.33
67.00	9.10	3.42	0.29	4.44	0.49
84.00	10.74	3.95	0.25	3.54	0.33
84.00	7.10	2.52	0.40	3.78	0.53
74.00	5.94	1.82	0.54	6.80	1.14
75.00	1.84	1.16	0.86	2.36	1.28
92.00	9.44	2.93	0.34	9.76	1.03
63.00	8.56	2.93	0.34	8.76	1.02
64.00	3.42	1.47	0.68	2.72	0.80
78.00	4.68	2.72	0.37	1.20	0.26
86.00	9.84	3.32	0.30	8.34	0.85
84.00	8.22	2.89	0.35	6.34	0.77
85.00	9.56	4.19	0.24	3.92	0.41
97.00	13.92	5.16	0.19	7.98	0.57
83.00	9.16	4.16	0.24	2.34	0.26
79.00	4.22	1.28	0.79	4.74	1.12
87.00	0.64	0.63	1.59	2.32	3.62
46.00	4.54	2.34	0.42	5.26	1.16
79.00	4.28	1.34	0.75	10.38	2.43
74.00	5.96	2.71	0.37	8.66	1.45
80.00	11.52	3.43	0.29	6.02	0.52
82.00	7.72	3.51	0.29	5.40	0.70

TABLE 6 cont'd

resting potential mV	mean action potential mV	mean rise rate V/sec	rise factor	qtr.decay time msec	decay factor
86.00	8.32	3.62	0.28	7.58	0.91
97.00	15.28	4.42	0.23	5.50	0.36
79.00	1.00	0.53	1.89	2.48	2.48
86.00	3.96	1.57	0.63	2.50	0.63
94.00	6.12	1.92	0.53	7.92	1.29
69.00	13.08	3.57	0.28	7.96	0.61
94.00	3.70	1.29	0.77	2.64	0.71
83.00	8.36	2.21	0.40	10.56	1.25
81.00	7.96	2.19	0.47	8.12	1.02
87.00	6.90	1.95	0.51	7.58	1.10
87.00	7.20	2.93	0.34	2.74	0.38
100.00	5.52	2.17	0.46	2.12	0.38
81.00	8.90	2.65	0.37	4.66	0.52
91.00	5.26	1.55	0.66	5.46	1.04
85.00	14.16	6.05	0.16	2.82	0.20
80.00	6.46	4.25	0.24	1.54	0.24
64.00	4.62	1.94	0.52	4.08	0.88
87.00	12.44	4.64	0.22	4.24	0.34
42.00	1.94	1.08	0.92	1.44	0.74
84.00	4.66	3.11	0.32	2.16	0.46
95.00	9.82	3.72	0.27	6.78	0.69
95.00	10.64	4.84	0.21	5.08	0.48
80.00	1.38	1.13	0.88	1.88	1.36
63.00	13.40	4.29	0.23	4.82	0.36
96.00	5.34	2.19	0.46	3.72	0.70
88.00	2.12	1.34	0.75	2.18	1.03
81.00	5.88	4.45	0.22	1.36	0.23
87.00	13.62	5.36	0.19	4.06	0.30
70.00	9.98	4.23	0.24	3.68	0.37
77.00	12.70	6.17	0.16	3.26	0.26
70.00	3.76	2.21	0.45	1.64	0.44
92.00	6.80	2.52	0.40	4.54	0.67
81.00	8.30	3.99	0.25	3.84	0.46
84.00	9.68	3.27	0.30	6.70	0.69
90.00	9.96	3.51	0.29	8.28	0.83
83.00	9.36	4.03	0.25	6.78	0.72
80.00	3.32	1.98	0.42	2.76	0.83
89.00	3.56	2.25	0.46	3.02	0.85
58.00	3.56	2.34	0.43	5.72	1.61
80.00	16.56	4.76	0.21	7.04	0.43
70.00	10.76	3.84	0.26	2.86	0.27
78.00	14.70	5.36	0.19	4.54	0.31
88.00	4.92	2.37	0.42	2.22	0.45

TABLE 6 cont'd

resting potential mV	mean action potential mV	mean rise rate V/sec	rise factor	qtr.decay time msec	decay factor
91.00	11.02	4.79	0.21	2.58	0.23
90.00	8.48	3.89	0.26	8.70	1.03
92.00	7.76	3.06	0.33	6.00	0.77
82.00	6.36	2.97	0.33	3.30	0.52
94.00	1.20	1.11	0.90	1.80	1.50
88.00	2.10	1.17	0.85	1.10	0.52

TABLE 7

The Effect of Fatigue on the Slow Response of Two muscle Fibres to Paired Neural Stimuli

STIMULI ELAPSED	SIZE OF FIRST POTENTIAL (mV [±] s.e.)		SIZE OF SECOND POTENTIAL (mV [±] s.e.)	
	(a)	(b)	(a)	(b)
0	13.05 ± 0.35	16.85 ± 0.40	13.30 ± 0.35	17.50 ± 0.45
100	11.80 ± 0.25	16.60 ± 0.40	13.20 ± 0.30	17.95 ± 0.60
200	11.60 ± 0.55	16.95 ± 0.40	12.85 ± 0.30	19.15 ± 0.40
300	10.45 ± 0.30	15.80 ± 0.35	12.55 ± 0.30	17.65 ± 0.35
400	9.80 ± 0.25	14.60 ± 0.25	12.85 ± 0.35	18.95 ± 0.40
550	10.10 ± 0.25	13.85 ± 0.45	12.15 ± 0.45	18.10 ± 0.30
750	10.00 ± 0.25	13.45 ± 0.40	12.20 ± 0.45	18.15 ± 0.35
1000	9.55 ± 0.30	14.35 ± 0.40	12.05 ± 0.35	17.70 ± 0.50
1400	9.35 ± 0.25	12.80 ± 0.30	11.80 ± 0.35	17.95 ± 0.30
1700	8.95 ± 0.50	13.55 ± 0.35	11.80 ± 0.35	16.50 ± 0.45
2000	9.00 ± 0.25	11.90 ± 0.40	12.20 ± 0.40	17.45 ± 0.40
2500	9.35 ± 0.30	12.15 ± 0.45	11.80 ± 0.30	16.35 ± 0.45

Columns (a) & (b) of this table correspond to graphs (a) & (b) of Figs 31 & 32 (Chapter 6)

TABLE 8

The Effect of Fatigue on the Facilitation of the Slow Responses of Two Muscle Fibres to Neural Stimulation

STIMULI ELAPSED	EXPERIMENT (a) FACILITATION \pm s.e.	EXPERIMENT (b) FACILITATION \pm s.e.
0	1.03 \pm 0.04	1.06 \pm 0.05
100	1.13 \pm 0.04	1.10 \pm 0.05
200	1.15 \pm 0.06	1.14 \pm 0.04
300	1.23 \pm 0.05	1.14 \pm 0.05
400	1.33 \pm 0.05	1.31 \pm 0.05
550	1.23 \pm 0.05	1.32 \pm 0.05
750	1.25 \pm 0.07	1.37 \pm 0.04
1000	1.30 \pm 0.07	1.27 \pm 0.07
1400	1.28 \pm 0.05	1.41 \pm 0.04
1700	1.41 \pm 0.10	1.23 \pm 0.05
2000	1.39 \pm 0.07	1.51 \pm 0.07
2500	1.30 \pm 0.07	1.39 \pm 0.09

The experiments (a) & (b) of this table correspond to graphs (a) & (b) of Fig. 32.

TABLE 9

Mechanical and Electrical Muscle Responses to Repetitive Stimulation of the Slow Axon

STIMULUS GAP msec.	MEAN ACTION POTENTIAL SIZE mV. \pm s.e.	BASE DEPOLARISATION mV.	MECHANICAL RESPONSE mgm
1000	11.20 \pm 0.25	0	0
150.	12.65 \pm 0.35	0	3
125	13.05 \pm 0.20	0.2	10.
100.	13.30 \pm 0.20	0.5	15
90	13.05 \pm 0.25	0.8	25
80	14.65 \pm 0.30	0.8	40
70	14.60 \pm 0.40	1.0	90.
60	15.45 \pm 0.25	1.4	150
50	15.65 \pm 0.30	2.0	310
45	16.15 \pm 0.35	2.6	400
40	16.20 \pm 0.35	3.4	580
35	17.60 \pm 0.40	4.4	760.
30	17.55 \pm 0.45	5.2	1000
25	18.40 \pm 0.40	8.0	1275
22.5	18.15 \pm 0.35	8.8	1500
20	18.00 \pm 0.35	10.8	1775
17.5	17.90 \pm 0.30	12.6	1975
15	17.70 \pm 0.30	14.6	2150
12.5	14.90 \pm 0.25	24.0	2700
10	13.10 \pm 0.20	25.0	2600
9	12.30 \pm 0.35	26.0	3250.
8	12.15 \pm 0.35	26.0	3400
7	10.60 \pm 0.30	28.5	3700

The data in this table relates to

Fig. 39.

TABLE 10The Mechanical Response of Coxal Muscle to PairedNeural Stimuli

STIMULUS GAP msec	MECHANICAL RESPONSE TO SECOND STIMULUS mgm	RATIO TO MEAN RESPONSE TO FIRST STIMULUS (12.5mgm)
2	390	31.2
2.5	380	30.4
3	355	28.4
3.5	350	28.0
4	325	26.0
5	290	23.2
6	275	22.0
8	255	20.4
10	235	18.8
12	230	18.4
14	200	16.0
16	180	14.4
20	150	12.0
25	135	10.8
30	125	10.0
40	80	6.4
50	80	6.4
60	50	4.0
80	35	2.8
100	25	2.0
125	20	1.6
150	15	1.2

The data in this table relates to Fig.42.

TABLE 11

Individual Mechanical Responses to Repetitive Stimulation of the Slow Axon and the

Corresponding Electrical Base Depolarisation

STIMULUS GAP msec.	MECHANICAL RESPONSE (mgm) TO EACH STIMULUS & RATIO TO LST					BASE DEPOL- ARISATION mV
	RESPONSE 1	RESPONSE 2	RESPONSE 3	RESPONSE 4	RESPONSE 5	
1000	6.5	5.5 (0.9)	6.5 (1.0)	5.5 (0.9)	3.5 (0.5)	0
150	6.0	15 (2.4)	19 (3.3)	22 (3.6)	23 (3.8)	1.30
125	6.5	22 (3.5)	35 (5.5)	36 (5.7)	44 (7.1)	1.80
100	5.0	26 (5.2)	42 (8.4)	50 (10.0)	52 (10.4)	2.30
90	5.5	35 (6.7)	57 (10.7)	64 (12.0)	66 (12.5)	2.30
80	5.0	39 (7.8)	68 (13.6)	76 (15.2)	76 (15.2)	2.80
70	5.5 *	44 (7.8)	80 (14.3)	85 (16.1)	90 (16.6)	2.70
60	5.5 *	52 (9.3)	93 (16.6)	94 (16.8)	93 (16.6)	3.35
50	5.5 *	59 (10.6)	116 (20.7)	112 (20.0)	105 (18.7)	4.05
45	5.5 *	64 (11.3)	112 (20.0)	111 (19.8)	103 (18.4)	4.55
40	5.5 *	83 (14.9)	150 (26.7)	146 (26.0)	122 (21.8)	6.00
35	5.5 *	94 (16.8)	176 (31.3)	161 (28.7)	135 (24.0)	6.70
30	5.5 *	97 (17.3)	177 (31.6)	176 (31.5)	120 (23.1)	7.80
25	5.5 *	110 (19.5)	205 (36.2)	185 (32.7)	158 (28.1)	9.30

* -- estimated value (oscilloscope settings too wide-range to measure accurately). All figures and ratios computed, then rounded to nearest 0.5mgm, 0.1 ratio. This data relates to Figs. 43b & 44b.

TABLE 12
Individual Mechanical Responses to Repetitive Stimulation of the Slow Axon and the
Corresponding Electrical Base Depolarisation (Second Experiment)

STIMULUS GAP msec	MECHANICAL RESPONSE (mgm)	TO EACH STIMULUS & RATIO TO 1ST RESPONSE 1	RESPONSE 2	RESPONSE 3	RESPONSE 4	RESPONSE 5	BASE DEPOL- ARISATION mV
400	23	23 (0.9)	19 (0.8)	17 (0.7)	18 (0.7)	0	0
200	23	23 (1.2)	24 (1.2)	22 (1.1)	20 (1.0)	0	0
150	28	28 (1.7)	30 (1.8)	29 (1.8)	31 (1.9)	0.35	0.35
125	30	30 (1.9)	32 (2.0)	32 (2.0)	33 (2.1)	1.00	1.00
100	35	35 (2.3)	49 (3.1)	50 (3.2)	52 (3.3)	1.45	1.45
90	42	42 (2.5)	70 (4.2)	69 (4.2)	56 (3.4)	2.05	2.05
80	61	61 (4.3)	82 (5.8)	76 (5.4)	80 (5.7)	2.80	2.80
70	67	67 (5.0)	120 (8.9)	100 (7.4)	82 (6.1)	3.30	3.30
60	92	92 (6.5)	145 (10.4)	125 (8.9)	109 (7.8)	4.15	4.15
50	101	101 (7.2)	192 (13.7)	173 (12.3)	159 (11.3)	5.30	5.30
45	130	130 (9.3)	239 (17.0)	231 (16.4)	197 (14.0)	7.05	7.05
40	163	163 (11.6)	275 (19.6)	255 (18.1)	252 (17.9)	8.50	8.50
35	183	183 (13.0)	306 (21.8)	315 (22.4)	252 (17.9)	9.55	9.55
30	212	212 (15.1)	365 (25.9)	381 (27.1)	377 (26.8)	11.15	11.15

* - estimated value (oscilloscope settings too wide-range to measure accurately). All figures and ratios computed, then rounded to nearest 0.5mgm, 0.1 ratio. This data relates to Figs. 43a & 44a.

TABLE 13

The Effect of Doubled (10-20mM/l) Saline Calcium on the Electrical Response to Paired Stimuli and the Mechanical Response to the Second of these Stimuli

TIME AFTER SALINE CHANGE minutes	RESPONSE TO FIRST STIMULUS (mV) \pm s.e.	RESPONSE TO SECOND STIMULUS (mV) \pm s.e.	FACILITATION \pm s.e.	MECHANICAL RESPONSE (mgm)
0	7.55 \pm 0.35	8.25 \pm 0.25	1.14 \pm 0.07	45
5	23.50 \pm 0.50	19.60 \pm 0.40	0.84 \pm 0.03	365
30	23.95 \pm 0.50	20.40 \pm 0.35	0.86 \pm 0.03	430
35	24.60 \pm 0.45	20.35 \pm 0.40	0.83 \pm 0.02	445
60	26.55 \pm 0.50	21.45 \pm 0.40	0.82 \pm 0.02	465
90	26.30 \pm 0.85	22.20 \pm 0.55	0.86 \pm 0.03	450
120	29.00 \pm 0.45	22.00 \pm 0.35	0.76 \pm 0.01	440
150	25.55 \pm 0.45	20.70 \pm 0.75	0.81 \pm 0.03	430
180	23.20 \pm 0.65	19.15 \pm 0.30	0.84 \pm 0.03	355

This data relates to Fig.52.

TABLE 14

Electrical Responses to Single Stimulation in Various External Calcium Concentrations

of Two Fibres of the same Muscle and the Corresponding Mechanical Response of the

Whole Muscle to Each Stimulus of a Pair, 25msec gap

CONCENTRATION OF CALCIUM (mM/l) SINGLE ELECTRICAL RESPONSE ± s.e. MECHANICAL RESPONSE TO EACH OF TWO STIMULI (mgm)

	(a)	(b)	FIRST	SECOND
3	3.25 ± 0.20	1.89 ± 0.10	0	0
6	6.80 ± 0.30	5.00 ± 0.25	0	8
9	16.65 ± 0.45	11.20 ± 0.30	4	65
12	23.85 ± 0.30	15.90 ± 0.30	30	265
15	26.70 ± 0.45	18.10 ± 0.30	50	380
18	31.20 ± 0.60	18.35 ± 0.25	80	485
21	31.55 ± 0.45	18.40 ± 0.35	95	535

This data relates to Fig.53.

TABLE 15

The Effect of Changes in External Calcium Concentration
on the Facilitation of the Second of Two Action Potentials
Evoked by Paired Stimuli, 25msec gap

CONCENTRATION OF CALCIUM (mM/l.)	SIZE OF FIRST RESPONSE (mV) ± s.e.	SIZE OF SECOND RESPONSE (mV) ± s.e.	FACILITATION ± s.e.
3	1.90 ± 0.10	2.00 ± 0.15	1.09 ± 0.10
6	5.00 ± 0.25	5.30 ± 0.20	1.09 ± 0.06
9	11.20 ± 0.30	11.10 ± 0.35	1.00 ± 0.03
12	15.90 ± 0.50	13.95 ± 0.35	0.88 ± 0.03
15	18.10 ± 0.30	14.45 ± 0.35	0.80 ± 0.02
18	18.35 ± 0.25	15.00 ± 0.35	0.82 ± 0.02
21	18.40 ± 0.35	13.40 ± 0.25	0.73 ± 0.02

This data relates to Fig.54.

TABLE 16

Individual Mechanical Responses to Repetitive Stimulation in Various External

Calcium Concentrations

RESPONSE OF THE WHOLE MUSCLE TO EACH STIMULUS OF A TRAIN (mgm)

CONCENTRATION OF CALCIUM (mM/l)	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>	<u>5th</u>	<u>6th</u>	<u>7th</u>	<u>8th</u>
3	0	0	0	2	4	6	10	10
6	0	5	20	25	30	35	25	25
9	10	60	70	60	40	40	30	30
12	20	130	100	70	50	45	40	40
15	70	200	130	100	70	60	50	50
18	150	260	130	100	70	60	50	50
21	200	300	150	110	80	70	50	50

This data relates to Fig.55.

APPENDIX B

THEORY OF THE DETERMINATION OF MEMBRANE CONSTANTS

THEORY OF THE DETERMINATION OF MEMBRANE CONSTANTS

The constants to be determined are :

- (A) The space constant - λ (mm.)
- (B) The time constant - τ_M (msec.)
- (C) The effective resistance between fibre inside and out - R_E (ohms)
- (D) The specific resistance of the fibre membrane - R_M (ohms/sq.cm.)
- (E) The specific capacity of the fibre membrane - C_M (μF /sq.cm.)

The determination of these contents is carried out using the following interrelations between them (terminology and equations from Fatt & Katz, (1951); Hodgkin & Rushton, (1946)) :-

$$\tau_M = R_M \cdot C_M \quad (1)$$

$$\lambda^2 = \frac{R_M}{R_i} \times \frac{\rho}{2} \quad (2)$$

Where ρ = fibre radius

R_i = specific internal resistance (i.e. axoplasm resistance)(ohms/cm.)

$$V = \frac{I}{2} \sqrt{(r_M \cdot r_i)} \cdot \exp\{-x/\sqrt{(r_M/r_i)}\} \quad (3)$$

Where V = voltage observed at recording electrode.

I = current passed at stimulating electrode.

x = distance between electrodes

r_M = transverse resistance of the membrane x unit length.

r_i = longitudinal resistance of the fibre per unit length.

$$\lambda = \sqrt{(r_M/r_i)} \quad (4)$$

$$R_E = \frac{1}{2} \sqrt{(r_M r_I)} \quad (5)$$

From equation (3), substituting in equations (4) & (5) we have

$$V = I R_E \exp(-x/\lambda)$$

$$\text{thus } \exp(-x/\lambda) = \frac{V}{R_E I}$$

rewritten as

$$\frac{-x}{\lambda} = \log_e \frac{V}{R_E I} = \log_e V - \log_e (R_E I) \quad (6)$$

V , I & x are directly determinable. If, therefore, I is fixed and the distance apart of the two electrodes is altered (x to x'), there will be a consequent change in observed voltage (V to V'). We will then have

$$\frac{-x}{\lambda} = \log_e V - \log_e (R_E I)$$

$$\frac{-x'}{\lambda} = \log_e V' - \log_e (R_E I)$$

By simultaneous equations :

$$\frac{x' - x}{\lambda} = \log_e V - \log_e V' = \log_e (V/V')$$

$$\lambda = \frac{x' - x}{\log_e (V/V')} \quad (7)$$

Equation (6) can be rewritten as

$$e^{-x/\lambda} = \frac{V}{R_E I}$$

$$R_E = \frac{1}{e^{-x/\lambda}} \times \frac{V}{I}$$

$$\text{thus } R_E = e^{x/\lambda} \times \frac{V}{I} \quad (8)$$

and R_E can be derived using the known value of λ .

The diameter of the fibre can be measured optically, or by sideways

movement of the electrode if micrometer-mounted. Alternatively, Fatt & Katz (1951) give the formula :

$$d = \sqrt{\left(\frac{4}{\pi} \times \frac{R_i}{r_i}\right)} \quad (9)$$

They assumed R_i to be 250 ohms/cm., based on measurements from Bozler & Cole (1935) and Katz (1948); r_i is determined from equations (4) & (5):

From (4) we have

$$\lambda^2 = \frac{r_M}{r_i}$$

$$r_M = \lambda^2 \cdot r_i$$

From (5) we have

$$4R_E^2 = r_M \cdot r_i$$

$$r_M = \frac{4R_E^2}{r_i}$$

Thus $\lambda^2 \cdot r_i = \frac{4R_E^2}{r_i}$

$$r_i^2 = \frac{4R_E^2}{\lambda^2}$$

$$r_i = \frac{2R_E}{\lambda} \quad (10)$$

R_M can be obtained from equation (2):

rewriting :

$$\frac{R_M}{R_i} = \frac{2\lambda^2}{\rho} = \frac{4\lambda^2}{d}$$

$$R_M = \frac{4\lambda^2 R_i}{d} \quad (11)$$

R_i must again be assumed.

To obtain T_M it is necessary to determine the velocity of propagation

of the half-value potential, either in the rising or the falling phase.

Hodgkin & Rushton (1946) give the velocity of propagation to be $2\lambda/T_M$. Thus,

by measuring the velocity, T_M can be determined from the known value of λ .

Equation (1) now gives

$$C_M = \frac{T_M}{R_M} \quad (12)$$

The author is grateful to Mr. D. Routledge for assistance in understanding these derivations.

APPENDIX C

THE CORRELATION COEFFICIENT

APPENDIX CTHE CORRELATION COEFFICIENT

A graph plot of two sets of variables which are directly related to each other will produce a straight-line graph. If either or both variables is subject to statistical variation there will be appreciable scatter in the points of the graph even though the relation remains linear. Fig.25a is a good example of this type of graph.

The statistical factor known as the correlation coefficient gives an indication of the linearity of the relationship between two sets of variables without plotting of graphs, and where there is extreme scatter calculation of the correlation coefficient will indicate whether the observed scatter is produced by random variation, or whether there is a true relationship.

For a number of pairs of variables $u(1)$ & $v(1)$, $u(2)$ & $v(2)$... $u(n)$ & $v(n)$, the correlation coefficient r is derived by :-

$$r = \frac{(\sum uv)/n - \bar{u}\bar{v}}{\sigma_u \cdot \sigma_v}$$

where σ_u & σ_v are the standard deviations of u & v
and \bar{u} & \bar{v} are the mean values of u & v .

The resulting coefficient is a number within the limits -1 to 0 to +1. The sign of the coefficient indicates the direction of the gradient of the line which best fits the

points of the graph produced by plotting the two variables against each other.

A perfect relationship between two variables, giving a straight-line graph with all points placed on the line, has a correlation coefficient of either 1.0 or -1.0, depending on the direction of the gradient. A relationship in which there is some scatter of the points, but still a good relationship (e.g. Fig.25a) will have a coefficient rather less than 1.0 (or more than -1.0).

The actual value of the correlation coefficient does not itself give a direct indication of the significance of a relationship, unless it has the values 1.0, 0 or -1.0, because in less-than-perfect relationships its magnitude is a function of the number of pairs of variables (i.e. graph points). Calculated coefficients must be referred to tables of significance, such as are to be found in Fisher & Yates (1963) for example, in which the significance of each value of correlation coefficient can be determined, entering the table at the relevant number of pairs of variables ("degrees of freedom").

Thus, for example, in a correlation of ten pairs of values the table shows that a coefficient having a magnitude greater than 0.63 (or less than -0.63) indicates a probability of less than 0.05 that the relationship has occurred randomly; with 50 pairs of variables a relationship is correspondingly

significant ($p = 0.05$) if the correlation coefficient exceeds 0.27.

The correlation coefficient, when entered into the tables, can indicate relationships even in very scattered graphs. For example, the graph of Fig.27a has a coefficient of 0.48, and appears very scattered, but the probability of the pattern having occurred randomly is less than 0.05. In the graph of Fig.25a, where the relationship is obvious, the coefficient is 0.91, and the relationship is of very high significance.

The significance of a non-linear relationship (e.g. a rectangular hyperbola such as that of Fig.25b) cannot be determined by the calculation of the correlation coefficient unless the graph is first transformed into a linear one. For example, two functions (u & v) may be related by some exponential function. The linear correlation is likely to be low. However, the correlation between each value of u and the corresponding value of v transformed in some way (e.g. $\log v$; e^v ; $1/v$; \sqrt{v}) may be significantly high. It can then be said that there is a significant exponential, or logarithmic, relationship between the two.

Correlation coefficients were calculated in many of the experiments in this study. Calculation was carried out by electronic computer which effected a great saving in time. The program, and a brief description of it, appear in Appendix E.

APPENDIX D

THE CALCULATION OF FACILITATION

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THE CALCULATION OF FACILITATION

A pair of numbers, A & B, can be related to each other by the ratio B:A, or by the fraction B/A, which expresses the ratio as a fraction or decimal number.

If instead of a single number pair we have a series of pairs of numbers A(1) & B(1), A(2) & B(2) ... A(n) & B(n), then the overall relationship of the series can be expressed in two different ways :-

The ratio of the means :-

$$\frac{\sum_{i=1}^n B}{n} \bigg/ \frac{\sum_{i=1}^n A}{n} \quad (1)$$

The mean ratio

$$\frac{\sum_{i=1}^n (B/A)}{n} \quad (2)$$

i.e. the ratio of the two mean values (equation 1) or the mean value of the individual ratios between each pair (equation 2).

These two expressions only give the same numerical result when applied to the same set of figures if one or more of the following conditions is fulfilled by that set :-

$$\sum_{1}^n B = B(1) = B(2) = B(3) = \dots = B(n) \quad (3)$$

$$\frac{B(1)}{A(1)} = \frac{B(2)}{A(2)} = \frac{B(3)}{A(3)} = \frac{B(4)}{A(4)} = \dots = \frac{B(n)}{A(n)} \quad (4)$$

i.e. if the value of B (or A) is constant in all the individual pairs of numbers (equation 3) or if all the values of B/A are the same regardless of the actual values of A & B (equation 4).

If neither of these conditions is fulfilled, then the value obtained by equation 2 (the mean ratio) is always greater than that obtained by equation 1 (the ratio of the means) from the same data.

The following examples illustrate this :-

(a) In the set of figures :-

	13	8	ratio = 1.625	
	15	8	ratio = 1.875	
	17	8	ratio = 2.125	
	14	8	ratio = 1.75	
	11	8	ratio = 1.375	
÷ 5	70	40	8.750	÷ 5
(means)	14 : 8			
(ratio of means)	1.75		1.75	(mean ratio)

(b) In the set of figures :-

	10	8	ratio = 1.25	
	12.5	10	ratio = 1.25	
	15	12	ratio = 1.25	
	17.5	14	ratio = 1.25	
	20	16	ratio = 1.25	
÷ 5	75.0	60	6.25	÷ 5
(means)	15 : 12			
(ratio of means)	1.25		1.25	(mean ratio)

(c) In the set of figures :-

	10	8	ratio = 1.25	
	12.5	10	ratio = 1.25	
	15	12	ratio = 1.25	
	17.5	15	ratio = 1.25	
	6	12	ratio = 0.5	
+ 5	61.0	56	5.5	÷ 5
(means)	12.2 : 11.2			
(ratio of means)	1.089		1.1	(mean ratio)

(d) In the set of figures :-

	10	8	ratio = 1.25	
	12.5	10	ratio = 1.25	
	15	12	ratio = 1.25	
	17.5	14	ratio = 1.25	
	12	6	ratio = 2.0	
+ 5	67.0	50	7.0	÷ 5
(means)	13.4	10		
(ratio of means)	1.34		1.4	(mean ratio)

The sizes of individual action potentials show considerable variation and analysis shows that the variation follows a normal distribution (see Fig.23b), the standard deviation of which can be as much as a quarter of the mean. When pairs or trains of responses are evoked, the size of each potential of the train is subject to this large statistical variation. As a result, all quantitative results must be expressed statistically. When a measure of the facilitation of any action potential by the preceding one is required, this also must be expressed statistically since the facilitation (expressed as a ratio of the size of

the second potential to that of the first) varies greatly with the changes in potential size. The two methods of comparison of sets of pairs of figures, detailed above, can both be applied but, as has been demonstrated, different values are obtained with the two methods.

Since both values are clearly valid, it is necessary to establish criteria to determine which method to use. It is clear that the only facilitation likely to be caused by an action potential will be on potentials which immediately succeed it - there is not likely to be any interaction between successive pairs of responses provided there is sufficient time lag between the two pairs. The use of the mean ratio (Equation 2) was therefore thought to be the most suitable in this case, since it takes account of individual events.

Since both values, and also the ratios, are subject to variation, it is necessary to calculate the statistical significance of the resulting mean value for the ratio. The standard error is obtained from the following equation :-

$$\sqrt{\frac{\sum_{1}^{n} B/A - B/A}{n^2}}$$

APPENDIX. E

THE USE OF THE ELECTRONIC COMPUTER

APPENDIX E

THE USE OF THE ELECTRONIC COMPUTER

An electronic computer has been used in this study for three purposes :-

- (1) Speedier processing of raw data measured from oscilloscope trace photographs into statistically meaningful form, including the lengthy process of calculation of facilitation (Appendix D):.
- (2) Calculation of, and comparison of, the parameters of the action potential, as described in Chapter 5.
- (3) The correlation of results calculated in (1) above to discover any meaningful relationships between variables, as described in Appendix C. Computation enabled all possible (or all likely) relationships to be rapidly explored without extensive graph-plotting.

In this study the computer has simply been used as a rapid and automatic calculating machine; in statistical calculations the time saved has been of the order of 75%, and in the other applications mentioned above the saving has been much greater (90% +).

The machine used was an Elliott 803, though it was not quite a standard model. The programs were written in Elliott Algol, a dialect of the internationally-devised computing language Algol-60.

It is not necessary to describe the logical mechanics of the programs used which are themselves merely intermediaries between the operator and the extremely long and repetitive logical processes of the machine itself. The descriptions of the four main programs used, which follows, will give some indication of the way in which electronic computation is organised. The exact meaning of all the symbols used, some of which are peculiar to Elliott Algol, but many of which are in the general specification of Algol-60, appear in Woolridge & Ratcliffe (1963).

In the following description of the programs, the accompanying figures are copies of (a) the text of the program, (b) the lay-out of data to be processed, (c) an example of a print-out of results. Each part of each figure represents the typed copy produced by the tape-punching machine as the tape is read or produced; numbers follow each other on the tape, and are presented to, or produced by, the computer, strictly in the order in which they appear in the copy, i.e. in conventional reading order.

Program (a) "Fatigue Responses"

This program is the basic one evolved for statistical calculation of all experiments in which paired stimuli were applied. The only difference between this program, used in the experiments of Chapter 6, and others used for the same sort of purpose, was the title and units

of the first column of the print-out (Fig. E 1c), the other versions used being "Time in Minutes" (in the experiment of Fig.52, Ch.8) and "Saline Calcium mM/l" (in other experiments in the same chapter).

Fig. E 1a is the text of the "Fatigue Responses" program. This program produces values for the mean sizes of two successive action potentials (evoked by paired stimuli) and for the mean facilitation of the second potential by the first. It also produces a mean value for the mechanical response, converting the result it obtains from millivolts (in which the data was read from the oscilloscope) into milligrams. The program will process any number of sets of results, each set being the activity at (in this case) a particular stage (or, in other applications, a time after changing the saline, or at a particular level of saline calcium - see above). For each set of results the program processes either one or two groups of results at will, depending on whether simultaneous observation of two fibres has been carried out (see Chapter 6).

The data is organised as in Fig. E 1b. The first number read by the computer from the data ("2" in Fig. E 1b) tells it whether one or two groups of results are set out in each set (depending on whether single observation or two simultaneous ones were made). The second number is the identifying tag of the experiment (the first experiment if

FIGURE E 1

- (a) The text of the program "Fatigue Responses"
- (b) A sample of the data in the organisation accepted
by the program
- (c) A sample of the print-out of results produced by
the program

```

begin
  real suma, sumb, summ, ratb, sqa, sqb, sqc, mecr, gram;
  integer count, l, m, n, p, iden1, iden2, totno, indic;
  read indic, iden1, p, gram;
  print 'Stimuli?Experiment?', sameline, iden1;
  if indic = 2 then
    begin
      read iden2;
      print 'Experiment?', sameline, iden2;
    end;
  if gram>0 then print 'Mechanical?';
  print 'Elapsed?mean 1.s.e.mean 2.s.e.s??, facilitation.s.e.s??';
  if indic = 2 then print 'mean 1.s.e.mean 2.s.e.s??, facilitation.s.e.s??';
  if gram>0 then print ' mean mech,? else print '??';

  begin
    real array mean11[1:p], mean12[1:p], mean21[1:p], mean22[1:p], fac11[1:p], fac12[1:p], mech[1:p];
    integer array numb[1:p];
    switch s:=rpt;
    for l:=1 step 1 until p do
      begin
        read numb[l];
        print digits(6), numb[l];
        count:=0;
rpt:  read n;
        count:=count + 1;
        if count = 1 then totno:=n else totno:=totno + n;
        begin
          real array a[1:n], b[1:n], rb[1:n];
          suma:=sumb:=ratb:=sqa:=sqb:=sqc:=0;
          for m:=1 step 1 until n do
            begin
              read a[m], b[m];
              suma:=suma + a[m];
              sumb:=sumb + b[m];
              rb[m]:=b[m]/a[m];
              ratb:=ratb + rb[m];
            end;
          for m:=1 step 1 until n do
            begin
              sqa:=sqa + ((a[m] - (suma/n))2);
              sqb:=sqb + ((b[m] - (sumb/n))2);
              sqc:=sqc + ((rb[m] - (ratb/n))2);
            end;
          end of electrical calculation;

          if count = 1 then
            begin
              mean11[1]:=suma/n;
              mean12[1]:=sumb/n;
              fac11[1]:=ratb/n;
              print sameline, aligned(4,2), 's??, mean11[1], 's??, (sqrt((sqa/(n-1))/n)), 's??,
                mean12[1], 's??, (sqrt((sqb/(n-1))/n)), 's??, fac11[1], 's??, (sqrt((sqc/(n-1))/n));
            end;
          if count = 2 then
            begin
              mean21[1]:=suma/n;
              mean22[1]:=sumb/n;
              fac12[1]:=ratb/n;
              print sameline, aligned(4,?), 's??, mean21[1], 's??, (sqrt((sqa/(n-1))/n)), 's??,
                mean22[1], 's??, (sqrt((sqb/(n-1))/n)), 's??, fac12[1], 's??, (sqrt((sqc/(n-1))/n));
            end of electrical printout;
          if indic = 2 and count = 1 then goto rpt;

          if gram>0 then
            begin
              summ:=0;
              for m:=1 step 1 until totno do
                begin
                  read mecr;
                  summ:=summ + mecr;
                end;
              mech[1]:=entier(((summ/totno)*gram)/5)+0.5)*5;
              print sameline, digits(5), 's??, entier(mech[1]);
            end;
          end of mechanical calculation and printout;

          print 'p', p;
          for l:=1 step 1 until p do
            begin
              print '?', sameline, numb[l], mean11[1], mean12[1], fac11[1];
              if indic = 2 then print sameline, mean21[1], mean22[1], fac12[1];
              if gram>0 then print sameline, mech[1];
            end;

          end of summary printout;

        end of program;

```

<u>Stimuli</u>	<u>Experiment</u> 404				<u>Experiment</u> 405				<u>Mechanical</u> mean mech.				
	mean 1.	s.e.	mean 2.	s.e.	facilitation	s.e.	mean 1.	s.e.		facilitation	s.e.		
0	13.08	0.36	13.30	0.35	1.03	0.04	16.86	0.40	17.51	0.44	1.06	0.05	260
100	11.82	0.26	13.18	0.31	1.13	0.04	16.62	0.38	17.97	0.61	1.10	0.05	260
200	11.59	0.54	12.86	0.28	1.15	0.06	16.97	0.40	19.16	0.39	1.14	0.04	240

2
404
3
3.125
405

0

20

16.0 14.6 11.6 13.0 14.6 11.4 14.2 15.0 12.8 15.4 15.0 11.4 12.4 13.2
12.2 14.2 11.8 13.8 12.4 16.6 14.8 10.8 13.6 12.0 10.2 12.8 15.4 14.6
13.0 12.2 12.0 11.6 12.4 13.6 12.0 14.4 10.6 12.0 14.8 13.4
20
17.4 19.0 18.6 19.2 17.6 16.8 18.8 15.0 18.0 14.0 17.6 17.0 17.8 15.2
16.8 19.0 13.4 18.0 15.2 18.8 16.6 16.2 17.2 18.6 14.0 20.0 20.8 14.0
15.2 19.8 15.6 19.4 15.4 17.0 15.6 19.6 16.8 18.4 18.8 15.2
108 90 90 88 90 84 86 84 88 84 84 82 82 86 80 80 90 84 86 86
86 84 82 78 80 80 82 80 80 78 74 80 80 80 78 80 80 80 80 76

100

20

15.0 10.2 11.4 15.2 11.6 13.8 11.6 15.2 10.2 13.0 10.6 13.8 10.8 12.0
11.6 12.4 12.2 13.4 11.8 13.0 12.2 14.6 12.0 16.0 13.0 12.4 11.4 11.0
12.0 12.4 10.0 12.4 11.6 13.6 13.0 12.6 13.4 13.4 11.0 13.2
20
15.6 16.6 17.0 19.4 13.0 24.0 18.4 15.6 19.5 19.7 14.6 10.2 16.0 17.0
17.6 17.6 15.6 21.0 16.8 17.0 18.8 18.2 18.0 20.4 17.0 17.4 15.0 17.0
18.4 18.8 14.8 18.4 15.0 19.4 17.6 17.6 18.0 14.8 15.6 19.4
80 78 82 80 84 80 80 80 86 82 82 78 76 80 82 78 80 80 80 80
86 82 88 86 82 84 86 86 88 84 86 86 84 88 90 84 90 88 90 86

200

20

8.4 13.2 10.8 12.0 11.6 14.6 11.8 12.0 12.0 12.8 10.0 14.0 9.8 13.4
11.0 14.0 9.6 15.0 20.4 12.4 12.8 11.6 12.0 10.4 10.4 13.6 12.4 11.8
12.8 11.2 9.2 14.6 11.0 13.6 11.0 12.4 12.0 11.4 12.8 13.2
18
16.4 20.2 17.6 19.4 15.2 17.4 15.8 17.4 15.6 21.4 20.0 13.4 19.8 15.6
17.0 21.6 14.4 21.2 17.4 17.6 15.2 18.6 18.0 20.2 18.0 17.6 16.4 19.0
17.4 20.8 14.8 19.6 17.2 21.2 19.2 19.0 14.0 17.8
76 78 72 80 74 74 76 74 78 74 74 76 78 80 76 74 76 80 76 78
76 76 76 80 80 80 76 76 78 78 72 74 76 76 80 72 72 74 78

two simultaneous ones are to be dealt with). The third number tells the computer how many sets of results there are; the fourth is the factor by which the mechanical data (in mV) must be multiplied in order to convert to mgm. The fifth number is the identifying tag of the second experiment (if any) and only appears if the very first number is "2".

The computer cycles as many times as there are sets of results (determined by the third number). In each cycle the first number read is the number of stimuli elapsed (or the time elapsed, of the calcium content of the saline, see above); the next number gives the number of individual pairs of results there are to be averaged in that sample. This determined, the results are read in pairs, the members of each pair being the first and second responses to a pair of stimuli; means and standard errors are calculated for each member of the pair, and for the ratio (see Appendix D). If there is a second experiment, this process is repeated; individual mechanical results are then read, averaged, and converted to milligrams.

This process will be much clarified by inspection of Fig. E 1b where the data layout can be seen to follow from the description above; the initial five numbers are followed by three cycles of results, each forming a set; in each set there are two initial numbers followed by the results obtained

from each experiment, and the mechanical responses.

A sample print-out appears in Fig. E 1c; the results presented in this figure are in fact computed from the data of Fig. E 1b.

Program (b) "Responses from Repetitive Stimulation"

This program is an extension of program (a) above and was used in the experiments of Chapter 7 to calculate results from traces like those of Fig. 43. The text of this program appears in Fig. E 2a; it operates on only one set of results, there being no provision for calculation of simultaneous sets. Each set of results corresponds to one value for the stimulus gap; within each set the program calculates the mean value of any (specified) number of action potentials, express the value of each as a mean ratio to the first of the string, and perform similar calculations on the string of individual mechanical responses corresponding to the action potentials, converting each set of results from millivolts to milligrams.

In the data of Fig. E 2b, the first number read in by the computer ("302") is the experiment identifying tag, the second indicates the number of sets of results to be computed (4 in this case). The third number states how many potentials were produced in each string, and the fourth the number of samples of this string measured (to be averaged).

FIGURE E 2

- (a) The text of the program "Responses from Repetitive Stimulation"
- (b) A sample of the data in the organisation accepted by the program.
- (c) A sample of the main print-out produced by the program
- (d) A sample of the summary print-out produced by the program

```

begin
  real sum, ras, sqe, sqr, meca, mech, z;
  integer a, l, m, n, p, c, count, iden, indic, check;
  switch ss:=nbg;
  read iden, p, c, n;
  print %f12?%s30?experiment?, sameline, iden;
  begin
    real array pot[1:p,1:c], sepot[1:p,1:c], mrat[1:p,1:c], semrat[1:p,1:c], mecrr[1:p,1:c],
      ramech[1:p,1:c], depol[1:p], totm[1:p], gap[1:p], pota[1:n];
    switch sss:=n2;

    for l:=1, 1 + 1 while buffer (1,%*) and l<=p do
      begin
        read gap[l];
        for count:=1 step 1 until c do
          begin
            real array elec[1:n], rat[1:n];
            sum:=ras:=sqe:=sqr:=0;
            for m:=1 step 1 until n do
              begin
                read elec[m];
                sum:=sum + elec[m];
                if count=1 then pota[m]:=elec[m];
                rat[m]:=elec[m]/pota[m];
                ras:=ras + rat[m];
              end;
            for m:=1 step 1 until n do
              begin
                sqe:=sqe + ((elec[m] - (sum/n))^2);
                sqr:=sqr + ((rat[m] - (ras/n))^2);
              end;
            pot[l,count]:=sum/n;
            sepot[l,count]:=sqrt((sqe/(n-1))/n);
            mrat[l,count]:=ras/n;
            semrat[l,count]:=sqrt((sqr/(n-1))/n);

            sum:=0;
            for m:=1 step 1 until n do
              begin
                read mech;
                sum:=sum + mech;
              end;
            mecrr[l,count]:=sum/n*3.125;
            if count=1 then meca:=mecrr[l,count];
            ramech[l,count]:=mecrr[l,count]/meca;
          end;

          sum:=0;
          for m:=1 step 1 until n do
            begin
              read z;
              sum:=sum + z;
            end;
          depol[l]:=sum/n;

          sum:=0;
          for m:=1 step 1 until n do
            begin
              read z;
              sum:=sum + z;
            end;
          totm[l]:=sum/n*3.125;
          check:=1;
        end of calculation;

        if check<=p then
          begin
n2:   for a:=1, a + 1 while not buffer (1,%*) do read z;
        print %f12?Data Error in Stimulus Gap ?, sameline, gap[check], %Msec.??;
        a:=check + 1;
        if a<=p then
          begin
            for l:=a, 1 + 1 while buffer (1,%*) and l<=p do
              begin
                read gap[l];
                for count:=1 step 1 until c do
                  begin
                    real array elec[1:n];
                    for m:=1 step 1 until n do read elec[m], mech;
                  end;
                for count:=1 step 1 until c do read z, z;
                check:=1;
              end;
            end
          else check:=a;
          if check<=p then goto n2;
          goto nbg;
        end of data error detection;

        for indic:=0, indic + 10 while p>indic do
          begin
            if p<(indic + 10) then a:=(p - indic) else a:=10;
            print %f16?Stimulus Gap Msec. ?;
            for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), gap[l], %f12?%;
            print %f13?Potential 1. mV. ?;
            for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), pot[l,1], %f12?%;
            print %f17?%s3?s.e.%s22?%;
            for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), sepot[l,1], %f12?%;
            for count:=2 step 1 until c do
              begin
                print %f12?Potential ?, sameline, count, %f. mV. ?;
                for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), pot[l,count], %f12?%;
                print %f17?%s3?s.e.%s22?%;
                for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), sepot[l,count],
                  %f12?%;
                print %f17?Ratio to Potential 1. ?;
                for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(3,2), mrat[l,count],
                  %f12?%;
                print %f17?%s3?s.e.%s22?%;
                for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), semrat[l,count],
                  %f12?%;
              end;
            print %f13?Mechanical 1. mgm. ?;
            for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), mecrr[l,1], %f12?%;
            for count:=2 step 1 until c do
              begin
                print %f12?Mechanical?, sameline, count, %f. mgm. ?;
                for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), mecrr[l,count],
                  %f12?%;
                print %f17?Ratio to Mech. 1 %f11?%;
                for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(3,2), ramech[l,count],
                  %f12?%;
              end;
            print %f12?Depolarisation at%Initiation of %s14?%;
            for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), depol[l], %f12?%;
            print %f17?Potential ?, sameline, c, %f. mV.?, %f12?Overall Mechanical %s9?%;
            for l:=(indic + 1) step 1 until (indic + a) do print sameline, aligned(4,2), totm[l], %f12?%;
            print %f17?Response mgm. ?;
          end of main printout;

          print %f16?%s20?Summary.%f12?%;
          for l:=1 step 1 until p do
            begin
              print %f11?%, sameline, aligned(4,2), gap[l], %f12?%, depol[l], %f12?%, totm[l], %f12?%;
              for count:=1 step 1 until c do print sameline, aligned(4,4), mrat[l,count];
              print %f12?%;
              for count:=1 step 1 until c do print sameline, aligned(4,4), ramech[l,count];
            end of summary printout;
          end;
        nbg: end of program;

```

200.00	0.00	30.63	1.0000	0.9650	1.0073	0.9635	1.0428	1.0000	2.0000	1.8889	2.0370	1.8148
150.00	0.00	43.75	1.0000	1.0364	1.0217	1.0451	1.0190	1.0000	1.9677	2.1935	2.2903	2.2581
125.00	0.14	81.87	1.0000	1.1323	1.1557	1.3030	1.1896	1.0000	2.8966	3.4138	3.8621	3.7586
100.00	0.32	124.37	1.0000	1.0424	1.0474	1.1460	1.0950	1.0000	4.5000	5.4167	5.4167	5.5833

Summary.

302;

4;
5;
5;

200;

11.2;	11.9;	11.8;	9.8;	10.8;	9;	5;	5;	4;	4;
10.4;	10.5;	11.9;	9.2;	11.2;	14;	11;	9;	9;	11;
10.4;	12.6;	11.2;	10.8;	10.4;	12;	10;	9;	10;	10;
11.1;	11.2;	10.6;	9.2;	11.0;	10;	12;	11;	16;	12;
11.2;	11.6;	9.8;	11.6;	13.1;	9;	10;	10;	9;	11;
0;	0;	0;	0;	0;					
8;	10;	10;	9;	11*					

150;

12.0;	10.7;	8.6;	8.9;	10.8;	9;	7;	5;	6;	4;
10.2;	11.0;	9.6;	10.9;	10.4;	14;	13;	12;	12;	10;
8.9;	10.0;	10.0;	11.3;	9.9;	14;	14;	14;	12;	14;
8.6;	11.4;	12.0;	10.4;	8.6;	16;	13;	15;	14;	14;
10.4;	10.0;	10.7;	10.0;	10.0;	14;	14;	14;	14;	14;
0;	0;	0;	0;	0;					
14;	14;	14;	14;	14*					

128;

7.6;	9.6;	8.6;	10.0;	8.3;	6;	7;	7;	5;	4;
8.8;	11.0;	9.3;	11.8;	9.1;	18;	17;	16;	18;	15;
9.8;	10.9;	10.5;	10.0;	9.4;	23;	20;	18;	18;	20;
12.8;	11.2;	9.6;	10.9;	12.1;	24;	23;	19;	23;	23;
10.6;	10.0;	10.9;	11.1;	10.1;	23;	20;	20;	25;	21;
0.1;	0.2;	0;	0.2;	0.2;					
27;	28;	25;	26;	25*					

100;

10.0;	9.2;	9.6;	7.1;	11.3;	5;	4;	4;	6;	5;
9.2;	9.4;	9.7;	10.2;	9.3;	22;	21;	20;	24;	21;
8.8;	10.4;	11.2;	9.6;	8.0;	26;	26;	27;	25;	26;
11.8;	9.2;	9.9;	11.6;	10.0;	26;	26;	24;	27;	27;
8.4;	11.0;	10.8;	10.4;	8.6;	26;	26;	27;	29;	26;
0.4;	0.2;	0.2;	0.5;	0.3;					
40;	38;	39;	43;	39*					

Experiment 302

Stimulus Gap	Msec.	200.00	150.00	125.00	100.00
Potential	1. mV.	11.04	10.20	8.82	9.44
S.E.		0.43	0.64	0.44	0.63
Potential	2. mV.	10.64	10.42	10.00	9.56
S.E.		0.46	0.26	0.59	0.18
Ratio to Potential 1.		0.96	1.04	1.13	1.04
S.E.		0.03	0.00	0.02	0.10
Potential	3. mV.	11.08	10.22	10.12	9.60
S.E.		0.41	0.27	0.26	0.27
Ratio to Potential 1.		1.01	1.02	1.16	1.05
S.E.		0.04	0.08	0.05	0.11
Potential	4. mV.	10.52	10.40	11.32	10.50
S.E.		0.37	0.61	0.55	0.51
Ratio to Potential 1.		0.96	1.05	1.20	1.15
S.E.		0.02	0.11	0.12	0.13
Potential	5. mV.	11.40	10.22	10.40	10.64
S.E.		0.58	0.14	0.19	0.47
Ratio to Potential 1.		1.04	1.02	1.19	1.09
S.E.		0.08	0.07	0.06	0.13
Mechanical	1. mgn.	16.37	19.38	18.12	15.00
Mechanical	2. mgn.	33.75	36.13	52.50	67.50
Ratio to Mech. 1		2.00	1.97	2.30	4.50
Mechanical	3. mgn.	31.33	42.50	61.33	61.25
Ratio to Mech. 1		1.89	2.19	3.41	5.42
Mechanical	4. mgn.	34.33	44.33	70.00	81.25
Ratio to Mech. 1		2.04	2.29	3.36	5.42
Mechanical	5. mgn.	30.63	43.75	66.13	83.75
Ratio to Mech. 1		1.81	2.28	3.76	5.58
Depolarization at Initiation of Potential	5. mV.	0.00	0.00	0.14	0.32
Overall Mechanical Response	mgn.	30.63	43.75	81.87	124.37

In this data there were five potentials in each string (as in Fig. 43) and five samples were evoked, measured, and averaged.

In each set of results, the first number is the stimulus gap value relating to the set; the data is then set out in the following order :- electrical string sample 1, mechanical string sample 1, electrical string sample 2, mechanical string sample 2 etc. followed by the corresponding values for base depolarisation for each string, and the total mechanical response of each string.

The print-out appears as in Fig. E 2c, and is largely self-explanatory. Fig. E 2d shows the summary print-out, produced as well as the full print-out; it is a precis of the more elaborate version, and can be used almost directly as the input data for program (d) below. The other programs have a similar facility, for the same purpose.

Program (c) "Action Potential Relations"

This program was specially written to calculate the data collected in the surveys of Chapter 5 and to evaluate the various factors there described.

The first number of the data (Fig. E 3b) is the number of penetrations which have been made in the survey, the second is the number of successive action potentials observed and recorded at each locus (the means of the factors

FIGURE E 3

- (a) The text of the program "Action Potential Relations"
- (b) A sample of the data in the organisation accepted by the program

Correlation Coefficients of U or log.e U, V or log.e V. C.M. Malpus;

```
begin
  real sumu, sumv, sumuv, squ, sqv, sdu, sdv;
  integer iden, a, b, l, m, n, p, x, array, wu, wv;
  read p, array;
  for l:=1 step 1 until p do
    begin
      read iden, n;
      print 'Experiment?', sameline, iden,
        'U:V log.e U:V log.e U:log.e V?';
      begin
        real array z[1:array,1:n];
        for m:=1 step 1 until n do
          begin
            for b:=1 step 1 until array do
              begin
                read z[b,m];
                z[b,m]:=z[b,m] + 1;
              end;
            end;
            for x:=1, x + 1 while not buffer(1,x) do
              begin
                real array u[1:n], v[1:n];
                switch ss:=start, two, three, four, infin;
                read wu, wv;
                for m:=1 step 1 until n do
                  begin
                    u[m]:=z[wu,m];
                    v[m]:=z[wv,m];
                  end;
                for a:=1 step 1 until 4 do
                  begin
                    sumu:=sumv:=sumuv:=squ:=sqv:=0;
                    for m:=1 step 1 until n do
                      begin
                        if a=2 then goto two;
                        if a=3 then goto three;
                        if a=4 then goto four;
                        goto start;
                      two:
                        u[m]:=ln(z[wu,m]);
                      three:
                        u[m]:=z[wu,m];
                        v[m]:=ln(z[wv,m]);
                      four:
                        u[m]:=ln(z[wu,m]);
                      start:
                        sumu:=sumu + u[m];
                        sumv:=sumv + v[m];
                        sumuv:=sumuv + (u[m]*v[m]);
                      end;
                    for m:=1 step 1 until n do
                      begin
                        squ:=squ + ((u[m] - (sumu/n))2);
                        sqv:=sqv + ((v[m] - (sumv/n))2);
                      end;
                    sdu:=(sqrt(squ/n));
                    sdv:=(sqrt(sqv/n));

                    if a=1 then print ' ', sameline, digits(2), wu, ' & ', wv, ' ';
                    if sdu<10-6 or sdv<10-6 then
                      begin
                        print 'none';
                        goto infin;
                      end;
                    print ' ', sameline, aligned(1,2), ((sumuv/n) - ((sumu/n)*(sumv/n)))/(sdu*sdv), ' ';
                  infin: end of calculation of each coefficient;

                    end of calculation of set of four coefficients;

                end;
              end of calculation of coefficients for each set of data;
            end of program;
          end;
        end;
      end;
    end;
  end;
end;
```

Action Potential Relations. C.M. Malpus;

```

begin
  real rp, ap, sump, sumris, sumdec, swap, lag;
  integer a, l, m, n, p, count, change;
  boolean anychange;
  read p, n;
  rp:=ap:=0;
  begin
    real array out[1:p,1:6], order[1:p], size[1:n], srise[1:n], sdecay[1:n];
    integer array mark[1:p];
    switch ss:=again;

    for l:=1 step 1 until p do
      begin
        sump:=sumris:=sumdec:=0;
        for m:=1 step 1 until n do
          begin
            read size[m], srise[m], sdecay[m];
            sump:=sump + size[m];
            sumris:=sumris + srise[m];
            sumdec:=sumdec + (sdecay[m] - srise[m]);
          end;
        read out[l,1];
        rp:=rp + out[l,1];
        out[l,2]:=sump/n;
        ap:=ap + out[l,2];
        out[l,3]:=((sump/n)/(sumris/n));
        out[l,4]:=(out[l,3]/out[l,3]);
        out[l,5]:=sumdec/n;
        out[l,6]:=(out[l,5]/out[l,2]);
      end of calculation;

      print 'Original Order', resting, mean action, mean rise, rise?,
            'qtr. decay', decay, 'potential', mV, 'potential', mV, 'time', V/sec. ?,
            'factor', time, msec, 'factor?';
      for l:=1 step 1 until p do print ' ', sameline, aligned(2,2), out[l,1], ' ',
            out[l,2], ' ', out[l,3], ' ', out[l,4], ' ', out[l,5], ' ', out[l,6];
      print 'Mean Resting Potential = ', sameline, aligned(3,1), rp/p,
            'Mean Action Potential = ', ap/p;

      for a:=1, 2, 4, 6 do
        begin
          for l:=1 step 1 until p do
            begin
              mark[l]:=1;
              order[l]:=out[l,a];
            end;
          again:anychange:=false;
          for l:=1 step 1 until (p-1) do
            begin
              if order[l+1]<order[l] then
                begin
                  swap:=order[l];
                  order[l]:=order[l+1];
                  order[l+1]:=swap;
                  change:=mark[l];
                  mark[l]:=mark[l+1];
                  mark[l+1]:=change;
                  anychange:=true;
                end;
            end of sort procedure;
          if anychange then goto again;

          if a=1 then print 'Ascending Order - Resting Potential';
          if a=2 then print 'Ascending Order - Action Potential';
          if a=4 then print 'Ascending Order - Rise Factor';
          if a=6 then print 'Ascending Order - Decay Factor';
          for l:=1 step 1 until p do
            begin
              count:=mark[l];
              print ' ', sameline, aligned(2,2), out[count,1], out[count,2],
                    out[count,3], out[count,4], out[count,5], out[count,6];
            end of printout;
          end of sort and printout;
        end;
      end of program;

```

4
5

11.6 2.4 5.6 11.4 2.4 5.8 11.9 2.3 5.7 12.0 2.5 5.5 11.0 2.4 5.7
89

9.6 2.5 7.6 9.8 2.7 7.6 2.4 2.4 7.8 9.9 2.7 8.0 9.1 2.5 7.7
81

4.4 1.8 4.4 4.5 1.7 4.3 4.2 1.7 4.2 4.7 1.5 4.5 5.0 1.8 4.4
86

15.0 3.1 9.0 15.2 3.2 9.5 15.4 3.1 9.7 14.8 3.0 8.9 14.6 3.1 9.0
76

of which will be calculated). In each of the sets, the computer reads in the stipulated number of sets of parameters which consist of the size of the action potential, the duration of the rising phase, and the quarter-decay time of the decay phase. The final, single number of each set (appearing below the sets of parameters, see Fig. E 3b) is the resting potential observed at that penetration.

The program is set out in Fig. E 3a; Table 6, Appendix A, which is the detailed result of one of these surveys, is an exact (even to the spacing) copy of the computer print-out produced by this program. In addition to this table, a print-out of the results is produced with each of the columns in turn sorted into ascending order of magnitude. This enabled a rapid appreciation to be made of any inter-relationship between columns.

Program (d) "Correlation Coefficient ..."

This program specifically evaluates the correlation coefficient of sets of data. The use of this parameter is described in full in Appendix C. The program derives logarithmically-transformed correlations of either or both variables in addition to the linear correlation (see Appendix C).

The mechanisms of the program (which appears in Fig. E 4a) are very similar to the other three as detailed above. Columns of data (with preliminary procedural

FIGURE E 4

- (a) The text of the program "Correlation Coefficient
of U or log.e U, V or Log.e V"
- (b) A sample of the data in the organisation accepted
by the program
- (c) A sample of the print-out produced by this program

Experiment 302

	U:V	log. ^o U:V	U:log. ^o V	log. ^o U:log. ^o V
1 & 2	-0.87	-0.96	-0.92	-0.98
1 & 3	-0.77	-0.91	-0.95	-0.99
2 & 3	0.97	0.93	0.95	0.99
1 & 8	-0.75	-0.77	-0.76	-0.77
1 & 10	-0.80	-0.98	-0.96	-0.99
1 & 11	-0.77	-0.90	-0.92	-0.99
1 & 12	-0.78	-0.91	-0.92	-0.98
1 & 13	-0.81	-0.93	-0.94	-0.99

numbers) such as are illustrated in Fig. E 4b are input. These columns were usually obtained from summary print-outs such as appear in Fig. E 2d. After these columns had been input, pairs of numbers were input, each number defined one of the columns (numbered from left to right in the data), the two numbers defining the two columns between which a correlation was required. Thus in the data of Fig. E 4b the first pair of figures call for the correlation coefficient between columns one and two.

For each pair of columns called, the program produced correlation coefficients between the two, linearly, between each column and the logarithmic transform of the other, and between the logarithmic transforms of the two. In the output results (Fig. E 4c) coefficients are tabulated, vertically by the stipulated columns, and horizontally by the type of transform.

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SUMMARY

SUMMARY

1. The functional anatomy and the innervation of the muscles involved in the movement of the mesothoracic coxa and trochanter are described in some detail.
2. Analysis of the haemolymph shows that there is no significant difference in ionic content between different instars of the same species, between the different species of the two genera investigated, or between the two genera themselves (Aeschna & Libellula).
3. The ionic content of the haemolymph in mM/l (\pm s.e.) was :-
$$\text{Na} - 133.4 \pm 2.8; \text{K} - 3.6 \pm 0.2;$$
$$\text{Cl} - 121.0 \pm 3.1; \text{Ca} - 10.3 \pm 1.1;$$
$$\text{Mg} - 7.2 \pm 0.6; \text{inorganic phosphate } 1.9 \pm 0.4$$
4. The pH of the haemolymph was 7.8 ± 0.2 (s.e.)
5. An artificial saline was formulated from these figures, taking into account the analytical results of other workers, to which it was found necessary to add 55gm/l saline (approx. 160mM/l) sucrose to maintain isotonicity.
6. Excised preparations of the coxal depressor of trochanter muscle (coxal muscle) and of the flexor tibialis muscle are described. These preparations were designed for investigation with intracellular micro-electrodes and

a mechano-electronic transducer.

7. Local active responses can be evoked in the membrane of the muscle fibres by high-intensity direct stimulation. The membrane parameters were determined to be of the same order of magnitude as other known arthropods, except the length constant; this was of the order of 0.4mm, its extreme shortness being ascribed to the relatively small diameter of the fibres (about 50 μ).
8. The coxal muscle preparation is innervated by a single axon, stimulation of which evoked a muscle response similar in principle to the generalised arthropod slow (tonic) response
9. The extensor tibialis is dually innervated via a single nerve trunk; stimulation of one axon gives a response identical to that of the coxal muscle in about half of the fibres, but none in the remainder; stimulation of the other gives a typical arthropod fast (twitch) response in all fibres.
10. The electrical appearance of both fast and slow muscle fibre responses is more easily referable to known crustacean response patterns than to those of other known insects
11. The slow mechanical response to repetitive neural

stimulation is compared with the electrical activity evoked simultaneously in representative fibres of the muscle. It is apparent that there is no simple relationship between the two, but an explanation in terms of current theory is attempted.

12. The size of the slow action potential varies considerably, not only from fibre to fibre within a muscle block, but also along any one fibre, and at a single locus on a fibre.
13. The size of the slow action potential as recorded randomly in a muscle block is not a function of the resting potential recorded at the same penetration. The variation in size of the action potential, it is suggested, is a product of multi-terminal origin on each fibre at end-plates which have an area small in relation to the length constant of the membrane, and to the inter-endplate region distance. It is also a product of the rapidly-decaying way in which the potentials spread from these end-plates.
14. Patterns of fatigue of the slow action potential suggest that there might be two types of muscle fibre, innervated by the one axon, in the coxal muscle; experiments have so far failed to show any differences in fibre properties other than rate of fatigue at constant activity

15. In fresh preparations the slow action potentials do not facilitate when successively evoked, except when the interval is so short that the two potentials appear to overlap, thought to be a result of interaction of released transmitter acting with transmitter still persisting from the previous release. Summation processes are normal.
16. In fatigued preparations facilitation occurs; it is roughly a function of stimulus frequency, but is very variable.
17. The size of the slow action potential and of the mechanical response are closely related to the amount of calcium present in the external medium. Both responses are rapidly abolished in a calcium-free medium and are greatly enhanced in a calcium-rich medium.
18. The evoked response to an increase in calcium concentration occurs in two phases, mechanical and electrical responses following the same time-course. It is likely that the initial rapid changes are due to the effect of the calcium concentration on the amount of neuromuscular transmitter released by nerve action, and that the secondary changes, which take place over a period of half an hour, are due to

enhancement of both mechanical and electrical responses by inward diffusion of calcium from the rich medium.

19. The effect of changes in the concentration of magnesium in the external medium is antagonistic to that of calcium with respect to the slow electrical response; increase in magnesium leads to a reduction in size, decrease to an enhancement.
20. The level of magnesium in the external medium is critical for optimum size of mechanical response. Both increase and decrease from the normal level (6 - 7mM/l) lead to a decrease in size of the mechanical response, though there is some indication of a momentary increase in size in reduced saline magnesium.
21. Appendices describe the mathematical basis of the determination of constants of membranes, the statistical method of calculation of facilitation ratios, the use of the correlation coefficient and of the electronic computer.

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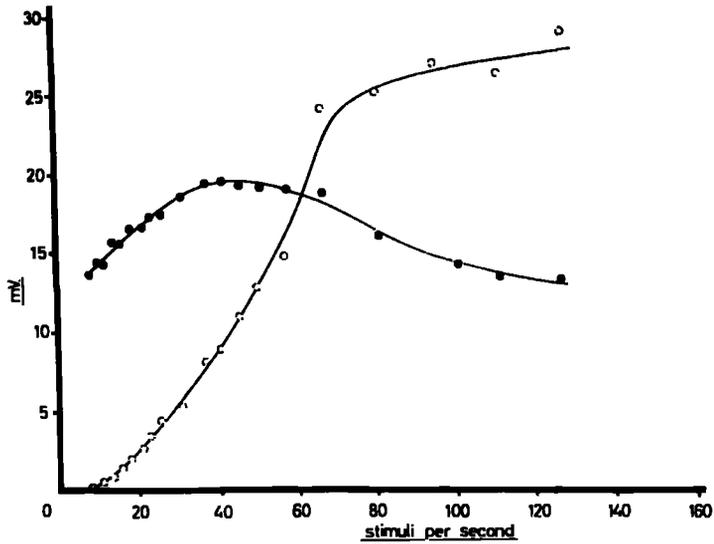
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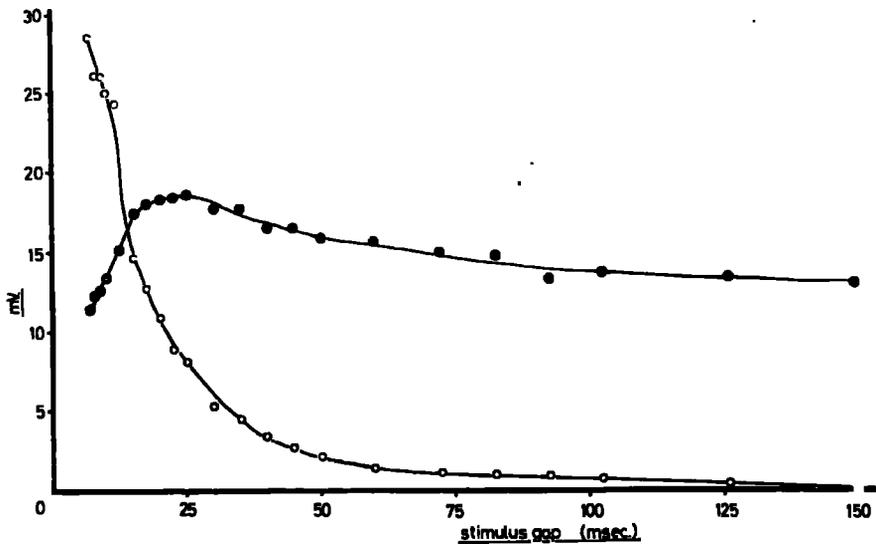
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(a)



(b)

The Response to Repetitive Stimulation of the Slow Axon.

(a) Depolarisation plotted against stimulation frequency.
 (b) Depolarisation plotted against the stimulus gap

○ - base depolarisation
 ● - mean potential size.

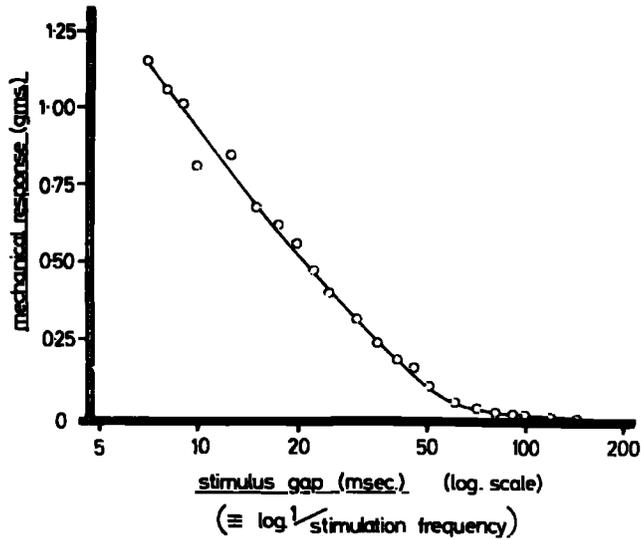
$$\text{stimulus gap (msec.)} = \frac{1000}{\text{frequency per second}}$$

Figure 38.

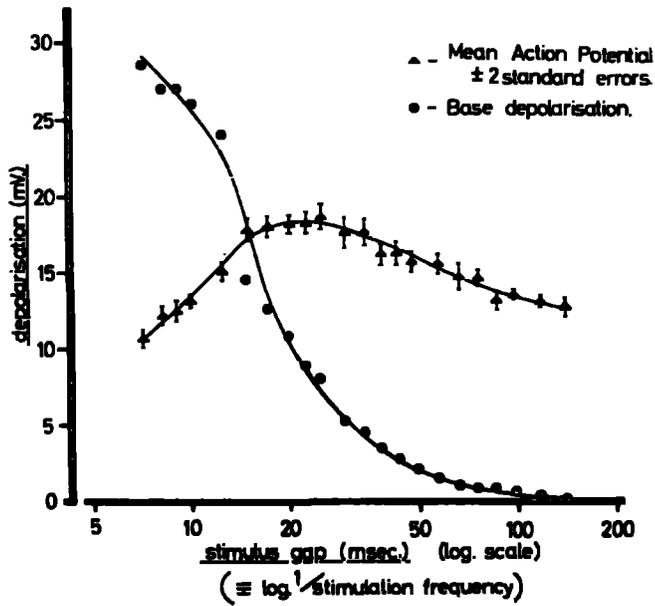
The mean potential size was obtained by averaging the sizes of the first 25 responses evoked by each train of stimuli. Inspection of Fig.37 reveals that when stimuli are applied in relatively rapid succession the individual responses do not summate equally; instead, within the first three or four responses, a steady state is reached in which each action potential commences at the same membrane polarisation level; at higher stimulation frequencies this steady state level is usually at a less-polarised level than is the resting level, from which single responses arise. The difference between the two levels has been termed the base depolarisation.

Fig.38 shows that the plotting of the same data in two ways, using frequency and stimulus gap, gives a rather different picture. It was found in this and other experiments that calculations and plots utilising values of stimulus gap were the more meaningful at sight, particularly in relation to the base depolarisation; in Fig.38b it is fairly evident by inspection that the relationship between base depolarisation and stimulus gap is exponential. Consequently the parameters of stimulus application have been referred to throughout in terms of the stimulus gap, in milliseconds.

In Fig.39 the results of a typical experiment of this type are given; detailed results appear as Table 9,



(A) mechanical



(B) electrical

The Response to Repetitive Stimulation of the Slow Axon.

Figure 39.