STUDIES ON THE NEUROMUSCULAR ANATOMY AND PHYSIOLOGY OF THE STICK INSECT, CARAUSIUS MOROSUS BR. (CHELEUTOPTERA).

by

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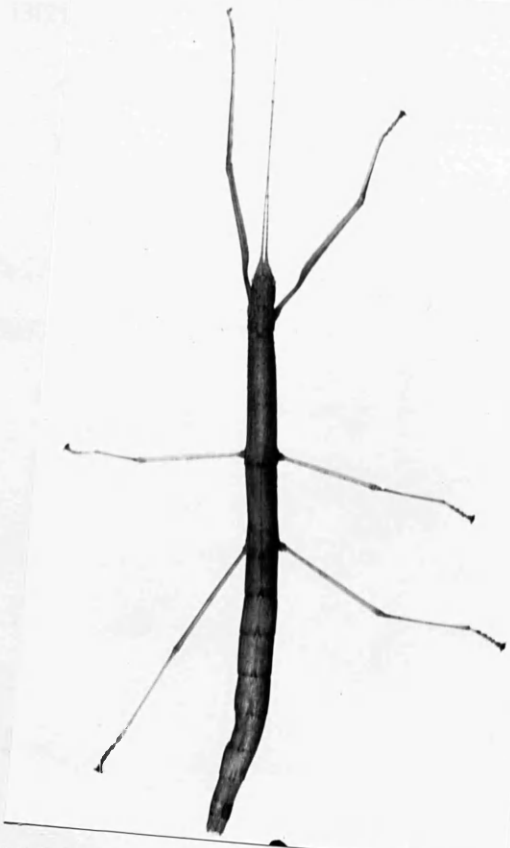
PUBLICATION.

Most of the material contained in Section III of this thesis has been incorporated in a paper which has been accepted for publication in the Journal of Physiology.

CARAUSIUS MOROSUS BR. ADULT FEMALE.



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INDEX.

GENERAL INTRODUCTION.

The most detailed studies of the nervous systems of animals have been made upon mammals. In mammals the skeletal muscles, which are the effector organs utilized in nearly all behavioural acts, are usually composed of thousands of muscle fibres innervated by hundreds of motor nerve fibres. These motor nerve fibres run from a central nervous system of great complexity. Nevertheless, the early work of the Sherrington School, which led to the publication of the classic "The Integrative Action of the Nervous System" (Sherrington, 1947, 2nd. ed.) showed that much could be learned about the functioning of the mammalian nervous system from studies of the sensory and motor apparatus. Following an increased interest in the detailed functioning of the sense organs, and the use of direct electrical recording methods in the study of central neurons, it may be expected that the early work of Sherrington will be progressively amplified and modified, and that our knowledge of the detailed working of the mammalian central nervous system will continue to grow.

In some ways, the nervous systems of insects appear

to be simpler than those of mammals or of vertebrates in general. Because they are so small, the muscles of insects contain relatively few fibres, and they are innervated by a very small number of motor axons - suggesting that peripheral control is of some importance. The central nervous system is less under the domination of the head; and this fact, coupled with the segmental organisation of the animal, enables a study of isolated parts of the body to be more easily made, e.g. of one thoracic ganglion and its associated limbs. As a result, it should be possible to arrive at an understanding of the quantitative basis of behaviour more easily in an insect than in a vertebrate, particularly a mammal. This is especially likely, since insect behaviour is largely instinctive. Insects appear, therefore, to offer considerable scope for neurophysiological studies which have as their general aim the ultimate description of behaviour in quantitative terms. In addition, they exhibit many interesting related features which make them attractive to the comparative physiologist.

This thesis describes work done in connection with the neuromuscular physiology of the common stick insect Carausius morosus Br. (Cheleutoptera). This insect is comparatively simple from a behavioural point of view.

It is wingless and parthenogenetic, lacking any behaviour associated with flight or reproduction. It is normally inactive during the day, but becomes active at night, when it feeds. In general, most activities seem to be linked to a diurnal rhythm, since moulting and hatching also normally occur only at night. When handled the animal may either struggle violently, or become immobile and rigid, often in the characteristic stick-like attitude. When disturbed other than by handling it often sways from side to side. It is hoped that it will prove possible in time to analyse all these activities.

In the present work, a start has been made by examining the neuromuscular mechanisms of one of the skeletal muscles, namely the flexor tibialis of the thoracic limbs. It is intended to extend this work in the future to all the limb muscles and to combine the results with those obtained from studies of the afferent impulses entering the ganglia during walking movements; and thence to make deductions about the central apparatus.

Apart from the general problem, the relationship between the ionic composition of the blood and neuromuscular transmission is of interest in Carausius. The work so far carried out upon insects (Hoyle, 1957) indicates that neuromuscular transmission in these

animals is similar in many respects to the corresponding process in vertebrates (see del Castillo and Katz, 1956). In particular, it appears that the cations bathing the muscles exert comparable actions upon neuromuscular transmission in the two groups. Most herbivorous insects, however, of which Carausius is one, exhibit blood ionic compositions which are markedly different from those of vertebrates or other insects (see Section III) and which might as a result be expected to have an adverse effect upon their neuromuscular transmission, and hence their general level of activity. This question has been examined.

The thesis falls naturally into three sections. In Section I, the anatomy of the limb muscles and their innervation are described. Coxal muscles are included with the femoral muscles for several reasons:

(a) to determine how far each leg could be regarded as similarly innervated;

(b) a small but important branch from one nerve running to the coxal muscles innervates the extensor tibialis of the locust (Hoyle, 1955b) in addition to its obvious innervation by the crural nerve, whose presence had not been suspected previously in spite of a detailed work on the anatomy of this insect (Albrecht, 1954); and

(c) to provide a background for the studies already mentioned which it is hoped to carry out in the future.

In Section II the neuromuscular mechanisms of the flexor tibialis muscle are described and discussed. In Section III, the results of blood analyses are given, and the effects of the four common cations upon the "fast" type of neuromuscular transmission are considered.

Hoyle (1957) has laid down certain requirements which he considers should be fulfilled if the results of studies on insect neuromuscular physiology are to be regarded as valid. These are:

(i) The anatomy and innervation of the muscles studied should be known.

(ii) The spacing of the motor end-plates along the muscle fibres should be known.

(iii) Axons supplying the muscle should be stimulated separately. Where differences in the properties of the axons are utilised for this purpose, impulses passing down the axons should be checked by monitoring.

(iv) Intracellular recording from the muscle fibres should be employed in adequate sampling from all parts of the muscle.

All these requirements have been met in the course of the work presented here.

SECTION I.

THE ANATOMY AND INNERVATION OF THE THORACIC LIMB MUSCLES.

INTRODUCTION.

Anatomy of thoracic muscles.

The thoracic muscles and nerves of Carausius morosus have been the subject of two previous accounts. The thoracic muscles were described by Jeziorski (1918), but he was more concerned with the body wall muscles which move the thoracic segments. His drawings of the muscles which move the coxae are sketchy, and as he made no attempt to separate them from one another, they give no idea of the origins and insertions of the muscles.

The work of Marquardt (1940) was much fuller. He showed all the thoracic muscles, coxal and body wall, well separated and labelled, and he described their innervation. He restricted himself to the muscles seen in the thorax, and did not attempt a detailed description of the femoral muscles. His purpose was to establish whether the pattern of nerves and muscles was similar in all three thoracic segments, and his work was sufficiently detailed for that purpose. Nevertheless, it was not felt to be

sufficient for the present work. A number of the nerve branches are shown by Marquardt as dotted lines, their exact destination being uncertain. Hoyle (1955b) found that a branch of nerve 3b (see later) innervated the extensor tibialis muscle of the jumping leg of Locusta migratoria. The existence of this branch had not been suspected previously, despite the publication of a monograph on the locust nervous system (Albrecht, 1954). This discovery emphasizes the necessity for tracing the course of all the thoracic nerves when studying any of the thoracic or limb muscles.

Innervation of insect muscles.

The number of axons supplying insect motor nerve endings has been the subject of a good deal of work. It is evident from this work that the muscles of most insects are supplied by a very small number of motor axons. Single nerve fibre innervation occurs in the sound muscles of cicadas (Hagiwara, 1953; Pringle, 1954), but it seems probable that in insects, as in Crustacea (e.g. see Wiersma, 1952) there is usually a minimum of two motor nerve fibres running to each muscle. A double innervation was described by Mangold (1905) in the thoracic leg and the "spring" muscles of Decticus, and in the body muscles

of caterpillars Ratpen. Montalenti (1928) found that the leg muscles of Hydrophilus were triply innervated; and Hoyle (1955b) also demonstrated that the extensor tibialis muscle of the jumping leg of the locust is triply innervated, although the homologous muscles of the other legs receive only two nerve fibres. The flexor tibialis muscles of Acanthacris ruficornis and Zonocerus sp. (Ewer, 1954), Romalea microptera (Ripley, 1954) and Locusta migratoria and Schistocerca gregaria (Hoyle, 1957) are innervated by four or more axons: carefully graded stimulus intensities applied to the motor nerve resulted in a stepwise increase in the tension developed by the muscle. However, it seems probable that in all these cases the flexor tibialis muscle is divided into units, each unit being composed of a compact bundle of muscle fibres and innervated by a separate "fast" nerve fibre (Hoyle, 1955b). Evidently, graded contractions can be elicited from such muscles, by the vertebrate method of varying the number of such units. Nevertheless, these muscles are not entirely comparable with those of vertebrates. In a vertebrate muscle the motor unit probably consists of muscle fibres scattered throughout the muscle (e.g. see Tiegs, 1953).

It will be apparent that insect muscles consisting of a number of units of this kind can be regarded as

essentially doubly innervated, since each separate unit is innervated by two axons of similar type.

Insect motor nerve endings.

The subject of insect motor nerve endings has received much attention in the literature, yet the most important facts remain peculiarly elusive. For a complete understanding of neuromuscular transmission, it is necessary to know the shape and structure of the motor nerve ending, its relationship to the sarcolemma and the contractile substance of the muscle, the area it occupies on the surface of the muscle, and its distance from neighbouring nerve endings on the same fibre. Few authors have been able to supply many of these details.

One reason for this lack of information is the extreme difficulty of differentiating insect motor nerve endings from surrounding structures. All methods used for staining nerve endings also stain muscle fibres and tracheoles. This is particularly true of silver stains. Critical examination of the figures given by Cajal (1890) and Marcu (1929), who both used silver techniques, gives rise to a strong impression that these workers erroneously described tracheoles as motor nerve endings. Marcu (1929) described the filiform endings he saw as penetrating the

contractile substance; and Tiegs (1955) has since made a similar claim. As Katz (1949) has pointed out, penetration of motor nerve endings into the contractile substance is most improbable, since the high internal potassium concentration of the muscle fibres would be expected to block conduction along the endings. The figure given by Tiegs (1955) is more difficult to assess than those of Cajal (1890) or Marcu (1929), as it consists of a transverse section across a muscle fibre. It seems significant to the author, however, that whenever nerve endings have been described as penetrating into the contractile substance, such endings appear in the figures, or are described, as filiform.

Only one other worker has described filiform endings in insects without stating that they penetrated into the contractile substance. Montalenti (1928) claimed that filiform motor nerve endings are present in Dytiscus muscles, the ultimate motor nerve branch running over a muscle fibre, and dipping at intervals to contact the sarcolemma. The nerve sheath stains much more readily than the axoplasm or motor nerve ending in locust, cockroach, and stick insect (personal observation); and if this is the case in other insects it might well be that Montalenti did not stain the nerve endings, but only the

nerve branches giving rise to them. While there is not necessarily any comparison between insects and crustaceans in this respect, it is of interest that Holmes (1943) has criticised the claim of van Harreveld (1939) to have stained filiform motor nerve endings in crustacean muscle fibres.

Although Montalenti (1928) described filiform endings in Dytiscus muscles, Mangold (1905) had already found endings of the Doyère-cone type in the same insect. Motor nerve endings of this kind are unmistakeably distinct from tracheoles, and they have been observed in a number of insects. Foettinger (1880) soaked the muscles of various Coleoptera in 85% alcohol for several days, and found cone-like motor nerve endings at the end of the period. Mangold (1905), using methylene blue, observed similar endings in a variety of insects. Morison (1927) working on the honey-bee, and Hoyle (1955b) on the locust, reported similar findings. Hoyle (1955b) was able to pull some of these endings off the muscle fibres, and found that they had an irregular, claw-like appearance, which showed a general resemblance to the figures given by Couteaux (1947) for amphibian motor end-plates. Hoyle (1957) has suggested that this Doyere-cone type of ending is the common type among insects.

It has long been known that most insect muscle fibres are innervated by a number of motor nerve endings distributed along their length. This is in contrast to mammalian muscle fibres, which are innervated by one, or at the most, two per fibre. Foettinger (1880) found that motor nerve endings occurred at intervals of about 100 μ along the muscle fibres of Chrysomela and a little farther apart than this in Passalus and Hydrophilus. Weiant (unpublished, cited in Roeder, 1953) saw endings 40 μ apart in cockroach muscles; and Hoyle (1955b) 60 μ apart in Locusta. If such figures are typical of insects in general, in a muscle fibre of 0.5 cm. length there would be 50 - 100 such endings.

Exceptions have been reported, however. Morison (1927) and Tiegs (1955) have claimed that in the muscles of Apis and Erythroneura respectively, there is only one motor nerve ending per muscle fibre.

Little information has been forthcoming concerning the spatial relationships between nerve endings on the same muscle fibre. Foettinger (1880) found that the endings he observed were mainly on one side of a fibre, but might sometimes be more unevenly distributed. The drawings given by other workers suggest that this is generally the case.

METHODS.

The anatomy and distribution of the coxal muscles inside the thorax and their motor nerves were determined by dissection under a binocular microscope. Freshly-killed insects were fastened to a wax base by strips of plasticene passing over their legs. They were opened by a median incision in the dorsal cuticle and the two sides of the thorax were eased apart and carefully cut down to the point of attachment of the coxal muscles with a dorso-lateral original. This provided a dorsal "window" into the thorax. The gut was now removed and the sides of the animal pinned down, keeping the pins clear of the muscles. This pulled down the sides of the thorax to some extent, exposing clearly the coxal muscles and thoracic ganglia.

In most cases the muscles were first dissected on one side under saline, and then the course of the nerves was traced on the other side under 70% alcohol. This proved a good standard procedure, as saline leaves the muscles flexible and easily handled, while alcohol, although it leaves the muscles brittle, whitens the nerves and helps to show them up.

It was often found helpful to give a prior wash in 1% methylene blue dissolved in saline. This was followed

after rinsing by a wash in 10% ammonium molybdate solution, to "fix" the colour. This made the nerves and muscles stand out more clearly from the surrounding fatty material. Muscles were removed and nerves severed once they had been noted.

Similar methods were used in dissecting the femoral muscles. In addition, serial sections of the femur and coxa were cut at 10 μ, as a means of checking the arrangement of the muscle fibres and their innervation. For this purpose recently moulted animals - in which the cuticle was soft - were chosen, and the legs were severed as close as possible to the thorax and cut in half across the femur. The tibiae were cut off near the femoro-tibial joint. Even when cut in this way, the pieces were still very long by comparison with their diameter, and so a fat-solvent fixative, in the form of Carnoy's fluid, was used to enable rapid penetration of the fixative to take place. It produced some shrinkage of the soft tissues, but this was considered less important than rapid fixation.

The legs were left in the fixative overnight and then passed slowly through 30%, 50% and 70% alcohols, after which they were transferred to a 1 : 1 mixture of 90% alcohol and monochlor-isothymol. From this they were

passed successively into pure monochlor-isothymol, 1 : 1 monochlor-isothymol and ester wax, and finally molten ester wax. After blocking in ester wax (Steedman, 1947) the blocks were trimmed and surrounded with a layer of paraffin wax to facilitate ribboning. Serial sections were cut at 5 μ on a Cambridge rocking microtome.

When the wax had been removed in the usual way, slides were stained either in Heidenhain's haematoxylin and van Gieson's stain, Ehrlich's haematoxylin and aqueous eosin, or Willis' method for nerve endings (Willis, 1945, with additional notes kindly supplied by the author).

Motor nerve endings were examined by removing coxal or femoral muscles and staining these in bulk after preliminary teasing or squashing. A number of staining methods were tried: unreduced and reduced methylene blue (Mangold, 1905; Opoczynska - Sembratowa, 1936; Smith, 1947; Harris and Peters, 1953); chlorantin blue, toluidine blue, gold chloride (Garven, 1925) and gold-toned silver (Willis, 1945).

RESULTS AND DISCUSSION.

(i) Anatomy.

The anatomy of the coxal and femoral muscles of the pro- and metathoracic limbs, is shown in Figs. 1, 2 and 3. The mesothoracic leg has been omitted: less anatomical work has been carried out upon this leg, and no physiological work. Nevertheless, sufficient dissections have been made to enable the conclusion of Marquardt (1940), that the musculature and nerves of each thoracic segment can be homologised according to the same basic plan, to be confirmed.

Each leg has a similar pleural and trochantinal type (Snodgrass, 1935) of articulation, and so this similarity is to be expected. However, the legs are inclined anteriorly or posteriorly to different extents both in resting, walking, and in the "stick" reflex: the pro- and mesothoracic legs tend to point forwards, the metathoracic legs backwards. In addition, the prothoracic segment is small and cramped by comparison with the other two thoracic segments; and the metathoracic segment is telescoped with the first abdominal segment. These facts appear to account for differences in size and orientation between the muscles of the pro- and metathoracic legs.

Apart from such differences the coxal muscles are,

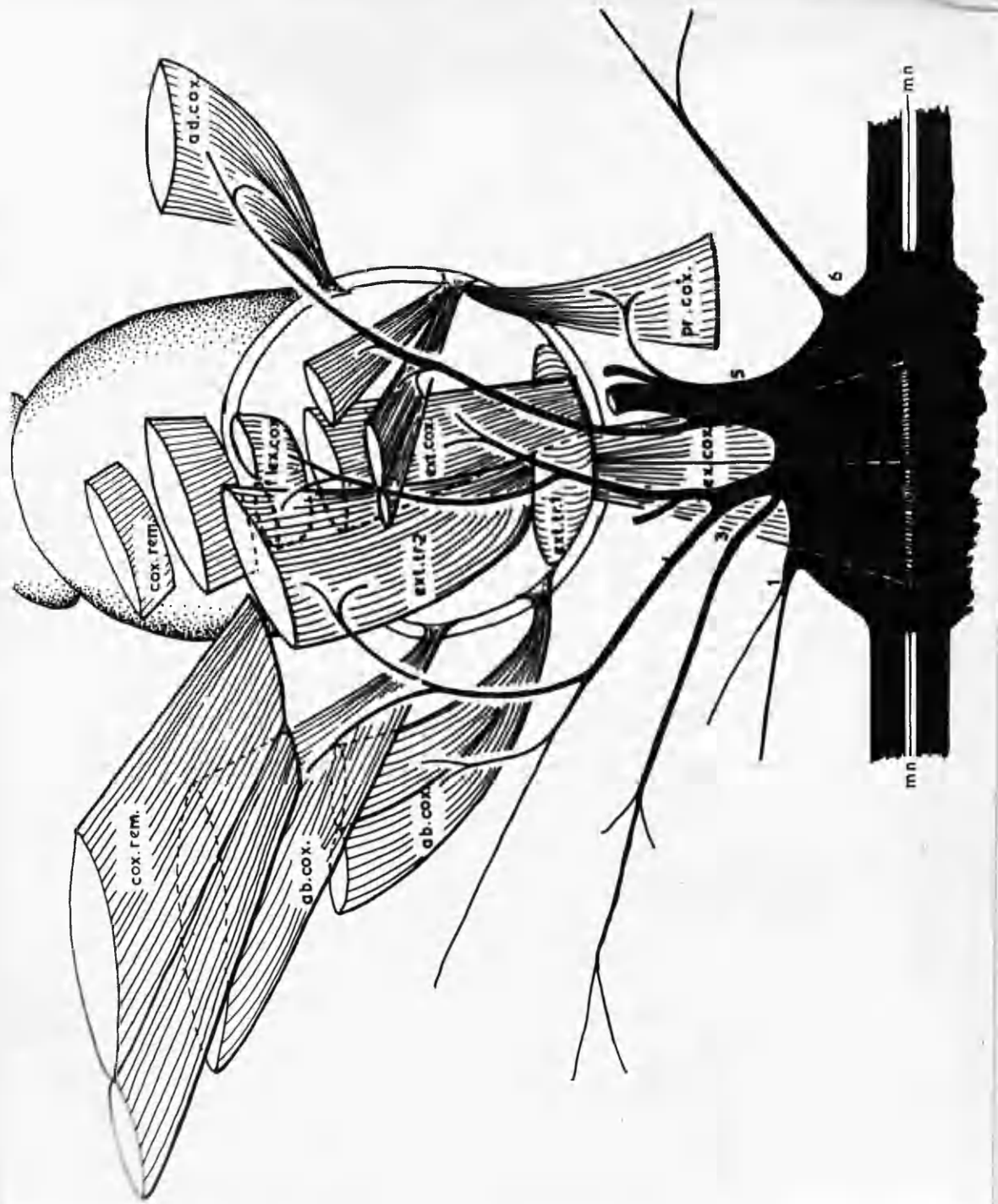


Fig. 1. Prothoracic nerves and muscles. The nerves are numbered according to Pringle (1939). mn; median nerve. cox. rem: coxal remotors; pr. cox: coxal promotor; ad. cox: coxal adductor; ab. cox: coxal abductors; flex. cox: coxal flexor; ex. cox: coxal extensor; ext. tr_1 and ext. tr_2: extensor trochanteris muscles.

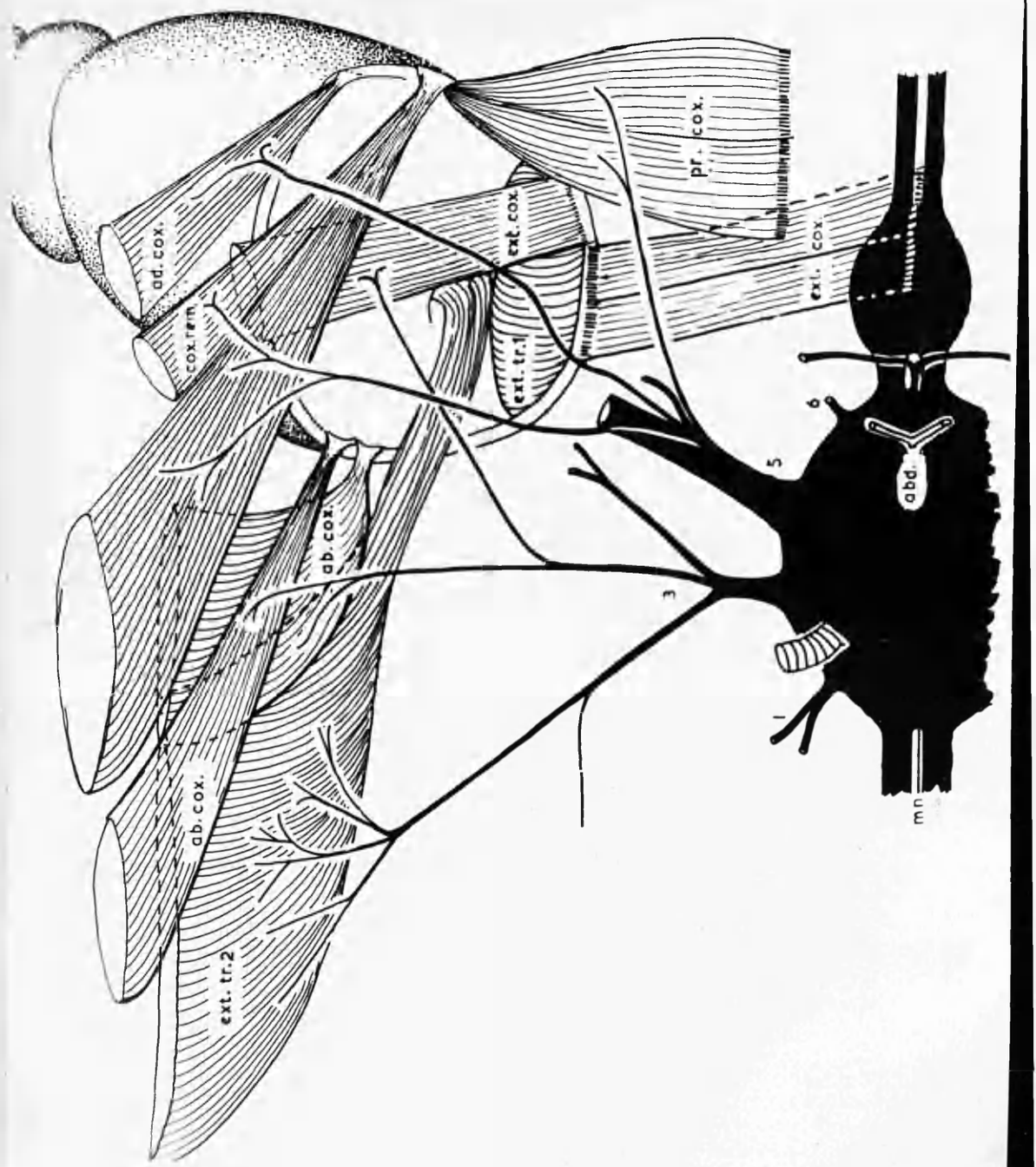


Fig. 2. Metathoracic nerves and muscles. abd: first pair of abdominal nerves. Other abbreviations as in Fig. 1.

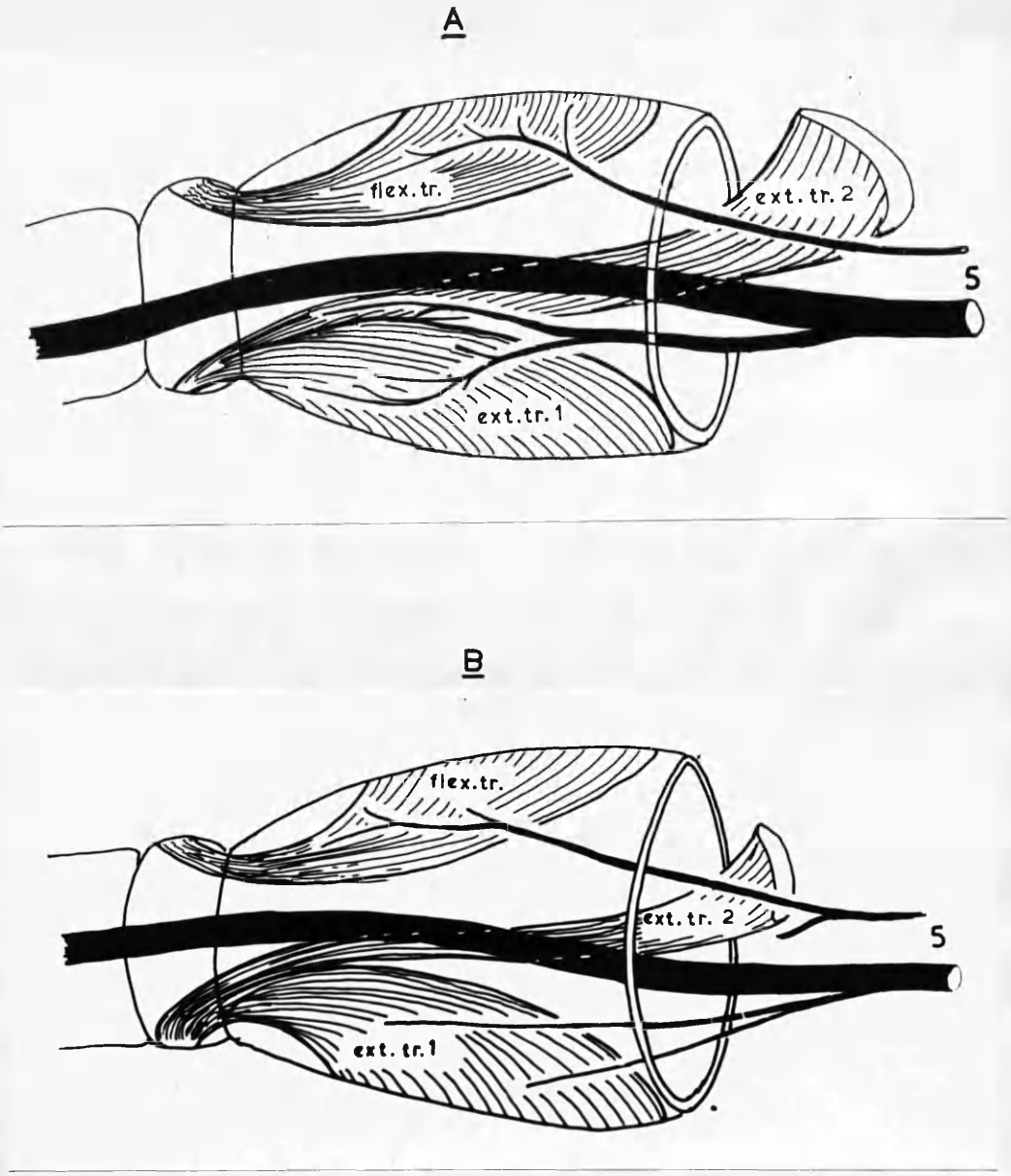


Fig. 3. Coxal muscles of A, prothoracic; and B, metathoracic, legs. flex. tr; flexor trochanteris; ext. tr; extensor trochanteris.

in general, similar in size in both legs. Marquardt (1940) however, figures prothoracic muscles which are uniformly more puny than their metathoracic counterparts. I find this unaccountable. In all, 15 insects have been dissected for anatomical purposes, and in no case did the corresponding proportions approach those given by Marquardt. The drawings by Jeziorski (1918) are not detailed, but do support the proportions given here, rather than those of Marquardt. Functionally, it is most improbable that the prothoracic legs should possess muscles which are small relative to those in the other legs. The prothoracic legs are used much more extensively in climbing and in struggling, a fact reflected in the much larger femoral muscles of this leg.

Both dissection and serial sections have confirmed Marquardt's (1940) description of the crural nerve as being the sole nerve innervating the flexor tibialis, extensor tibialis, and retractor unguis muscles. There is no branch of nerve 3 passing to the femoral muscles, as there is in the locust metathoracic leg (Hoyle, 1955b).

The flexor tibialis muscle consists of pinnately-arranged bundles of muscle fibres, having their origin on the median apodeme, and their insertion on the lateral cuticle of the femur (Fig. 4). The insertions of the

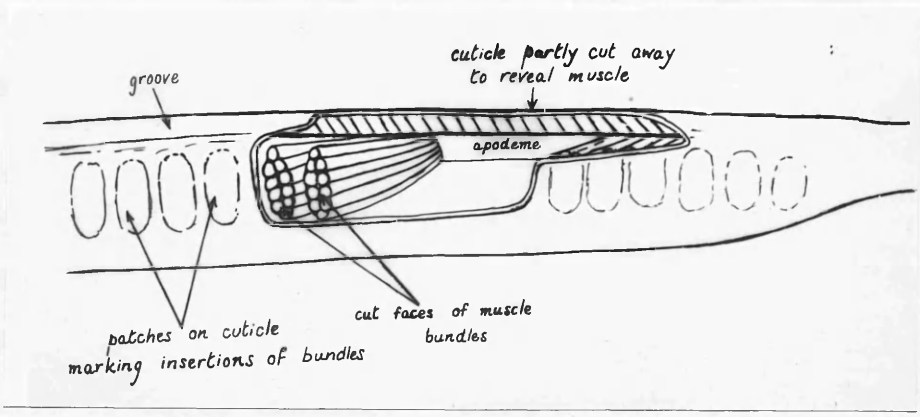


Fig. 4. Diagram of part of prothoracic leg with cuticle partly removed to show details of flexor tibialis muscle.

bundles are visible externally as patches on the cuticle. Because of the pinnate arrangement of the bundles, a number of them are cut through in transverse section at varying points between their origin and insertion (Fig. 5). Individual fibres vary in diameter along their length, becoming smaller near their insertion on the apodeme. They reach a maximum diameter of about 50-60 μ near their origin on the cuticle. Individual fibres are about 1.5 mm. in length in the prothoracic leg, somewhat shorter in the metathoracic leg.

Essentially then, the general organization of the muscle is similar to the metathoracic extensor studied by Hoyle (1955b), but the muscle is smaller, and there are accordingly fewer bundles and fewer fibres in the bundles. Hoyle proposed that the term muscle unit should be applied to these bundles. Such a term is purely anatomical, and not to be confused with either a vertebrate motor unit, or a vertebrate fasciculus.

When the crural nerve enters the femur, it divides to send branches to the extensor tibialis and retractor unguis muscles, but the main body of the nerve continues through the femur, adjacent to the flexor tibialis muscle (Fig. 5), which it innervates. In transverse section this nerve is seen to contain a number of large axons

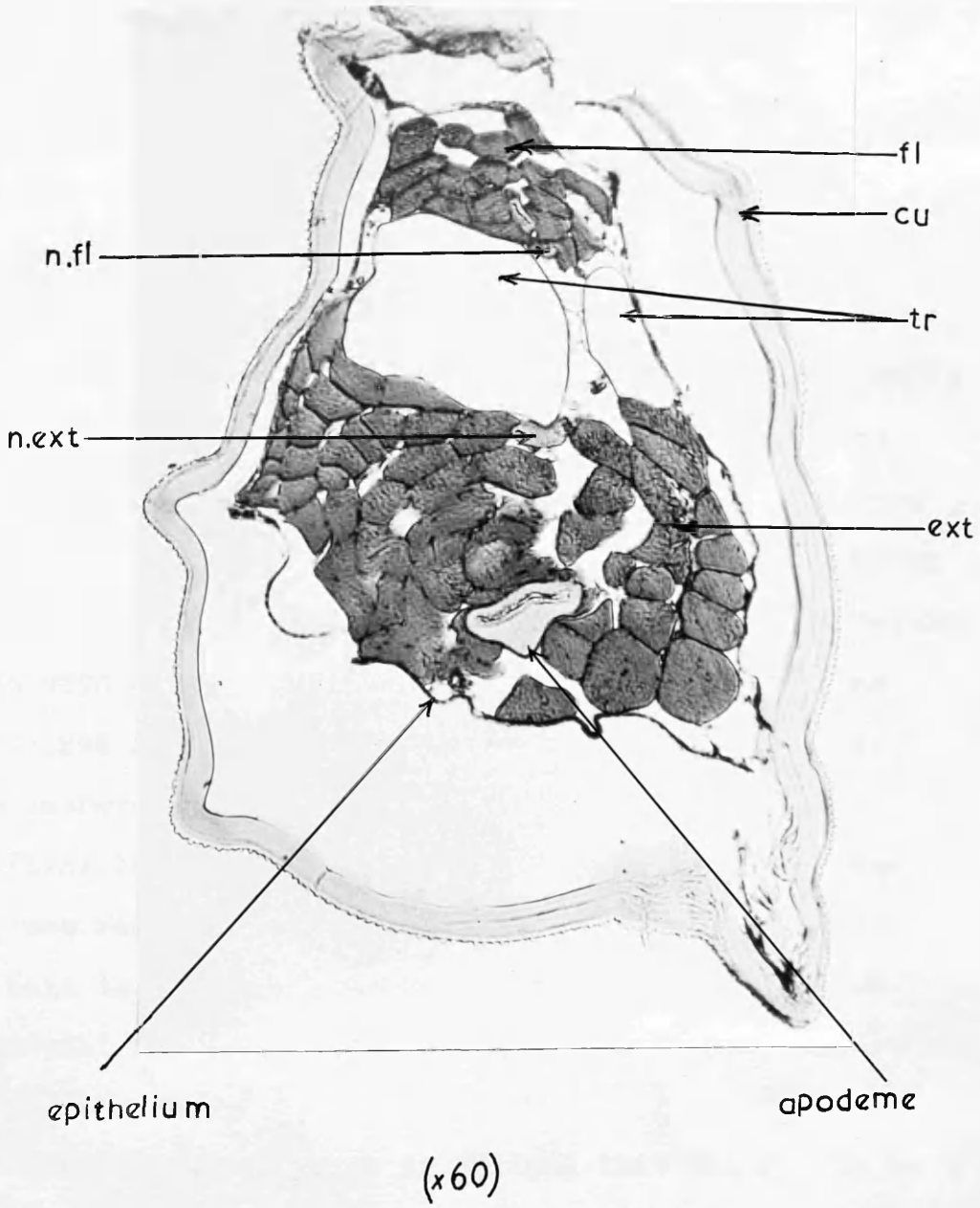


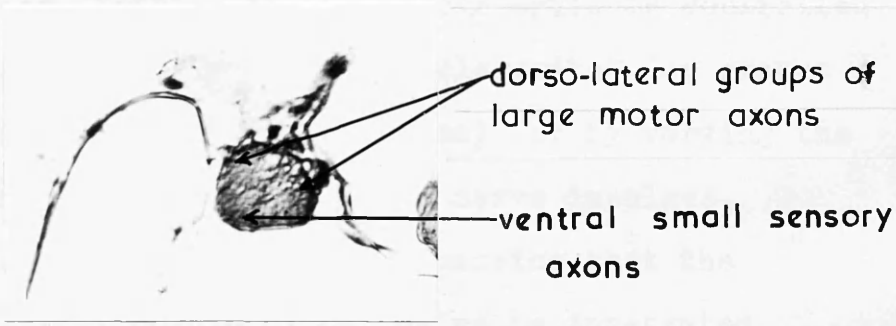
Fig. 5. Transverse section of prothoracic femur. cu: cuticle; fl: flexor tibialis; ext: extensor tibialis; tr: trachea; n.fl. and n. ext: nerves to flexor and extensor muscles respectively. Haematoxylin and eosin.

roughly dorso-lateral in position, and many smaller axons (Fig. 6). The small axons continue beyond the femur to the tarsi, and are presumed to be sensory axons running from the tarsal receptors. The large dorso-lateral axons are the motor axons innervating the flexor tibialis muscle.

As it passes through the femur, the crural nerve gives off branches at intervals on either side, in such a way that each branch runs to a pair of adjacent muscle units. Owing to the pinnate arrangement of the muscle fibres these branches are cut through obliquely in transverse section, and it is not possible to determine the number of axons in each. Nor has it been possible to determine this with certainty by following the number of axons contained in the main nerve through serial sections. The impression is gained, however, that each branch contains two of the largest axons, which are presumed to produce responses of the "fast" type (see Section II). If this is the case, it is probable that each of the pair will run to one of the bundles in the two innervated by the branch.

Physiologically, it is evident that the muscle is innervated by a number of axons possessing comparable properties (Section II) but which are separate from one

A



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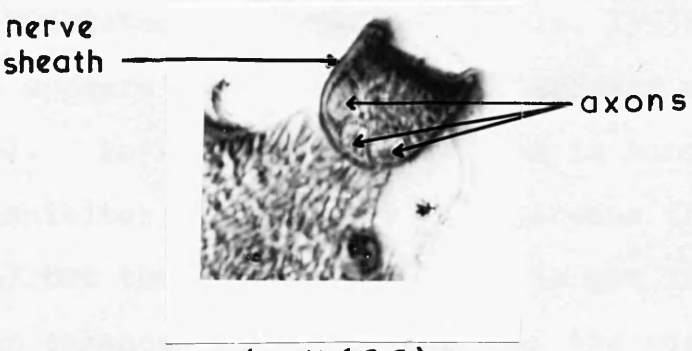


(ca. X 600)

Fig. 6. Transverse sections of nerves to: A, the flexor muscle; B, the extensor muscle. (Prothoracic leg).

another. The impression reported above suggests that a separate "fast" axon may run to each muscle unit, as it does in the flexor tibialis of the locust (Hoyle, 1955b): although it is also possible that the same axon could send branches to a number of different muscle units. In either case, tension in the muscle could be controlled both by varying the number of muscle units (or groups of them in operation at a given time), or by varying the frequency and pattern of the motor nerve impulses.

It is interesting to note in passing that the extensor tibialis muscle of Carausius is innervated throughout by the same three axons, which run along its length. There are three axons in the motor nerve to the locust metathoracic extensor (Hoyle, 1955b); and one of these appears to exert a hyperpolarizing action (Hoyle, 1955c). Hoyle suggests this axon is homologous with the inhibitor axon found in crustaceans (Fatt and Katz, 1953b) but that in the locust it is not inhibitory, but rather enhances the contraction of the muscle. The nature of the third axon in Carausius, which occurs in all three legs, should prove to be of considerable interest.

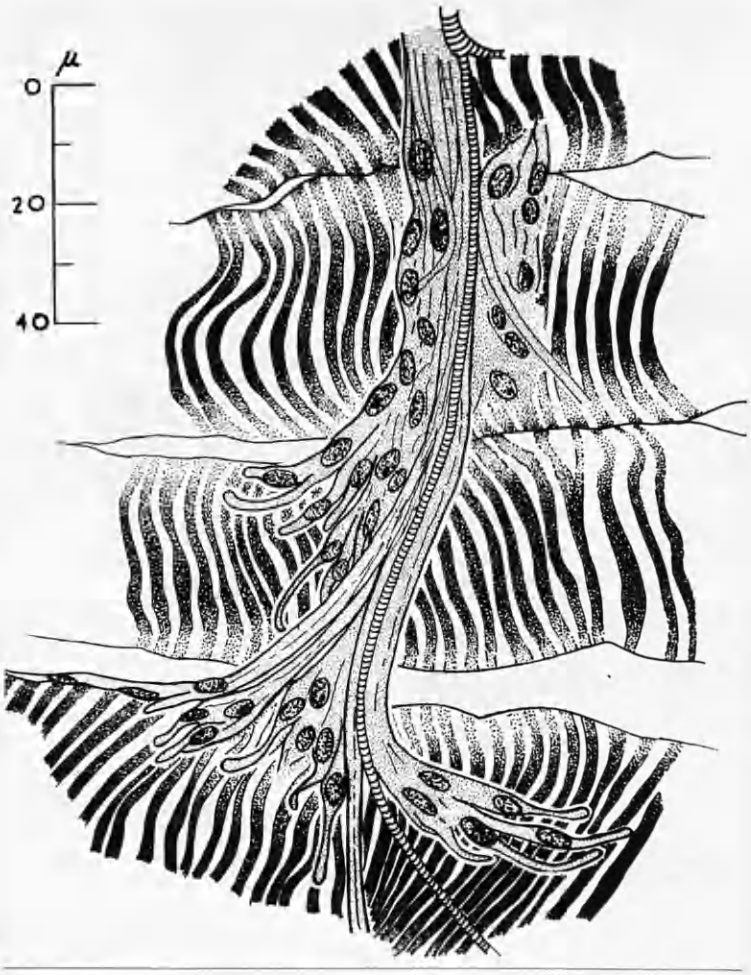


Fig. 7. Camera lucida drawing of a series of motor nerve endings on fibres of the flexor tibialis muscle. Squash preparation. Stained by gold chloride method (Garven, 1925).

(ii) Histology.

The motor nerve branch innervating a particular muscle unit passes to the centre of the unit, and there divides to send finer branches between and along the muscle fibres. The ultimate branches produce motor nerve endings on the muscle fibres at intervals of about 60 μ along the fibres.

The motor nerve endings are irregular claw-like structures branching over the surface of the muscle fibre (Fig. 7). Each ending covers a relatively large area of the muscle surface, so that the extreme edge of one ending may be only 10 μ away from that of an adjacent ending in some instances.

The individual motor axons can sometimes be determined right up to the point where the ending, or "motor end-plate" to use a vertebrate term, begins. Beyond this point dividing walls cannot be seen. In all cases where it has been possible to determine individual axons in the nerve leading to an end-plate, the number of axons has apparently been two, in the flexor tibialis muscle. The cellular sheath which surrounds insect nerves (e.g. see Hughes, 1953), and which appears to function as a selective ion barrier protecting the nerve from adverse ionic concentrations in the body fluid (see Section III)

terminates at the point where the end-plate structure begins.

Motor nerve endings in Carausius are thus typically of the "Doyere-cone" type. The cockroach P. americana was also examined at the same time as Carausius, and proved to have similar end-plates. These results add weight to the suggestion (Hoyle, 1957) that most, if not all motor nerve endings in insects are of this type.

Carausius is also similar to the locust (Hoyle, 1955b) in that the protective sheath surrounding the motor nerve terminates at the point where the end-plate begins; and axons lose their identity when they enter the end-plate. The first fact implies that the motor nerve endings are bathed in the external medium, like the muscle fibres, but unlike the nerve itself: this point is considered in Section III. The second fact implies that an endplate can produce two distinctly different muscle responses (Section II), but that the axons exciting the end-plate and ultimately responsible for these responses, are not separated in the endplate substance.

The latter aspect does not appear to have been commented upon in the literature, yet if the facts stated are correct, it is a remarkable one. It might be

supposed that the two axons do, in fact, retain their identity in the endplate substance, but that conventional staining methods will not differentiate them. Since the axons in the nerve leading to the end-plate can be differentiated with osmic acid, this view requires either that the material delimiting the axoplasm is different in the motor nerve and the end-plate respectively, which is improbable; or that osmic acid is unable to penetrate the surface membrane of the end-plate. The latter view receives some support from the fact that the end-plate is extremely difficult to stain. Nevertheless, in the few instances where relatively deep staining of the end-plate has been achieved, as in the preparation from which Fig. 7 was drawn, the endplate has always appeared to be a syncytial structure of completely homogeneous appearance. If this is in fact the case, the detailed properties of the impulses passing down the motor axons will need to be studied, to determine whether these are the operative factors in determining the type of muscle response evoked by the endplate. In this connection, direct stimulation of the muscle (i.e. of the endings themselves) might also yield valuable information.

Staining of the end-plates has been achieved in only two instances (both with gold chloride) despite prolonged

work using the stains outlined in the Methods section.

Examination of stained preparations of squashed muscles reveals that the axoplasm of the motor axons is equally difficult to stain: where stains are taken up it is the sheaths which are coloured, not the axoplasm.

The failure of silver stains is particularly striking. It is hard to avoid the thought that the differences in staining properties between insect and vertebrate nerves are a reflection of some important biochemical difference between them. An intensive study of insect nerve endings using modern methods - phase-contrast, interference, and electron microscopy - might yield important new facts. Certainly, many of the desiderata outlined in the introduction to this Section have not been obtained with the conventional staining methods used in the present work.

SECTION II.

NEUROMUSCULAR MECHANISMS OF THE FLEXOR TIBIALIS MUSCLE OF CARAUSIUS.

INTRODUCTION.

There is a potential difference across the membrane of a "resting"muscle fibre, such that the inside of the fibre is negative to the external medium.

In vertebrates, the arrival of an impulse at the motor nerve endings results in a partial reversal of the "resting" potential difference in the end-plate region of the membrane, i.e. the end-plate membrane is partly depolarized. This depolarisation is the end-plate potential. If the end-plate potential reaches a sufficient size it evokes an additional, active, response from the muscle fibre membrane in such a way that the latter becomes further depolarised. The additional depolarisation is the spike potential, and it usually overshoots the zero potential level to a considerable extent. The spike potential excites adjacent regions of the membrane to a similar reversal of charge and the process continues along the fibre so that the depolarisation started at the end-plate travels along the length of the fibre. This active,

travelling depolarisation is the propagated response. The vertebrate mechanism is discussed in detail by Fatt (1954) and del Castillo and Katz (1956).

The depolarisation of the membrane is the factor which activates the contractile material of the muscle fibre, causing contraction. Since there is only one end-plate on most vertebrate skeletal muscle fibres an active membrane response which will propagate along the fibre is essential if the whole muscle fibre is to contract. Without a propagated response, the end-plate potential simply dies away exponentially.

It is implicit in this mechanism that if the end-plate potential is sufficiently large to initiate a propagated response, the whole muscle fibre will contract: but if neuromuscular transmission is blocked so that the end-plate potential cannot give rise to a propagated response, it will not contract. Therefore, if a vertebrate muscle is to exhibit graded contractions, these must be brought about by varying the numbers of fibres contracting at a given time. A muscle of this kind needs to be innervated by many axons, each axon controlling only a few muscle fibres, if control of the whole muscle is to be at all delicate.

Insect muscles are innervated by a very small number

of axons, each axon supplying all, or many, of the fibres of a muscle; and there are a number of motor nerve endings distributed along the length of each muscle in most insects (Section I). These facts imply at once that muscular control in insects is likely to be different from that found in vertebrates. Yet earlier workers on insect muscles such as Kahn (1916), Solf (1931), and Friedrich (1933) treated their preparations in the same way as frog gastrocnemius/sciatic preparations. It was not until Pringle (1939) published certain observations which he had made upon the cockroach, that the neuromuscular mechanisms implicit in the insect type of organisation were realised, although work on the similar crustacean organization had been proceeding earlier (literature reviewed in Katz, 1949; Wiersina, 1953).

Pringle (1939) discovered two types of electrical and mechanical response in the metathoracic ~~flexor~~ extensor tibialis muscle of the cockroach Periplaneta americana. Mechanically, they were a quick powerful tetanus; and a smooth tonic contraction. Electrically, these mechanical responses appeared to be linked to the activity of two separate motor axons. Rijlant (1932) had already discovered two different amplitudes of nerve impulses passing to the muscles of Musca domestica.

one associated with active contraction, the other with tonus. Pringle realised that a double innervation of the muscles was present in these insects, similar to that which had already been found in certain crustacean muscles (van Harreveld and Wiersma, 1937). His experiments were carried out with external electrodes. When intracellular techniques were introduced, more insects were examined by other authors, and double innervation came to be recognised as characteristic of insects. The new techniques have allowed a more detailed examination to be made of the muscle responses produced by this double innervation (Hagiwara, 1953; Wilson, 1954; Hagiwara and Watanabe, 1954; and especially, Hoyle, 1955a, 1955c).

Pringle (1939) termed the two motor axons innervating the metathoracic flexor tibialis of the cockroach, "fast" and "slow". Hoyle (1953a) followed this terminology in his work on the locust. These terms are used in the present work. A "fast" axon is one which when stimulated by a single shock, produces a quick contraction of the muscle, like the single twitch of a frog gastrocnemius/ sciatic preparation. A relatively low frequency burst of stimuli applied to the axon results in a powerful tetanus in the muscle. A "slow" axon produces either

a very slight muscular movement, or none at all, upon single shock stimulation; but when it is stimulated repetitively at a relatively high frequency, a slow, smooth, contraction results. The higher the frequency, the greater the tension developed by the "slow" fibre over a wide range of stimulation frequencies. In the locust (Hoyle, 1955c) one type of slow motor nerve ending acts as a tonic "holding" force, serving to retain a muscular tension which has resulted from the activity of the other "fast" or "slow" nerve endings.

Sometimes the "fast" and "slow" axons appear to differ in diameter, e.g. in Geotrupes (Marcu, 1929) and in Cyclochila (Tiegs, 1955) in which one axon, presumably the "fast", is of greater diameter than the other; and in the mesothoracic legs of Schistocerca and Locusta (Hoyle, 1957), where the "fast" axon to the flexor tibialis measures 10 - 11 u and the "slow" axon 6 u in diameter, resulting in conduction velocities of 2.2 and 1.5 metres/sec. respectively. The condition is not invariable: in the metathoracic extensor tibialis of these last two insects the diameter of the two axons is very similar.

The locust metathoracic extensor tibialis receives three axons, one "fast", one "slow", and one which

Hoyle (1955c) has designated a hyperpolariser, the function of which he considers to be one of enhancing muscular contraction by increasing the extent to which the muscle fibre membrane is depolarised. As stated in Section I, the extensor tibialis of Carausius is also innervated by three axons; but the flexor, with which the present work is concerned, appears to receive only two. It was expected, therefore, that in the latter case the two axons would prove to be of the straightforward "fast" and "slow" types.

METHODS.

The animal was placed with its ventral surface uppermost on a bed of plasticene contained in a Petri dish, and fastened to this bed by small strips of plasticene passing over the femurs just behind the femoro-tibial joints in such a way that the tibiae were free to move. The particular leg to be examined was surrounded by plasticene to keep it supported, and a well was formed around it by a plasticene wall which passed over the coxa, keeping it fixed in position. The ventral cuticle was then removed from most of the femur, exposing the flexor tibialis muscle. The most

satisfactory way to do this was to make a transverse cut in the cuticle near the join with the tibia and then to pull back along the leg the small lip so formed, when the ventral cuticle came off in a single strip. This method seemed to cause less damage to the tracheae covering the muscle than a more careful dissection. In cases where these tracheae had been damaged by dissection, the activity of the muscle fibres appeared to be depressed.

The electrical properties of the muscle fibre membrane were studied by means of glass capillary intracellular micro-electrodes (Ling and Gerard, 1949; Nastuk and Hodgkin, 1950). These were made from pyrex tubing of 7 mm. diameter, drawn out in an oxy-coal gas flame to a capillary of about 0.5 mm. diameter. This was then further drawn out by bringing it near a small coal gas flame and pulling sharply when the glass became sufficiently soft. If correctly pulled a long tapering tip resulted. The tips were examined under the microscope for correct taper (Ling and Gerard, 1949) and to ensure that the tip remained open. The correct tip diameter of less than 0.5 u is beyond the resolution of the light microscope, but with experience the appearance of a closed tip could be recognised in most cases.

Electrodes which passed this inspection - about 50% of those pulled - were filled with 3M potassium chloride. In early experiments, filling was carried out by boiling the electrodes in the saline, but later another method was adopted, since boiling by the application of heat caused large bubbles which often broke the tips of electrodes, and Nastuk (1950) found that the method might also alter the electrical properties of the electrodes. The method adopted involves filling the electrodes with methyl alcohol by boiling them in the liquid under reduced pressure, and subsequent boiling under reduced pressure in the potassium chloride, when the alcohol boils off and is replaced by the chloride solution. These operations were carried out by suspending the electrodes in a beaker of the liquid concerned, which was stood in a "Pyrex" vacuum dessicator connected to a water vacuum pump (Fig. 8).

When filled, the electrical properties of the electrodes were examined by passing a square pulse from the stimulator through them and recording the result on the oscillograph screen. Undue distortion of the square pulse by the electrode led to its being discarded. If satisfactory in this respect its resistance was checked by switching a 20 megohm shunt between recording and ground electrodes. The extent to which the oscillograph

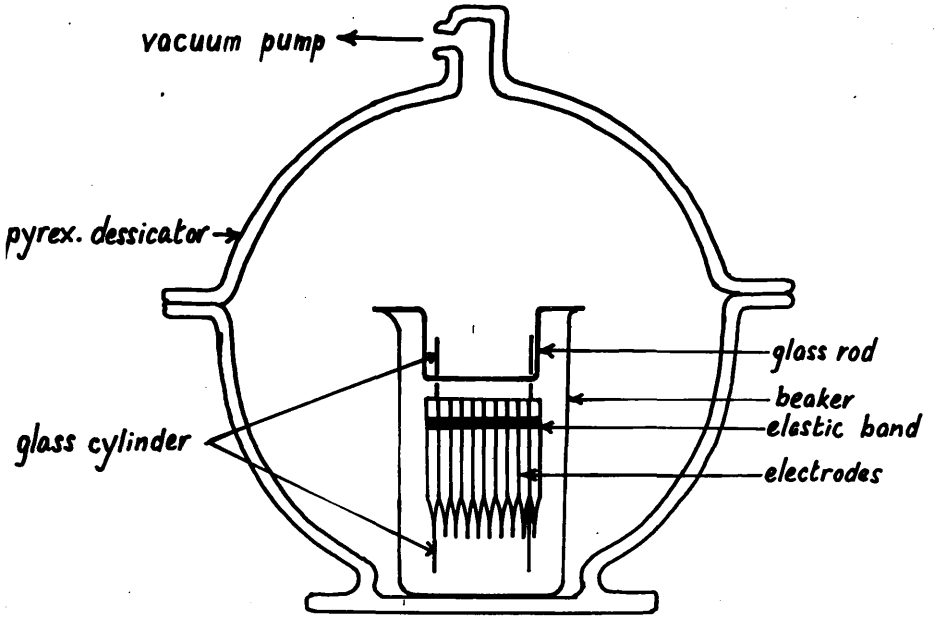


Fig. 8. Method of filling micro-electrodes.

trace was reduced by electrodes of 10 megohms resistance or more - the desired minimum (Nastuk and Hodgkin, 1950) - was known by substituting a 10 megohm resistor for the electrode and noting its effect. Typical test traces are shown in Fig. 9. It was found that some electrodes which gave a distorted trace when first tested were completely satisfactory if left to soak in the potassium chloride for a week or so. This was probably due to an initial imperfect filling of the electrodes, resulting in small bubbles of air remaining in the tip, which slowly diffused away in time. The distortion shown by such electrodes at their first test could be recognised in most cases and they were simply put on one side for later testing.

The recording arrangements are shown in Fig. 10. The recording leads from micro-electrode and muscle bath were led off to a cathode-follower input stage, which is essential in view of the very high impedance of this kind of electrode. The cathode-follower used was a modified version of the circuit designed by Bishop (1949), using instead of the original 954 acorn valves Mullard EF 37A pentodes at 33 v. H.T. and with heaters underrun at 4 volts. The 20 megohm shunt used for testing electrodes could be switched across the imputs of this unit.

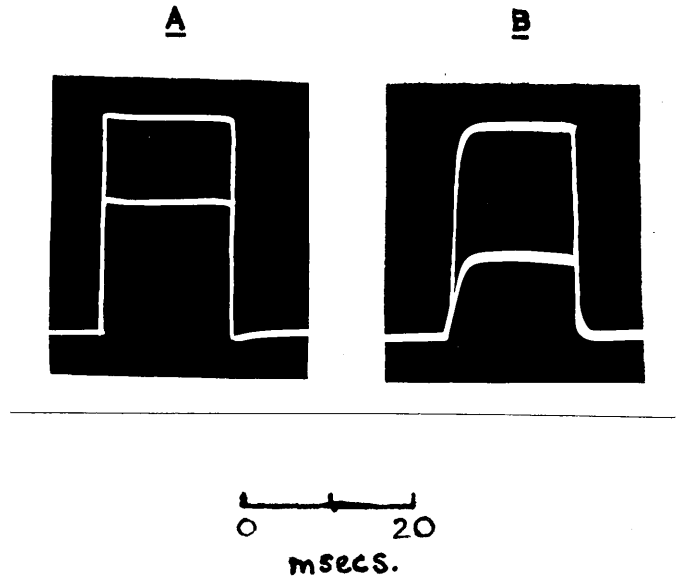


Fig. 9. Testing micro-electrodes. A, 10 megohm resistor in place of electrode. Two sweeps superimposed to show the drop in size of the square pulse trace when a 20 megohm shunt is switched across the inputs. B, recording through an acceptable electrode. Two sweeps superimposed to show the effect of switching in the 20 megohm shunt across the inputs. An electrode should show a resistance of 10 - 40 megohms for optimum results (Nastuk and Hodgkin, 1950). Note slight distortion of square pulse by the electrode.

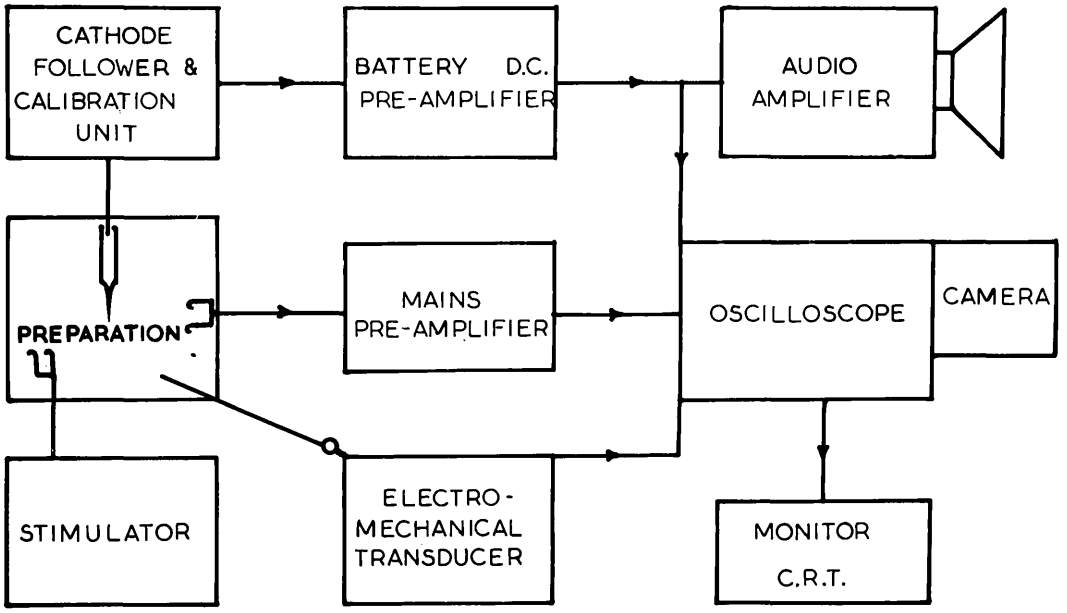


Fig. 10. Diagram of apparatus used. Arrows denote direction of signals.

Another switch enabled a calibration circuit to be switched into the ground side of the cathode-follower. This circuit was a simple one based on Ohm's Law, designed by Dr. G. Hoyle (unpublished). It will be seen (Fig. 11) that the current in the circuit, which is set to 100 μA, will fall slightly as soon as the decade resistance is switched in. The current level may either be reset to 100 μA at each increase in the decade resistance or the error allowed for in the recorded trace.

From the cathode-follower the signal was led into a D.C. pre-amplifier with balanced inputs (Copeland, 1952) and thence to a Cossor 1049 oscillograph. A lead from the oscillograph input to a simple audio amplifier with loudspeaker (Dickinson, 1951) also allowed the signal to be heard. A Cossor 1428 oscillograph camera was used to record the traces appearing on the oscillograph screen on Ilford DG 91 film or BP 1 paper. The camera could be driven by a Cossor camera drive unit.

The microelectrodes were used to impale single muscle fibres. In early experiments insertions were made by means of a micrometer assembly, but in most experiments either a Prior micromanipulator or a Zeiss sliding micromanipulator was used for this purpose.

The stimulator used was a double channel instrument

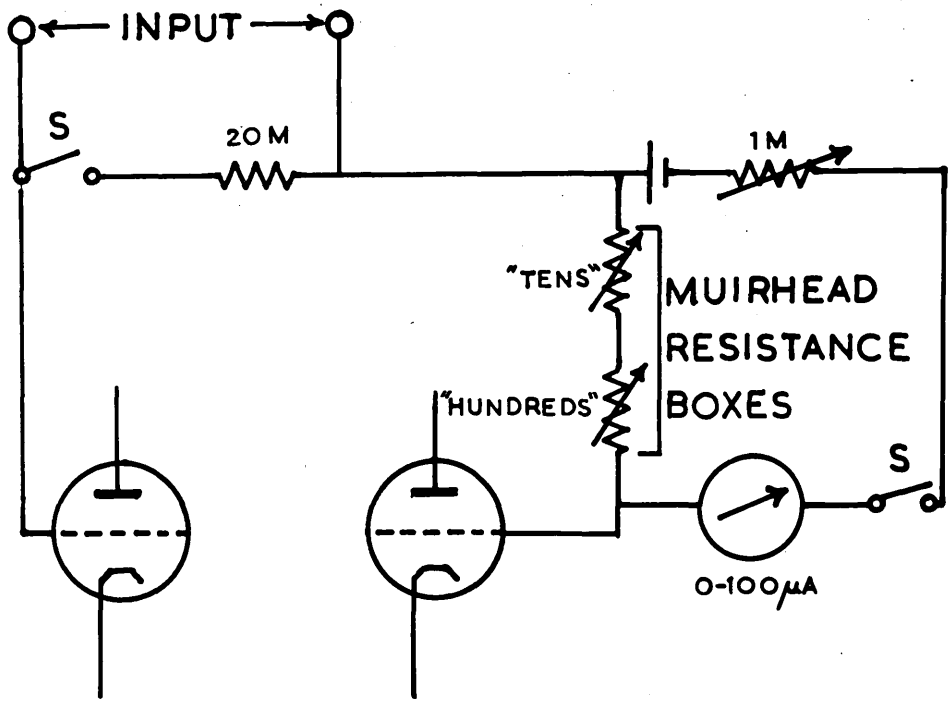


Fig. 11. Circuit diagram of calibration unit.

(The decade resistors could be switched into the circuit independently : both were variable in steps of $\frac{1}{10}$ to maximum value rising from zero Ω to maximum value).

of conventional design by the late Philip Parker (unpublished, Fig. 12). The oscillograph sweeps were triggered by this instrument. For stimulation of the crural nerve the square pulse from the stimulator was passed through a differentiating transformer and applied to the nerve through tapered silver wire electrodes. These were made by tapering 0.007 in. diameter silver wire by electrolysis in silver nitrate. The tapered wire was drawn quickly through molten polythene, becoming covered in an insulating film of the plastic. A small part of this film was scraped away from the very tip of the electrode, and the tip bent into a small hook with the bared metal on its inside bend. The hook could be manipulated under the nerve, using Palmer rack-work, and the nerve raised or lowered upon it.

Impulses passing down the crural nerve were monitored by means of similar electrodes, and fed to a mains pre-amplifier. The output from the mains pre-amplifier was connected to the A2 amplifier of the oscillograph from time to time, to check the impulses passing along the nerve.

Mechanical responses were recorded by means of a simple electro-mechanical transducer. This consisted of a resistance bath of glycerine with a small potential

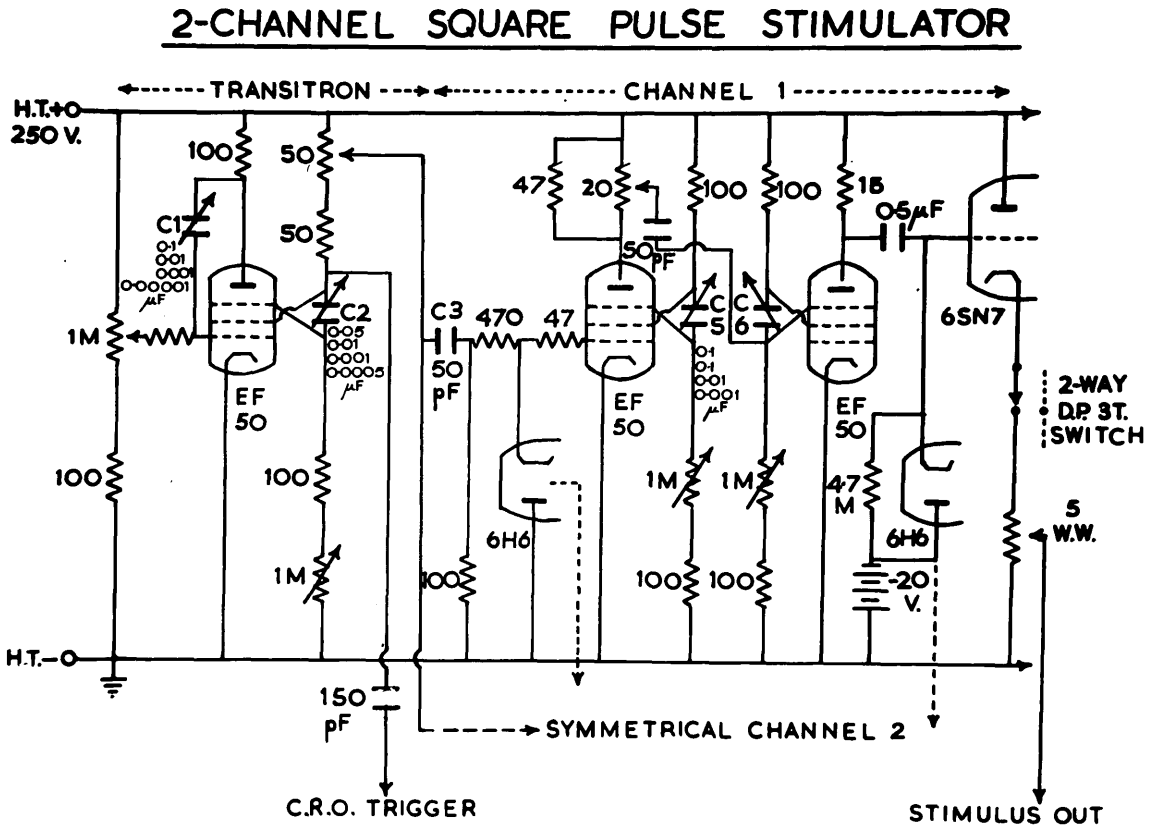


Fig. 12. Circuit diagram of stimulator. Only one of the two symmetrical channels is shown. (Except where otherwise stated, resistance values are in K ohms).

difference across it, into which dipped a movable piece of piano string wire of suitable thickness (Fig. 13). By attaching the tip of the tibia to the piano wire with cotton thread, and connecting the wire to a suitable circuit (Fig. 13) the movements of the tibia were converted to an amplified electrical signal which was fed to the A2 amplifier of the oscillograph.

A general view of the apparatus is shown in Fig. 14. The Cossor oscillograph and camera, and the Advance signal generator (for calibration signals) were purchased commercially, and the mains pre-amplifier was already available (without power-pack). The remaining apparatus was built by the author for the purpose of carrying out the work described in this thesis.

The two axons in the crural nerve were stimulated independently of one another by the technique of Kuffler and Vaughan Williams (1953). In this technique the nerve is stimulated with the anode leading, i.e. anode nearest the muscle. When the axons in the nerve are of different diameter, the impulses set up by the stimulus at the cathode propagate at different speeds. By choosing a suitable distance between the stimulating electrodes and a suitable time course for the original square pulse, it can be arranged that one impulse will arrive at the

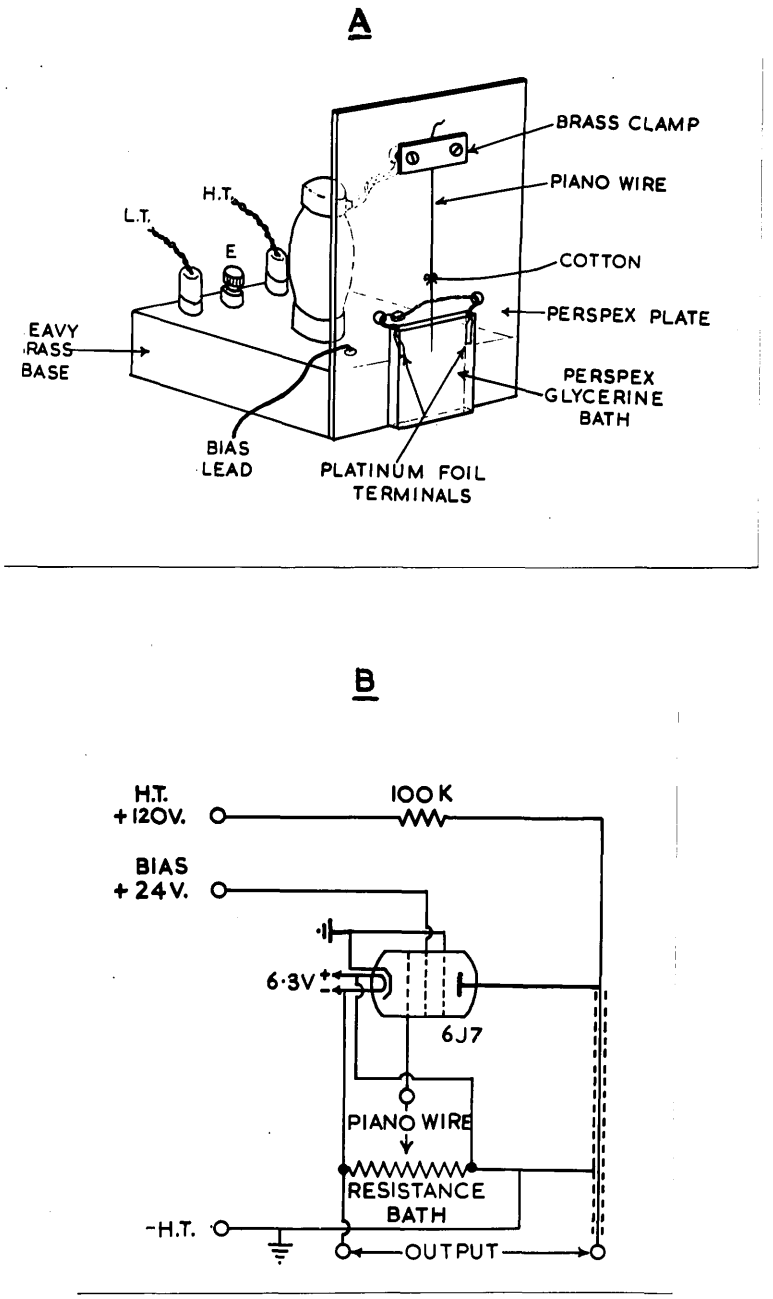


Fig. 13. Mechano-electric transducer. A, diagram of transducer. B, circuit diagram.

Fig. 14. General view of apparatus. On the left are the micromanipulator, electrode assemblies, transducer, lamp, and binocular microscope. The signal generator was used for time calibration signals.

anode at the same time as the anodal pulse is operative, and be suppressed. The other impulse either precedes or follows the suppressed impulse, and does not coincide with the anodal pulse. It thus continues on its course unaffected.

Criteria for electrode penetration.

Electrodes showing drift or erratic base-line variations were always discarded. As the electrode entered a muscle fibre there was a sharp drop in the zero base line, indicating a sudden change in potential. This was frequently, though not always, accompanied by a click from the loudspeaker. The sudden potential drop was taken as indicating the penetration of a fibre. If the electrode encountered any obstruction, such as a trachea, or the tip had become too large through breakage for easy penetration of a fibre, a slow drop in the zero baseline was observed as the electrode was lowered through the muscle. The baseline often showed marked fluctuations in these instances.

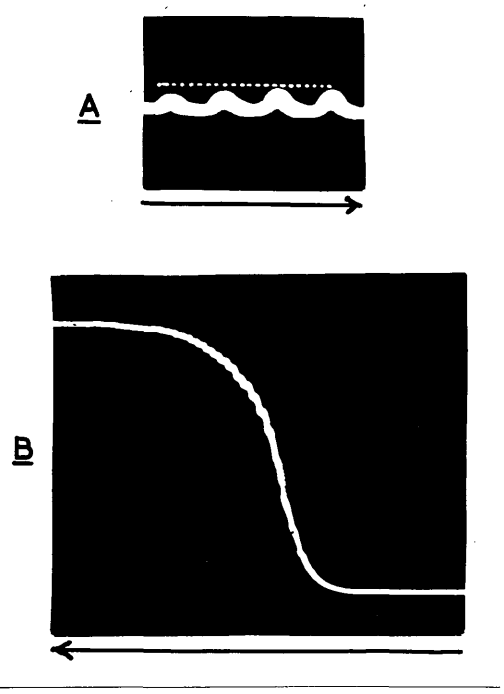
A further indication of correct penetration was due to the differences in shape and size of the muscle potentials recorded externally, with the tip of the electrode pressing into the muscle fibre membrane; and internally, with the electrode properly inserted. These two potentials were easily recognised, with experience.

RESULTS.

When a single stimulus was applied to the "fast" (F) axon at above threshold level, a quick twitch of the flexor tibialis muscle was observed. This was accompanied by a flexion of the tibia which, although brisk, was slight in the extent of its movement - much smaller than similar twitches of the locust metathoracic extensor tibialis. In Carausius the twitch is sufficient to produce only a very small movement of the tibia (Fig. 15A).

A progressive increase in stimulus intensity, beginning below the threshold level required for a mechanical response of any kind, resulted in an accompanying step-wise increase in the extent of the twitch contraction (Fig. 15A). The increase in stimulus intensity which evokes this response is small, and the tension addition at each step very slight, so that it is impossible to state with certainty the number of steps involved. However, the difference between the twitch at the beginning and that at the end is sufficiently marked to be obvious to visual inspection.

In the locust, single shocks of high intensity applied to the F axon are followed by a tetanic contraction of the extensor tibialis muscle (del Castillo, Hoyle and Machne, 1953). This effect has not been observed in



(A enlarged about 3x more than B)

Fig. 15. Tension records. A, effect of a progressive increase in stimulus intensity upon the twitch contraction. B, contraction obtained with repetitive stimulation of the F axon at 20 stimuli/sec.
(The dotted line in A has been drawn parallel to the resting base line).

Carausius. In this insect a single shock of any intensity applied to the F axon results in a single twitch of the muscle.

The resting potential of the individual fibres of the flexor tibialis muscle was measured by the insertion of a micro-electrode. In a saline approximating in ionic composition to the insect's own haemolymph, and containing 18 m. equiv. potassium ions per litre the resting potential averaged about 41 mV (see Section III) from a range of values between 37 - 45 mV.

On single-shock stimulation of the F axon, a depolarisation of the resting membrane - the "action potential" - occurred. This was always associated with a contraction of the muscle fibre impaled. Often, when the stimulus was near threshold level, not all the fibres showed action potentials or twitches, and sometimes small action potentials were picked up from neighbouring active fibres when non-active fibres were impaled. The latter exhibited action potentials and twitches when the stimulus intensity was raised a little.

Fig. 16 shows two different action potentials recorded on different time bases. The rising phase of the F response occupies about 6 m. secs. and is followed by a long decay phase of 100 to 150 m.secs. In many cases

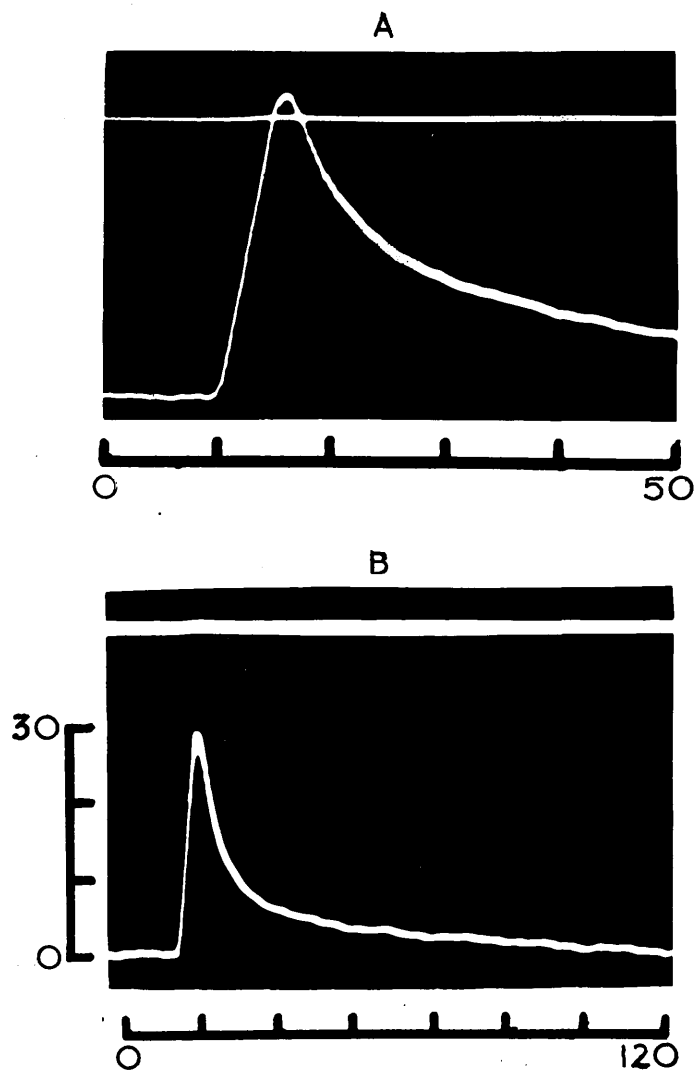


Fig. 16. Two examples of the normal electrical response of Carausius muscle to single shock stimulation of the F axon, on different time bases. Calibration: mV and msec.

small overshoots of the zero potential line were seen. In the "normal" saline these never amounted to more than about 8 mV. in size, the majority being only a few mV above the zero line. Overshoots appeared to be more common in young adults (judged by the appearance and feel of the cuticle) than in old adults. It is of interest that del Castillo, Hoyle and Machne (1953) linked the absence of overshoots with deterioration of their locust preparation. Ling Roth (1917) observed that as Carausius adults aged they became less active and weaker in movement.

Effect of temperature on the action potential.

The temperature of the saline bathing the muscle was varied between 5° C. and 30° C., and the action potential measured at different temperatures within this range. The electrode was kept in the same fibre throughout the experiment. Records from a typical experiment are shown in Fig. 17. It will be seen that alterations in the temperature of the external medium result in changes in the time course, and to a lesser extent, the amplitude, of the action potential.

As the temperature is lowered, the time course of the action potential lengthens, and it becomes slightly

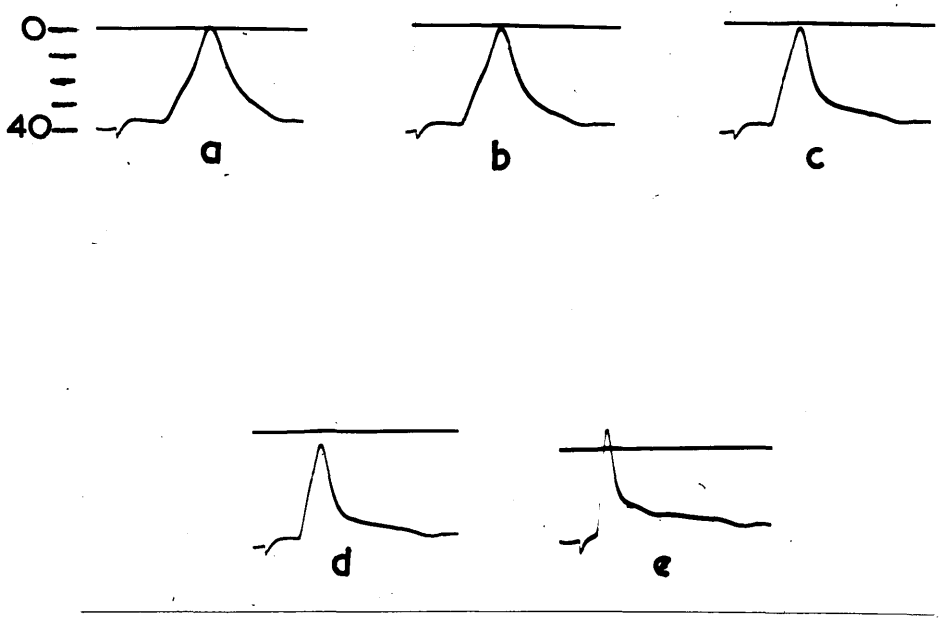


Fig. 17. The effect of temperature upon the F response. Records at (a) 5°, (b) 10°, (c) 15°, (d) 20°, and (e) 30° C. Calibration: mV. Note that the zero potential line did not stay in the same position during this experiment, owing to drift.

smaller in magnitude. It will be noted that the latent period between the stimulus artifact and the onset of the action potential also increases. When the temperature is increased the reverse changes occur.

Coincident with the lengthening of the time course of the action potential is an alteration in the shape of its rising phase. When the temperature is progressively lowered an inflexion appears in the rising phase which becomes increasingly marked (Fig. 17). The inflexion demonstrates that the action potential is the sum of two events, the end-plate or junctional potential, and the spike potential, or active membrane response. This fact is further confirmed in Section III. It is evident from Fig. 17 that the rate of rise of the two components remains comparable: the inflexion is due to the increasing time taken to initiate an active membrane response once the junctional potential has reached a sufficient size, at low temperatures.

Variation in size.

In a few cases it was possible to impale the same fibre at several positions along its length, and to record the F response at each position. In no case was a variation in size recorded, which was greater than 10%

i.e. there was little variation in the size of the action potential along a muscle fibre.

Effect of pharmacological drugs on the F response.

In some experiments, substances which are known to have definite effects upon neuromuscular transmission in vertebrate muscles were added to the normal saline, and the F response recorded. The substances used for testing in this way were: acetylcholine 1 : 100 to 1 : 100,000; physostigmine 1 : 100; atropine 1 : 100 to 1 : 1,000; and adrenalin hydrochloride 1 : 10,000 to 1 : 1,000. The test salines were allowed to bathe the muscle for at least 30 minutes, and in some cases up to several hours. In the case of acetylcholine and physostigmine salines, "hyalase", a commercial preparation of hyaluronidase kindly supplied by Messrs. Bengers, was added to the salines at a strength of 1,000 units per 100 ml. This enzyme has the property of digesting some connective tissues, and has been used successfully to avoid artifacts arising from stained connective tissue in the histological examination of sensory endings in the human skin. (Weddell et al., 1954).

No significant alteration in the size or time course of the action potential occurred in any of these experiments.

Paired stimulation of the F axon.

Further information concerning the nature of the F response was obtained by applying pairs of stimuli to the F axon, the interval between each of the pair being progressively reduced in successive experiments.

In Fig. 18A the stimuli are far enough apart, for the F response which each evokes to be separate from the other. In records B - D the second stimulus was applied at various times during the decay phase of the first response. It resulted in a second response, which summated with the first. This second response was never larger than the first: indeed, it decreased in size as the stimulus producing it was applied nearer the beginning of the decay phase of the first response. In records E - F, the second stimulus was applied during the upper part of the rising phase of the first response, and in G, H and I during the lower part. In E - F the second response continued to decay in size as it approached the peak of the first response. A faint hump remains on the decay phase in record G (arrowed), but in H and I the second response has completely disappeared. Nevertheless, there is evidence that the second stimulus, when applied in the early part of the rising phase of the first response, does exert an effect. The single response

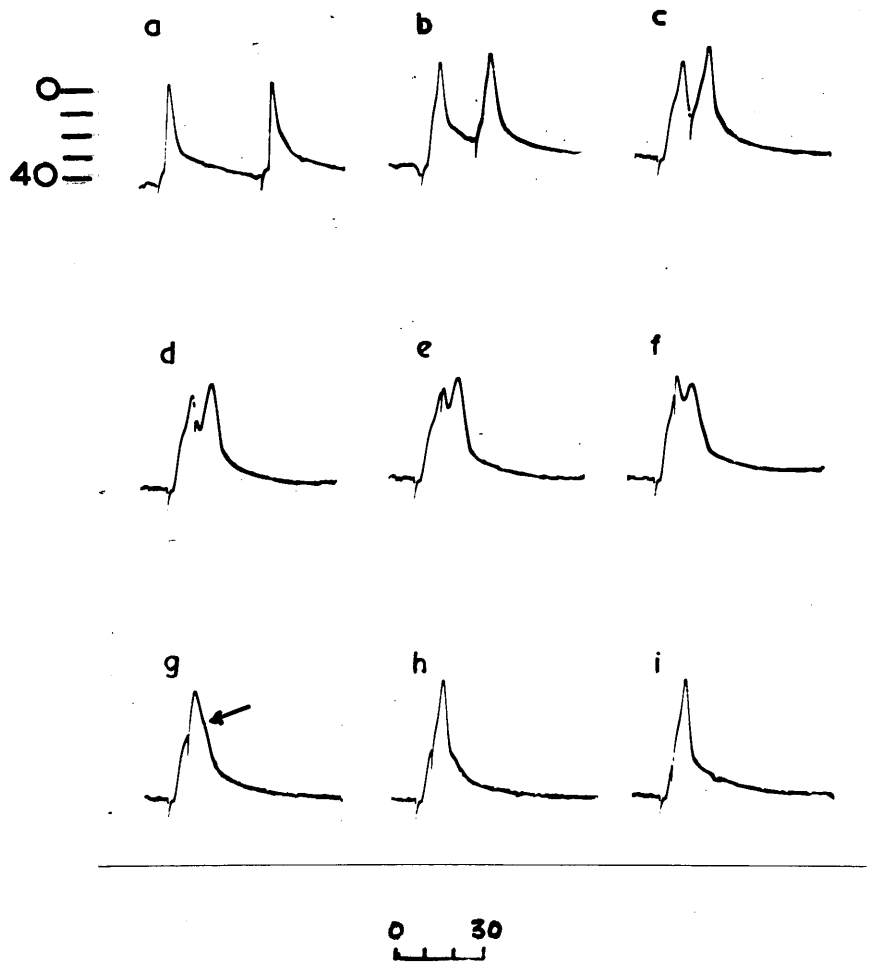


Fig. 18. The effect of paired stimuli upon the F response. From a - i, the interval between the two stimuli was progressively reduced. The stimulus artifacts appear as small downward strokes. Calibration: mV, and msec.

resulting from two shocks in G, H and I is larger in size than the responses due to single shocks in A, and the first responses seen in records A - F. The single shock responses in A - F have a magnitude of about 42 mV. The combined response in record G measures about 53 mV, and in records H and I about 60 mV.

Owing to the presence of an inflexion in the rising phases of the first responses in records B - F, and in the combined response in G - I, it is possible to determine which component of the action potential is enhanced in the latter records. The active membrane response remains constant in size, or very nearly so, throughout the records, at about 20 mV. It is the junctional potential which is increased in size in records H - I, and by inference, therefore, in record G.

Repetitive stimulation of the F axon.

When the F axon is stimulated repetitively at frequencies above about 5/sec., a tetanic contraction of the muscle is observed (Fig. 15). At lower frequencies a degree of mechanical summation is sometimes seen, but usually individual twitches occur separately. A smooth mechanical fusion takes place at 25 stimuli per second, but full flexion occurs at any frequency which will

produce a tetanus: below 25 stimuli/sec. small twitch elements are simply superimposed on a steady development of tension, similar to a frog gastrocnemius/sciatic preparation at low tetanic stimulation frequencies.

Fusion of the electrical response begins at higher frequencies than fusion of the mechanical response. The action potentials are discrete entities at 10 stimuli/sec., and fusion does not begin until stimulation frequencies of the order of 50/sec. are approached (Fig. 19).

Figs. 19B, C, are records obtained when the F axon was stimulated by a burst of shocks at a frequency of 10/sec. In B, successive sweeps were recorded on a slowly moving film; in C the film was allowed to remain stationary, and successive sweeps were allowed to superimpose themselves upon one another. The action potentials recorded in this way evidently show no variation in height, i.e. successive responses are not influenced by preceding responses. This conclusion has been confirmed many times during experiments involving repetitive stimulation of the fast axon at various frequencies.

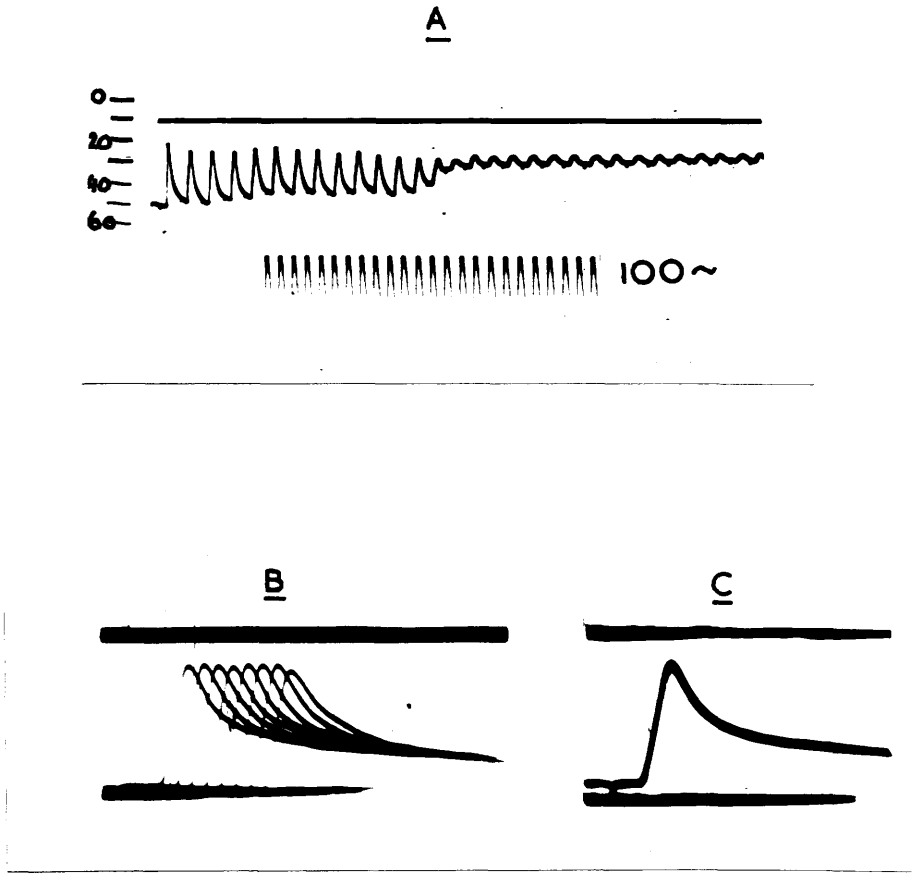


Fig. 19. Repetitive stimulation of F axon. A, stimulation at 50/sec. Note gradual fusion of electrical responses, presumably through a gradual lengthening of the decay phase. B, C, a burst of stimuli at 10/sec. In B, the film was slowly moving, in C it was stationary. The responses are all of equal size. Calibration: mV.

Mechanical performance during repetitive stimulation.

Hoyle (1955c) measured the tension developed by the metathoracic extensor tibialis of a locust hopper under conditions of natural stimulation (tickling the abdomen). He found that this muscle developed a maximum tension of 20,000 g./g. ~~body~~ muscle weight, compared with a figure of 1,000 g./g. for frog muscle, and 2,000 g./g. for human muscle. However, in terms of tension per unit area of cross-section of the fibres themselves, the performance was comparable to that of frog muscle. It is possible that an adult muscle might have given a slightly better performance. Hoyle concluded that the very large tension produced per gm. weight of muscle resulted from the pinnate arrangement of its fibres.

As the flexor tibialis of Carausius is also pinnate it was decided to obtain some figures for this muscle comparable with those of Hoyle for the locust. When lying on its back with its tarsi in the air, as it is in the preparation used in these experiments, Carausius is extremely passive and difficult to excite to cause it to move. Stimuli were therefore applied to the F axon at a moderate tetanic frequency of 20/sec. Weights were attached to the tip of the tibia by cotton thread, and the maximum weight which could be lifted during the

tetanus was determined by trial and error, using increasingly heavy weights.

In a typical case, the muscle was able to lift weights up to 2 gm. At the latter weight, the lift could be maintained only for a second or so, and a period of rest was necessary before the weight could be lifted as a result of another burst of stimuli. 2 gm. was accordingly taken as the maximum load which the muscle was capable of lifting.

The length of the tibia in this case was 1.8 cm., that of the femur 1.85 cm. The muscle weighed 9 mgm. Longitudinal sections through the femoro-tibial joint revealed that the attachment of the apodeme to the head of the tibia was about 0.3 mm. from the fulcrum. The lever factor is therefore approximately 60 : 1. Using this figure it is evident that the peak tension developed by the muscle in lifting 2 g. was about 13,000 g./g. muscle weight.

The "slow" response.

Spontaneous movements of the tibiae due to activity in the flexor muscle were observed during some experiments, though in general this was rare. Some of these movements were associated with F responses in the muscle, and were quick and jerky in character. Others were associated with

muscle responses which were quite different in character from F responses. In these cases the movements of the tibia were either smooth and slow in character, or the flexor muscle was maintained at a constant level of contraction, without obvious movement of the tibia. The electrical responses concerned are termed "slow" or S responses.

Responses of this type are shown in Fig. 20. They are smaller than F responses, and were found to vary between about 5 to 20 mV in magnitude. They appear to be similar in type to junctional or end-plate potentials (cf. p. 91.): the decay phase appears to be exponential, and no inflexion has ever been observed in the rising phase.

Repetitive stimulation of the S axon.

When the S axon is stimulated at a sufficiently high frequency the S responses in the muscle fibre show a marked tendency toward facilitation (Fig. 21.A); successive responses are larger than preceding ones. Active membrane responses have not been observed during facilitation.

At frequencies above about 10/sec. the S responses begin to fuse together (Fig. 21B). The level of

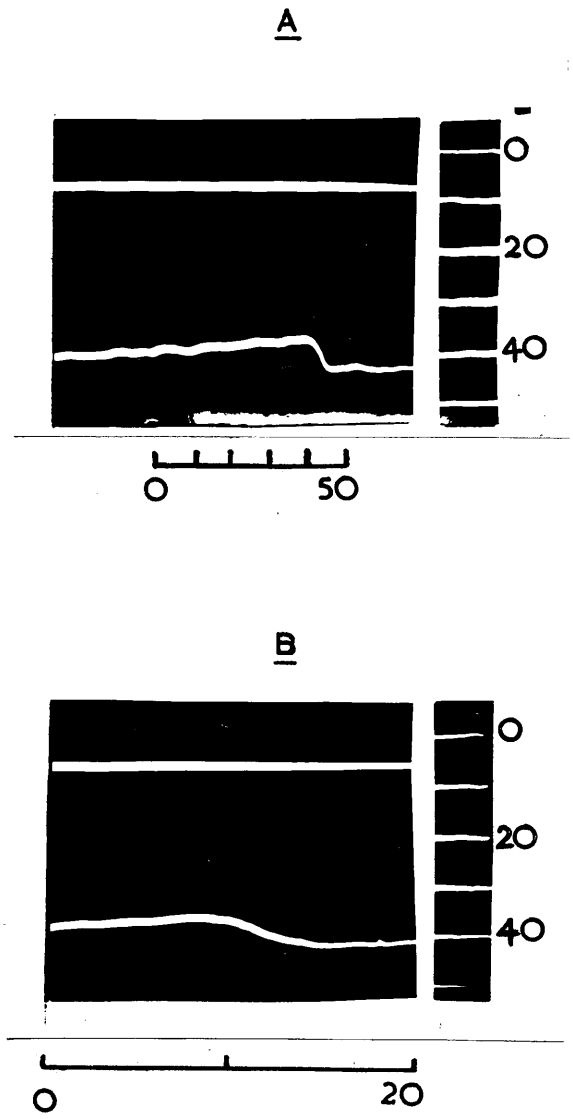


Fig. 20. Slow responses, recorded A, during spontaneous activity; b, during single shock stimulation of the S axon. Calibration: mV and msec. (Trace reading from right to left.)

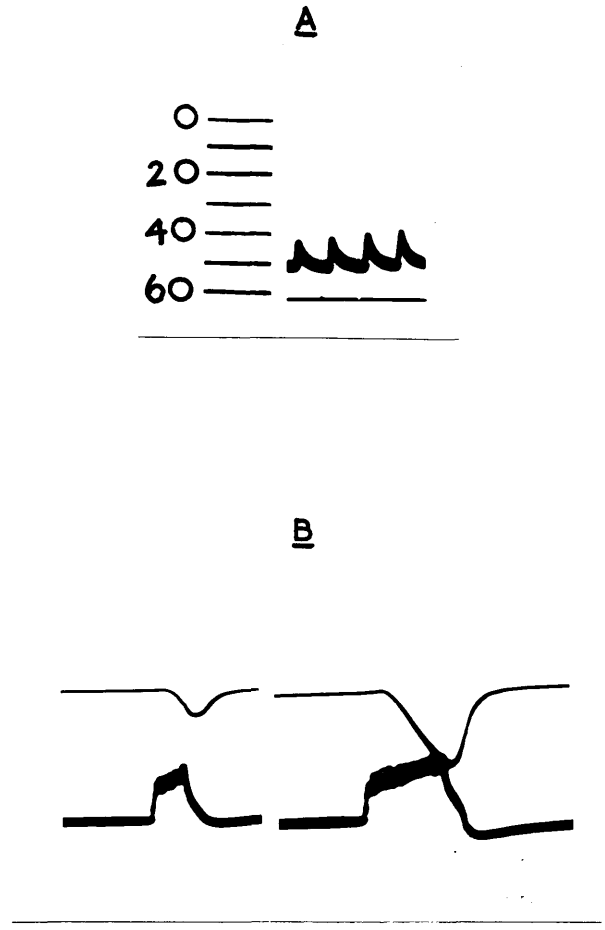


Fig. 21. Repetitive stimulation of S axon to show A, facilitation at low frequency stimulation; B, fusion of responses at high frequency (50/sec.) stimulation. Upper recording: tension. Calibration: mV.

depolarisation of the membrane increases with the frequency of stimulation. This is coupled with an increase in the extent of the contraction of the muscle. The contraction appears to be smooth at all frequencies, and is quicker at higher frequencies of stimulation. Tibial twitches have not been observed following single shock stimulation of the S axon, though very feeble twitches of the fibres do occur: presumably several successive responses are necessary in the muscle fibres before a contraction will occur which is able to move the tibia.

Combined F and S responses.

It is evident both from spontaneous activity and from artificial stimulation that F and S responses can occur together. When this happens, the two responses simply summate (Fig. 22). F responses are simply superimposed upon the S depolarisation.

Occurrence of F and S responses.

During the course of the work presented in this thesis over 60 experimental animals have been used for the electrophysiological work, and in some of them several legs have been examined. Over 4,000 separate muscle fibres have been impaled, about 1,000 in the metathoracic

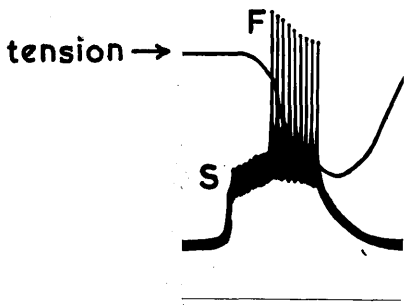


Fig. 22. Combined stimulation of S and F axons. The S axon was stimulated first, then the stimulus width was altered to bring in the F response.

leg, and 3,000 in the prothoracic leg. The latter figure includes those examined during the work described in Section III, when S responses were not considered, and when F responses were examined only in superficial fibres. F and S responses have been looked for in about 800 prothoracic fibres at all positions and depths in the flexor muscle.

Thus, the occurrence of F and S responses can only be considered tentatively. Conclusions are safer in the case of F responses. Nevertheless, it is interesting to note that F responses have been found in every fibre impaled, and S responses in every fibre in which they have been looked for. It is therefore very probable that all the fibres of the flexor tibialis muscle are innervated by an F axon; and it is possible that in addition they may all be innervated by an S axon.

DISCUSSION.

The resting potential of the flexor tibialis fibres of Carausius is low in magnitude by comparison with values given for the muscle fibres of other insects. The figure of 41 mV for Carausius is well below that of 60 mV found in Locusta and Schistocerca (Hoyle, 1957) in a saline containing 10 m. equiv. potassium per litre.

Similar values to the locust were obtained by Hagiwara and Watanabe (1954) from the wing muscles of Gampsocleis and the sound muscles of Platypleura in a saline with 2.7 m. equiv. potassium per litre; but they observed resting potentials of only 42 mV in Mecopoda and Graptosaltria, in the same saline. Wilson (1954) obtained an average value of 45 mV for the resting potential of muscle fibres of the cockroach Periplaneta americana, in a similar saline. His figures ranged from 30 - 70 mV however, and Hoyle (1955c) has suggested that the higher figure is probably more representative of the true resting potential in the intact animal.

Comparable figures for vertebrate muscle fibres are much higher, generally falling within the range of 85 - 95 mV (see Hodgkin, 1951). Unfortunately, a direct comparison between such figures and those given for insects is not possible from a functional point of view. This is because most of the insect data has been obtained from experiments utilising salines of an arbitrary ionic composition which may not resemble that of the body fluids of the animals. Nevertheless, the general position is certainly that insect resting potentials are lower than those of vertebrates; and the figure obtained for Carausius muscle fibres is among the lowest insect values

so far recorded. This may be partly related to the potassium content of the haemolymph (see Section III).

In the locust, the magnitude of the end-plate potential is directly proportional to that of the resting potential (del Castillo et al., 1953); and in the muscles of Acrididae in general the size of the action potential is related to that of the resting potential, so that there appears to be less chance of an overshoot of zero potential occurring in muscle fibres with low resting potentials (Hoyle, 1957). In view of this, it was surprising to find that overshoots are common in Carausius, particularly in younger animals. These occurred even though there did appear to be a relationship between the sizes of the resting and action potentials. Thus, one or two fibres with low resting potentials were occasionally observed in apparently healthy muscles, and in these cases the action potential was small and without an overshoot.

It is possible that there is some measure of adaptation in Carausius, which ensures that the action potential reaches a suitable height for reasonable functioning of the muscle fibres. This would be useful, because it is evident that the extent of contraction of the muscle fibre is related to the degree of depolarisation

of its membrane (see Section III, also Hoyle, 1957).

But apart from any question of adaptation of this kind, it is apparent that the action potential in Carausius is smaller in size than it is in, say, the locust (Hoyle, 1955c). A comparison between the mechanical performance of the stick insect flexor and the locust extensor is therefore of interest. The tetanus tension of 13,000 g./g. body weight is less than that developed by the locust muscle. The best comparison, however, is made in terms of the mean cross-section area of the individual muscle fibre. When this is done, the figure for Carausius is about 800 gm./cm^2, not far below the figure of 1,000 g./cm^2 found in frog and locust muscles (Hoyle, 1955c).

Fibre for fibre, therefore, the flexor tibialis of Carausius is a little poorer mechanically than the muscle fibres of either of these animals. This may be related to the smaller action potential activating the contractile mechanism. It must be remembered, however, that the locust metathoracic extensor is a specialized muscle in which the development of big tensions for jumping is all-important. It would be interesting to have figures for other locust muscles.

The striking tension which the flexor can produce

is seen to be related to the pinnate arrangement of the fibres, as it is in the locust extensor (Hoyle, 1955c). The shortness of the fibres does not hamper their ability to develop tension, while the pinnate arrangement allows the optimum concentration of their power.

It would be of interest to compare the tetanus/twitch ratios of these two muscles. Unfortunately, Hoyle (1955c) gives no figure for the locust: he merely states that the tetanus/twitch ratio "is high". It is evident even from visual inspection that a locust twitch is more powerful than that of Carausius. In the latter the twitch is very small by comparison with the tetanic contraction. No direct measurement has been made, but it seems very probable that the ratio will be as high as, or even higher than, it is in the locust.

There are indications that the tetanus/twitch ratio of an insect muscle fibre is not dependent upon the same mechanism as a vertebrate skeletal fibre. Hill (1951) has produced evidence that in simple twitch excitation of a vertebrate muscle, relaxation begins before the tension mechanism has had time to overcome the influence of the series compliance. On this view, contraction is an all-or-none phenomenon, occurring in response to a single, complete depolarisation. Nagai (1953) stimulated single

crustacean muscle fibres by means of multipolar electrodes, and did not observe a maximal contraction with single shocks of any intensity. Hoyle (1955c) invoked this result to explain the high tetanus/twitch ratio of the locust extensor. He suggested that in arthropods the contractile material is not fully activated by single muscle depolarisations, however complete, but that more than one is required for full activation.

If this is the case, the low twitch tension in Carausius may be due simply to the kind of variation which might be expected to occur in such a system among different insects. If the small action potential were the sole cause of the small twitch, it is improbable that the muscle would be able to develop the tetanus tension recorded.

It is evident that the F response in Carausius is similar to that observed in other insects (see Hoyle, 1957) and also to certain "fast" type responses of crustacean muscle fibres (Furshpan, 1955). Our detailed knowledge of the insect F response rests almost entirely upon the work of Hoyle on the locust (del Castillo, Hoyle and Machne, 1953; Hoyle, 1955a; 1955c). In this insect, lowering the temperature of the external medium results in a lengthening of the time course and a reduction in the

size of the F response; and an inflexion becomes increasingly apparent in the rising phase of the response, as the temperature is progressively lowered (del Castillo et al., 1953). This indicates that the response is composed of two components: below about 8° C the upper of these two components disappears. These authors considered that the two components corresponded to the end-plate and spike potentials of vertebrate muscle. Hoyle (1957) has proposed that the terms junctional potential and active membrane response respectively, should be used in arthropods instead of the vertebrate terms.

Lowered temperatures affect the F response in Carausius in a similar way. But although the inflexion delimiting the junction between the two components became more apparent as the temperature was progressively lowered, the active membrane response (a.m.r.) did not disappear, even at 5° C. Carausius morosus is strictly a tropical species (Chopard, 1938) of the Far East, where temperatures, both diurnally and seasonally, remain at a fairly constant high level. It might be expected that low temperatures would depress neuromuscular transmission in Carausius as much as in the locust, but that is evidently not so. Qualitative laboratory observations also suggest that a culture thrives better in diurnally fluctuating

temperatures than in a constant high temperature. There may possibly be some acclimatization effect: this species has been bred in this country for many years in temperate conditions. Locusts are usually bred in the warm.

The failure of pharmacological substances known to alter vertebrate muscle response, to exert an observable effect upon the response in Carausius, indicates that any neuromuscular transmitter substance which may be involved in the production of the junctional potential is not acetylcholine, and may not even be very closely related to it. This is in agreement with findings in the cockroach (Roeder and Weiant, 1950) and the locust (Hoyle, personal communication). It is generally assumed that a non-cholinergic transmitter is present in all insects, although in fact there is little published evidence on the subject.

The results obtained by stimulating the F axon with paired stimuli are of great interest. It is evident that a stimulus applied during the decay phase of an action potential will evoke either a similar response, or if the second stimulus is sufficiently near the peak of the original response, a reduced second response. Refractoriness of this kind would fit well into the ionic

hypothesis generally accepted as explaining the active response in vertebrate muscles (see Hodgkin, 1951). On the basis of this hypothesis, it can be argued that refractoriness immediately following the spike potential in vertebrates is due primarily to the high potassium conductance which occurs at this point and to a lesser extent to the inactivation of the "sodium carrier" by the intense depolarisation of the propagated response. If the active membrane response is produced by a process comparable with the vertebrate one (additional evidence is presented in Section III that the two processes are similar but not identical), the refractoriness observed in Carausius during and immediately following the active membrane response is readily explicable.

When a second stimulus is applied during the rise of the junctional potential, a potentiation of the junctional potential by the second stimulus is observed. The most obvious explanation for this effect is that the second stimulus causes the liberation of a greater quantity of transmitter substance. A similar explanation has been advanced to explain the potentiation of the first few muscle impulses during repetitive stimulation of the motor nerve to the frog sartorius muscle (Eccles and MacFarlane, 1949).

A notable feature in this set of experiments was the size of the active membrane response, which remained virtually constant although the size of the junctional potential was increased. No information relating active response to junctional potential height (except that a critical j.p. height is necessary for initiation of the a.m.r.) is available for insects; but Furshpan (1955) found that in muscle fibres of the decapod crustacean Cambarus the spike height was proportional to the junctional potential. This is unlikely to be the case in the locust, since in this insect the active response is very variable in both magnitude and duration even when recorded from the same site (Hoyle, 1957). Variations of this kind have not been seen in Carausius, where the a.m.r. appears to remain virtually constant in height at any one site. Nevertheless, there are similarities between the locust active response and that of Carausius. In the locust, the spikes appear to have an upper limit in size of about 25 mV. Evidence is given in Section III of this thesis that the active response in Carausius varies little in magnitude, and that most of the variations observed can be attributed to corresponding changes in the size of the resting potential in different salines. Thus in both insects there seems

to be a limitation to a surprisingly low level of the amount of charge which can be transported across the membrane. This is particularly striking in the locust, where the saline used contained a quantity of sodium similar to that used in vertebrate salines (Hoyle 1955a). On the basis of the ionic hypothesis, it would be expected, other things being equal, that the locust active response would therefore approach that of the vertebrates in size.

This limitation in size of the active response emphasizes the importance of the multiple end-plate distribution along single insect muscle fibres. Hoyle (1957) considers that the largest active responses he found in locust muscle fibres might just be capable theoretically of setting up propagation, but not in most cases. The smaller active responses in Carausius would certainly not be able to do so. If the whole muscle fibre is to contract then a series of end-plates, each producing local contractions which sum together along the length of the fibre, is a method of ensuring that it does so, in the absence of a propagated response.

The terms "facilitation" and "summation" are often used interchangeably in neurophysiological literature. Summation is often regarded as one method whereby

facilitation occurs. The author would prefer to use summation, as now, to denote arithmetic summation of mechanical or electrical responses; and to reserve facilitation for instances in which a potentiating effect by one response upon another is observed, i.e. where a response is actually enhanced by a previous response. Thus, the potentiation effect observed by Eccles and MacFarlane (1949) in frog sartorius fibres, which is referred to above, would be facilitation.

Facilitation in this sense is not a property of the F response in Carausius. When the F axon is stimulated repetitively at a frequency sufficiently low for discrete responses to occur, each response is either equal in size to, or smaller than, the preceding response. On the other hand, two responses sufficiently close together will summate, as stated previously.

The small extent of the twitch contraction implies that in normal functioning of the F axon, movement of the flexor tibialis muscle is brought about by trains of impulses passing down the axon, which evoke a tetanus from the muscle. The extent of the contraction, and the speed with which is occurs, will depend upon the frequency of these impulses. This is one way of carrying out graded movements of the tibia. In Carausius there is an additional method available for this purpose. The

possession of a number of F axons running to different groups of muscle fibres, and with different thresholds, will allow alterations to be made of the number of muscle fibres in action at a given time.

Nevertheless, there is an obvious limit to the delicacy of muscular contractions produced by F axons. F axons are certainly necessary to overcome the inertia of the femur/tibia system, particularly in a long-legged animal like Carausius. But for more delicate movements, and for maintenance of tonus, the S responses are necessary.

The S responses in Carausius are comparable to the S1b responses described by Hoyle (1955c) in the locust, and to the slow responses of Cambarus muscle fibres (Furshpan, 1955), although they more nearly resemble the latter. In all these animals these S responses are depolarisations of the muscle fibre membrane which look ~~rather~~ like end-plate potentials, and are usually rather smaller in size than the F responses. In the locust, however, an inflexion is visible in the rising phase of the S1b response, which is thought to denote the presence of an active response. In Carausius, as in Cambarus, there is no evidence of an active response.

In all three animals repetitive stimulation results in a tetanus of the slow responses, which exhibit marked facilitation, which is reflected in the extent of the

contraction of the muscle. Fine central control of the frequency of S impulses could result in very delicate or quite strong contractions, as required. In the locust there is a second slow motor response which was considered by Hoyle (1955c) to be ~~very~~ responsible for the very slow movements and the maintenance of tonus. Evidently in Carausius, the single S response does the work of the two locust S responses. On the evidence available, it is not certain whether additional control of S-evoked movements is possible because of the presence of a number of S axons of similar properties running to the muscle; but this may be so.

Two instances of nervous inhibition of insect muscles have been claimed. Ewer and Ripley (1951) claimed to have discovered peripheral inhibition in the levator tarsi of the locust. Hoyle (1955c) has shown that the phenomenon they observed was probably due to polarization of the stimulating electrodes. The other claim was made by Friedrich (1933) in respect of Carausius. He stimulated the flexor at a stimulus intensity just below that required for excitation and found that a slight further relaxation of the resting muscle occurred. He also stimulated the muscle at a similar intensity while the muscle was relaxing from a contraction and found an

increase in the relaxation rate. These effects, as shown by his records, were very slight. Similar effects have occasionally been observed in Carausius, but no change in electrical sign of the muscle membrane occurred. There is clearly an alternative explanation which would not have occurred to Friedrich, since he was unaware of the existence of S responses in insects. The stimulation might equally well have been of the S axon running to the extensor muscle. No other evidence of inhibition such as is found in crustacean muscles (Fatt and Katz, 1953b) has been observed in Carausius, and the histological evidence is against a third axon. Friedrich's claim must be regarded with great suspicion.

To sum up this Section, it is evident that distributed electrical responses of the membrane occur at a number of foci along each fibre of the flexor tibialis muscle. These are responsible for initiating local contractions which sum along the length of the fibre so that it contracts as a whole. The responses may be of the F or S type: the F responses are necessary for quick movements, while the S responses function in smooth slow movements, and in the maintenance of tonus. The pattern of innervation of this muscle is simpler than that of the locust metathoracic extensor tibialis, where there is a

second S response, and an additional hyperpolarizing axon. The locust muscle is a specialized one, and its innervation is different from the extensors of the other legs. It may be that the pattern seen in the Carausius flexor will prove to be more typical of insect muscles.

SECTION III.

BLOOD IONIC COMPOSITION AND THE EFFECT OF IONS ON THE FAST RESPONSE.

INTRODUCTION.

As a result of their analyses of the ionic composition of frog blood plasma and muscle cytoplasm, Boyle and Conway (1941) suggested that the ionic ratios they found were evidence of the existence of a Donnan equilibrium across the plasma membrane of the muscle fibres. The suggestion was further developed by Conway (see Hodgkin, 1951, for a restatement). Similar ionic ratios have since been found in many excitable tissues, including muscle fibres of the shore crab Carcinus maenas (Shaw, 1955). A Donnan equilibrium of the kind proposed requires that the resting potential across the membrane of a cell shall be directly proportional to the logarithm of the external potassium concentration. This relationship has been found wherever it has been looked for. Although no analytical figures are available for insect myoplasm, such a relationship has also been found in the locust (Hoyle, 1953b) and has been presumed to be due to the same mechanism. There is no reason to believe that other insects are different in this respect.

Since the extent of contraction of a muscle is related to the amount by which the resting membrane is depolarised, and since the size of the action potential is related to that of the resting potential, it follows that the external concentration of potassium may possibly influence the effectiveness of muscular contraction.

In vertebrates, the arrival of an impulse at the motor nerve terminals results in the liberation of a chemical substance, acetylcholine, from those endings. The liberated acetylcholine diffuses across the junctional region to become attached momentarily to special acetylcholine receptors on the muscle fibre membrane immediately beneath the nerve terminals. The attachment is extremely short, because the acetylcholine is rapidly hydrolysed by a specific cholinesterase concentrated at the end-plate surface. During its brief attachment, however, it causes a catastrophic breakdown of the membrane in the end-plate region, to render it completely permeable to all ions. The permeability is completely non-specific. A net inward flow of cations results, and the end-plate membrane becomes depolarised. This depolarisation is the end-plate potential. It leads to the depolarisation of adjacent areas of the muscle fibre membrane in much the same way as a depolarization of a nerve by an electrical

stimulus: a muscle spike is initiated when the inward sodium current due to sodium carrier activation just exceeds the outward potassium and chloride current (see Hodgkin, 1951; Fatt, 1954; del Castillo and Katz, 1956).

Sodium, calcium and magnesium ions have specific actions which modify or prevent the various steps in this process. Sodium ions are not essential for the development of the end-plate potential. Acetylcholine can still depolarize in a sodium-free medium, though much less effectively (Fatt and Katz, 1952a). Sodium ions increase the reactivity of the receptor with the transmitter. In normal functioning, therefore, they are nevertheless important in the development of the end-plate potential. Since the ratio of inward sodium current to outward potassium current is the determining factor in the production of an active membrane response, sodium ions must be present in a minimal quantity in the bathing medium, if an active response is to be produced. The concentration of these ions in the medium will influence the height and rate of rise of the active response.

Calcium ions affect the amplitude of the end-plate potential by altering the quantity of acetylcholine liberated from the motor nerve terminals (del Castillo and Stark, 1952). A higher concentration of calcium

results in the release of a greater amount of acetylcholine. In addition, it has been postulated that the "sodium carrier" responsible for transporting sodium ions across excitable membranes must be combined with a calcium ion in order to function (Hodgkin, 1951). Calcium ions would therefore be expected to alter the permeability of the membrane to sodium ions, thereby affecting the active membrane response. An appreciable effect by calcium ions on sodium permeability has, in fact, been demonstrated in the giant axon of the squid (Frankenhauser and Hodgkin, 1955).

An excess of magnesium ions blocks neuromuscular transmission by decreasing the amount of acetylcholine liberated by the motor nerve endings, i.e. magnesium ions are antagonistic to calcium ions in this respect (del Castillo and Engbaek, 1954). To a lesser extent, magnesium ions decrease the sensitivity of the end-plate to the depolarizing action of acetylcholine, and have a direct effect upon the excitability of the muscle fibres. The two latter effects, however, are relatively unimportant compared with that exerted upon the liberation of the transmitter substance.

It follows that the amount of acetylcholine liberated from the motor nerve terminals is a function of the

relative amounts of calcium and magnesium ions present. A large excess of magnesium or a considerable depletion of calcium in the bathing fliud will result in the liberation of such a small quantity of acetylcholine that the end-plate potential generated may not reach the critical size necessary for the initiation of an active response. Propagation of the depolarisation along the fibre will not then occur.

Hoyle (1955a) found that in the locust and cockroach an increase in magnesium ions reduced the size of the junctional potential and lowered the excitability of the muscle fibre membrane. Calcium ions antagonised the action of magnesium on the junctional potential and their presence was necessary for its development. He made no detailed study of the action of sodium ions in this work, but had previously noted in a qualitative observation (Hoyle, 1953b) that a reduction in the sodium level of the bathing medium appeared to result in a reduction in the action potential. Hoyle (1955a) considered that these results obtained with different ion concentrations, coupled with previous work on the F response (del Castillo et al. 1953), indicated that insect neuromuscular transmission was essentially similar in kind to the vertebrate process, although not cholinergic.

These results threw into relief certain facts

concerning herbivorous insects. It had been shown that the ionic composition of the haemolymph of most insects is closely related to their diet (Boné, 1944; Clark and Craig, 1953; Duchateau, Florkin and Leclerq, 1953). In herbivorous insects the blood sodium and potassium levels are similar in general to those of the food plant but magnesium is higher and calcium lower than in the food plant (Duchateau et al., 1953). The sodium/potassium and calcium/magnesium ratios of the haemolymph are both less than unity. In carnivorous and omnivorous insects, the proportions of the mineral constituents of the haemolymph are more comparable to those of vertebrate blood: there is a high sodium/potassium ratio, and the calcium/magnesium ratio approaches or exceeds unity. The locust examined by Hoyle (1955a) is anomalous in this respect. Although herbivorous in habit, its blood ionic composition is like that of omnivorous or carnivorous insects. The cockroach is omnivorous. We therefore lack any detailed study of neuromuscular transmission in an animal with the herbivorous type of blood composition.

It might be expected that in herbivorous insects there will be a low resting potential due to high blood potassium, and a small action potential due to a combination of low resting potential and low blood sodium.

Since the size of the mechanical response in arthropod muscle is directly related to that of the action potential, the muscles of herbivorous insects might be expected to contract less vigorously than those of carnivorous or omnivorous insects. Evidence has already been advanced in Section II to show that, although the muscles of Carausius, a herbivorous insect, are slightly inferior in mechanical performance to those of the locust, they nevertheless have considerable contractile powers.

Hoyle (1955a) found that a concentration of 20 m. equiv. magnesium per litre reduced the locust or cockroach junctional potential to a very small size, and led to the disappearance of the active membrane response. This magnesium level is only one-half to one-fifth of those found in the blood of many herbivorous insects (Clark and Craig, 1953; Duchateau et al. 1953). If all the magnesium found in the blood of these insects is present in the form of unbound ions, the muscles of herbivorous insects should be very weak.

Thus, the ionic composition of the blood of herbivorous insects would appear to be inimical to the proper functioning of the muscles of these animals. The problem is restricted to the muscles. Hoyle (1953b) found that the nerves of the locust are surrounded by a

sheath which probably acts as a selective ion barrier and which provides a more favourable ionic environment for them than the haemolymph. A sheath similar in appearance occurs in other insects (Hughes, 1953), certainly in Carausius, and probably in all insects. Evidence that it functions similarly in Carausius is provided indirectly by the experiment of Pflugfelder (1937). He removed the corpora allata from 1st or 2nd instars and found the sheath around the nerve cord degenerated. This degeneration could lead to paralysis, presumably due to the unfavourable environment around the nerves. Histologically, barriers of this kind are not observed around the muscle fibres.

Following these facts and inferences, it seemed desirable to examine the effects of the four common cations, sodium, potassium, calcium and magnesium on the F response of Carausius.

BLOOD COMPOSITION.

Blood analyses were carried out using small samples obtained by pricking the articular membrane between two of the thoracic segments, and taking up the resulting drop of blood in a haemocytometer pipette. A measured quantity of the blood was then pipetted into a known small quantity of distilled water. Animals could be

bled in this way every four days without evidence of discomfort. The insects used for these analyses lived for a normal span of time.

Sodium, potassium and calcium were estimated on the samples by means of an "Eel" Flame Photometer. Prior to magnesium and phosphate estimations, protein was precipitated with a small quantity of 10% trichloracetic acid, and centrifuged off. Magnesium was determined by the titan yellow method of Heagy (1948) and phosphate by the molybdate method of Snell and Snell (1949). For chloride and bicarbonate, Conway microdiffusion methods were used; the original method for bicarbonate (Conway, 1950), and a modified colorimetric method for chloride (Gordon, 1952). In all the colorimetric methods, the unknown was measured against known standards in an "Eel" Colorimeter.

The results of these analyses are given in Table 1. They show good agreement with the results of previous workers, although the magnesium value obtained was not quite so high as that of Duchateau et al. (1953). Nevertheless, the value of 106 m. equiv. obtained here is 20 to 30 times that found in vertebrate blood or in the haemolymph of a carnivorous insect.

There arises the question of how far the results

Table 1. The ionic composition of Carausius haemolymph.

Figures are given in m.equiv. per litre as the mean of a number (in brackets) of analyses. ± S.E. of the mean. The results of previous workers are included in the table. All figures are to the nearest whole number.

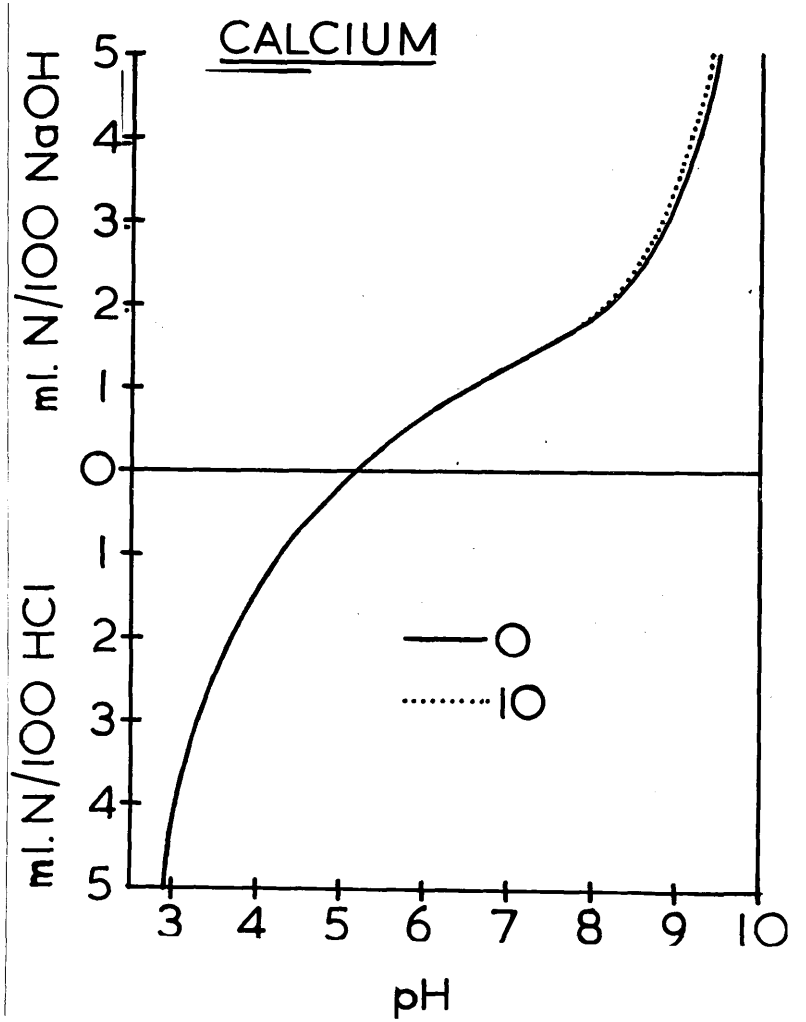
Na	K	Ca	Mg	Cl	Phosphate, as $H_2PO_4^-$	HCO_3	
15 ± 3 (17)	18 ± 3 (17)	15 ± 2 (12)	106 ± 12 (13)	101 ± 11 (17)	16 ± 3 (7)	5 ± 2 (3)	
21	25	-	-	-	-	-	Bone, 1944.
9	28	16	145	-	-	-	Duchateau et al., 1953.
14	17	-	-	-	40*	-	Ramsay, 1953; 1955.
11	18	7	108	87	13*	-	"serum": Ramsay, 1955.
				87-100			May (1955)

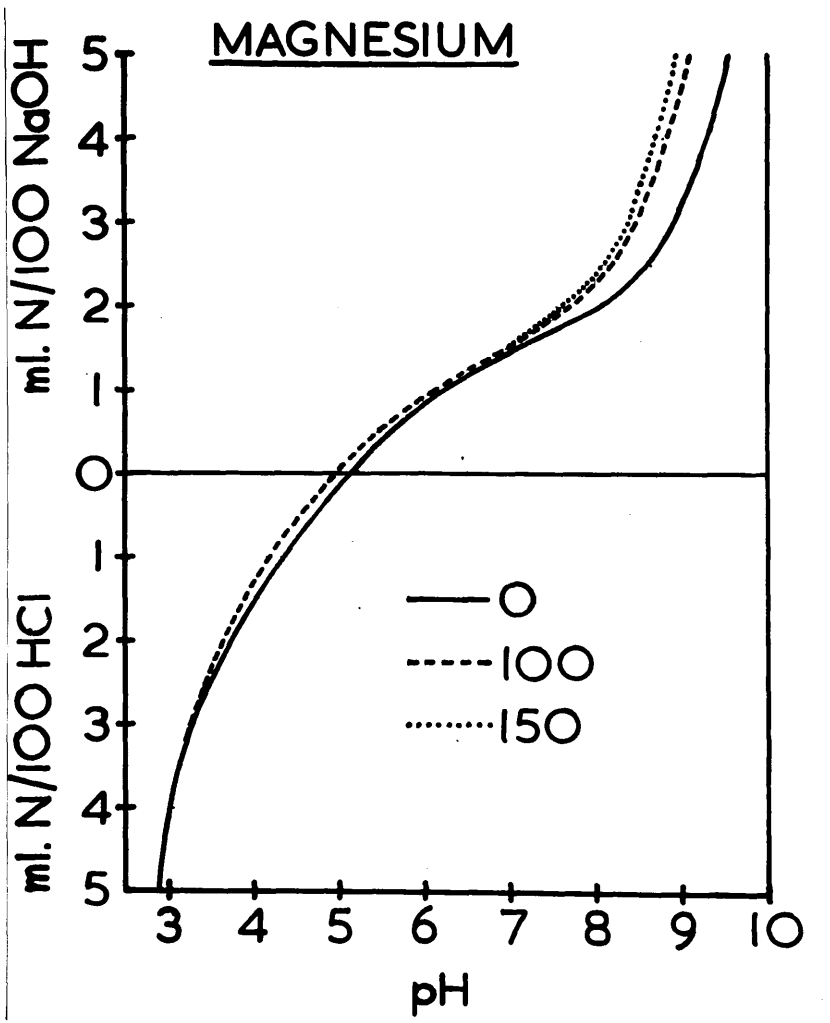
* as PO_4^{3-}

obtained from the analyses represent the concentrations of ions actually occurring free and unbound in the intact animal. The blood of insects is known to contain large quantities of amino-acids, which could have the property of binding some of the ions. The matter was investigated by testing the effect of varied concentrations of the different cations upon the titration curve of the blood amino-acids. This method has been used with success by Monnier (1949) in investigations on ion binding. The amino-acid content of Carausius haemolymph has been given by Duchateau, Sarlet, and Florkin (1952). The quantities given by these authors were made up in various salines, and the titration curves of the mixtures were found, using a Pye pH meter. The salines used were variants of the normal saline (see below) in which the concentration of one ion was increased or decreased.

The titration curves obtained are shown in Figures overleaf. It is evident that the only ion which is likely to be bound to any significant extent to the blood animo-acids at the concentration at which it normally occurs in the haemolymph is magnesium. This is in agreement with the investigations of Albert (1950) on the binding of magnesium by amino-acids.

A saline was prepared for experimental purposes


CALCIUM
ml. N/100 NaOH
ml. N/100 HCl
5
4
3
2
1
0
1
2
3
4
5
3
4
5
6
7
8
9
10
pH
0
10


MAGNESIUM
ml. N/100 NaOH
ml. N/100 HCl
5
4
3
2
1
0
1
2
3
4
5
3
4
5
6
7
8
9
10
pH
0
100
150

utilising the figures obtained by analysis, so as to approximate closely to the free ionic composition of the haemolymph. Some allowance was made for probable binding of magnesium by the blood amino-acids. The composition of the saline was as follows: Na 15; K 18; Mg 100; Ca 15; H_2PO_4 6; HPO_4 9; Cl 133 (all in m. equiv. per litre). This saline has a pH of about 6.6, similar to the pH value obtained from fresh blood using a Pye pH meter with a glass electrode micro-assembly. Experimental salines were prepared by addition or subtraction of the chloride of the ion under investigation. To the normal saline 63.3 g. of sucrose per litre were added, and the tonicity of the other saline mixtures was adjusted to that of the normal saline by a suitable alteration of the sucrose content.

For a sodium-free mixture, potassium phosphates were substituted for sodium phosphates, and the amount of potassium chloride adjusted to preserve the potassium level of the normal saline. It was found that the use of phosphate buffers resulted in the formation of precipitates, presumably of calcium phosphate, at low magnesium concentrations. Phosphate/bicarbonate buffering (12 m. equiv. H_2PO_4 -; 8 m. equiv. HCO_3- per litre) was accordingly used for zero and 50 m. equiv. magnesium

salines. Some experiments were carried out in which all the magnesium salines were buffered in this way, and the results were not found to differ from experiments in which phosphate-buffered salines were used.

Even in the normal saline a precipitate formed on standing. No saline has been used in this condition.

Tetraethylammonium, tetramethylammonium, and tetra-n-butyl ammonium iodides were converted to chlorides by dissolving them in distilled water and passing the solution through a column containing the Permutit ion exchange resin "de-acidite FF" in chloride form, until the liquid passing through showed no trace of iodide. The resultant chloride solutions were then titrated by the silver chromate method, and their strengths adjusted to a suitable level for incorporation in the salines. This method of conversion avoids the possible objection which can be made to that used by Fatt and Katz (1953a), in which sulphurous contamination of the quaternary ammonium compounds is a possibility.

EXPERIMENTAL PROCEDURE.

The preparation used has been described in Section II. Throughout these experiments the F axon was stimulated in the thorax by single shocks at the rate of about 1/sec. to produce simple twitch contractions.

The effects produced upon the F responses of the muscle fibres by salines of different composition were studied by changing the fluid in the plasticene well surrounding the leg. The routine procedure was to impale about ten fibres selected at random over the surface of the muscle in each saline, after a replacement time of 30 minutes. The electrode was left in the last fibre impaled in each saline to enable the effect of the next saline to be observed at regular intervals up to the standard replacement time. In addition, the effect of a series of salines was sometimes examined with the electrode left in the same fibre for the whole series. Only superficial fibres were used for sampling, but the effects described were checked on deeper fibres from time to time, and found to be similar to those seen in superficial fibres.

At least 3 separate sets of experiments, involving 3 separate animals, were carried out with each saline, usually more. The results given are those from one set of experiments only in each case, because it was felt that, owing to the variations observed between animals, statistical analysis of the results might give a false impression of the extent of scatter in the observations made. However, no results are given which

are not in accordance with those obtained in all experiments using the particular saline under review.

RESULTS.

If the experimental preparations are completed before the removal of the cuticle from the femur, it is possible to impale fibres of the flexor tibialis muscle within two minutes of covering the muscle with normal saline. The shortest time within which a change in the ionic composition of the bathing fluid was seen to produce an effect upon a superficial fibre was about one minute in high potassium saline. In most superficial fibres it was a little longer than this. If deeper fibres are impaled immediately after they have been covered in saline, it should therefore be possible to record the electrical responses of these fibres while they are still bathed in their normal medium. The two records already shown in Fig. 16 were obtained in this way. Mean values obtained from six such deeper fibres in the same muscle were 41 mV $\pm$ S.E. 1.5 for the resting potential and 39 mV $\pm$ 2.2 for the action potential.

The long decay time observed in these F responses was not a result of the appearance of a contraction artifact. The time course and resting potential level

did not vary when small vertical movements of the electrode were made: when a fibre is not properly impaled, such movements result in fluctuations of the apparent resting potential level and the time course varies. Furthermore, the initial part of the recovery phase occurred more quickly in high sodium saline, although the mechanical response was then greater. In low calcium, the recovery phase was slower, but the tension developed by the muscle was much smaller. Contraction artifacts were observed in some cases: they could always be distinguished from the time course of the action potential.

At first no attempt was made to see that the normal saline was isotonic with the muscle fibres. Roeder and Weiant (1950) and Wilson (1954) added a small quantity of sucrose to the saline they used for the cockroach, but Hoyle (1953a) and Hagiwara (1953) added none to the salines they used for various Acrididae. The osmotic pressure of insect blood is high, owing to the presence of large quantities of free amino-acids and reducing sugars (see Roeder, 1953). Figures for the osmotic pressure of the haemolymph of most of the insects studied by these authors are not available, but it is probable that some, at least, of the salines used by them were

hypotonic to the muscle fibres they examined. However, no adverse effects were reported as a result of their use. There was some swelling of locust muscle fibres in the saline used by Hoyle (1953a), but this had no effect upon their electrical and mechanical properties (Hoyle, personal communication).

This was not the case in Carausius. In the normal saline without added sucrose, the magnitude of the resting and action potentials showed a substantial decline (Fig. 23), but recovered when sucrose was added to the saline. Within limits, depending upon the normal action of the salt concerned, recovery could be induced by the addition of almost any salt to the saline. For example, the addition of a moderate quantity of potassium ions resulted in a small increase in the size of the action potential, although the resting potential declined further. Following these observations, the normal saline was made up with sucrose, as stated. The amount of sucrose to be added was determined by trial and error until resting and action potentials were obtained similar in size to those recorded from deeper fibres bathed in the insect's own body fluid.

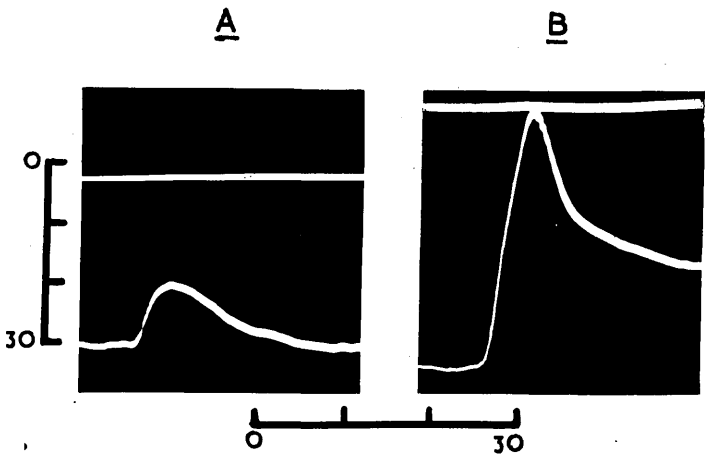


Fig. 23. A, normal response without addition of sucrose to saline; B, with sucrose (see text). Calibration: mV and m-secs.

The effect of sodium ions.

Changes in the external sodium concentration affected the magnitudes of both the resting and action potentials. Fig. 24 is a graph of the results from a typical experiment. Below about 100 m-eq. sodium the curves for the resting and action potentials follow an almost parallel course. The similar shape of the two curves may mean that at these sodium concentrations the change in size of the action potential is at least partly a result of the corresponding change in the resting potential: del Castillo et al. (1953) found that the size of the end-plate potential of locust muscle fibres was directly proportional to that of the resting potential.

At sodium concentrations above 100 m-eq. both curves rise much more slowly, but the curve for the action potential maintains a higher rate of rise than the curve for the resting potential. The differences are not statistically significant (*t* test, $P = 0.05$) but the same trend was present in all experiments. Some factor other than the magnitude of the resting potential may be involved. In both resting and action potential the change in magnitude in different sodium concentrations is relatively small: about 10 mV. in the resting potential, and 12 mV.

Table 2. Magnitude of resting and action potentials in different sodium concentrations.

M.equiv sodium per litre	Number of samples	Mean resting potential in millivolts ± S.E.	Mean action potential in millivolts ± S.E.
0	9	40.4 ± 0.85	39.8 ± 0.75
15	23	40.0 ± 0.68	41.7 ± 0.57
50	15	41.9 ± 0.72	44.0 ± 0.78
100	13	43.3 ± 0.63	47.4 ± 0.73
150	12	43.4 ± 0.56	46.4 ± 1.15
200	12	43.0 ± 0.58	50.1 ± 1.46

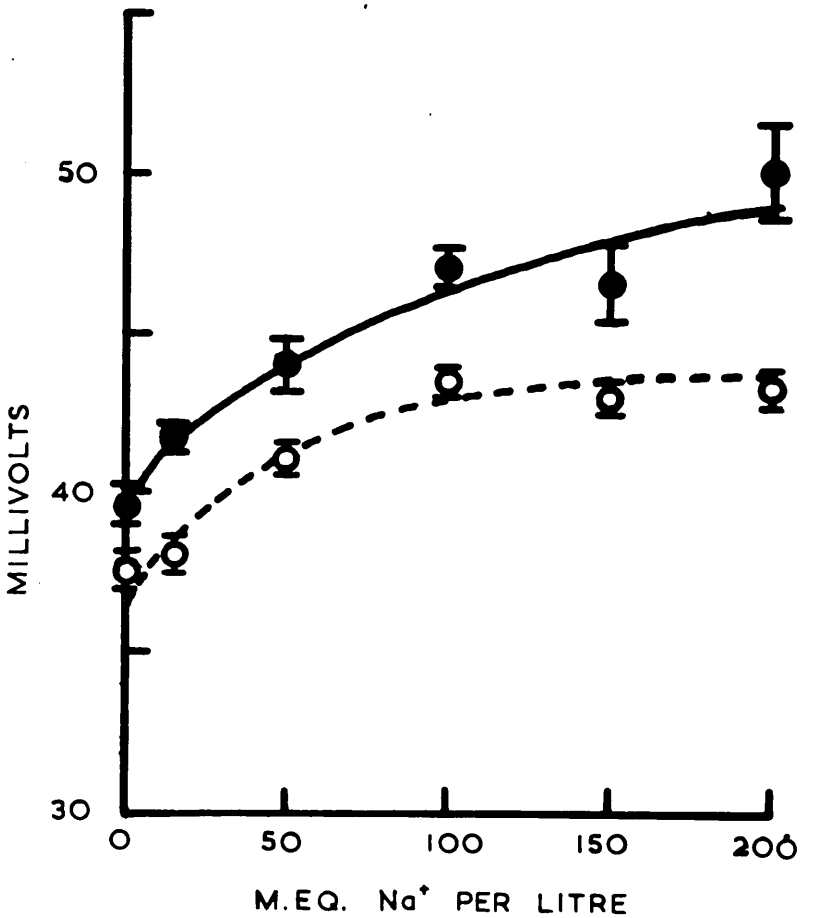


Fig. 24. Relation between external sodium concentration and size of resting potential (hollow circles) and action potential (full circles). Limits of S.E. of mean shown by horizontal bars where they exceed radius of circles, and similarly throughout this thesis.

in the action potential.

By employing a suitably fast time-base on the oscillograph, it is possible to determine the relative sizes of the end-plate component, which will hereafter be termed the junctional potential, and of the active membrane response. The junction between the two components then appears in some records as a slight bend in the rising phase of the action potential (the validity of this criterion is discussed on p. 91). Analysis of the figures obtained from such selected fibres shows that the curves for the two components follow a parallel course which is similar in shape to that of the action potential as a whole (Fig. 25).

In Fig. 26 records are shown which illustrate the effect of lowering the sodium in the bathing fluid from normal to zero, or raising it from normal to 200 m-eq. The record in zero sodium was taken 3 hours after transference of the fibre to this saline from normal saline: it was no different from the response recorded after 30 minutes. It is evident from these records that an alteration in external sodium concentration results in a corresponding alteration in the rate of rise of the action potential. The rate of rise of the junctional potential and active membrane response are affected in

Table 3. Magnitudes of junctional potential and active membrane response in different sodium concentrations.

M.Equiv. sodium per litre	Number of samples	Mean junctional potential in millivolts ± S.E.	Mean active Membrane response in millivolts ± S.E.
0	12	24.5 ± 1.7	14.5 ± 0.98
15	11	29.0 ± 1.4	17.5 ± 1.2
50	9	30.0 ± 0.93	18.0 ± 1.4
100	10	33.0 ± 0.90	19.5 ± 0.85
150	12	33.0 ± 1.2	20.0 ± 0.98

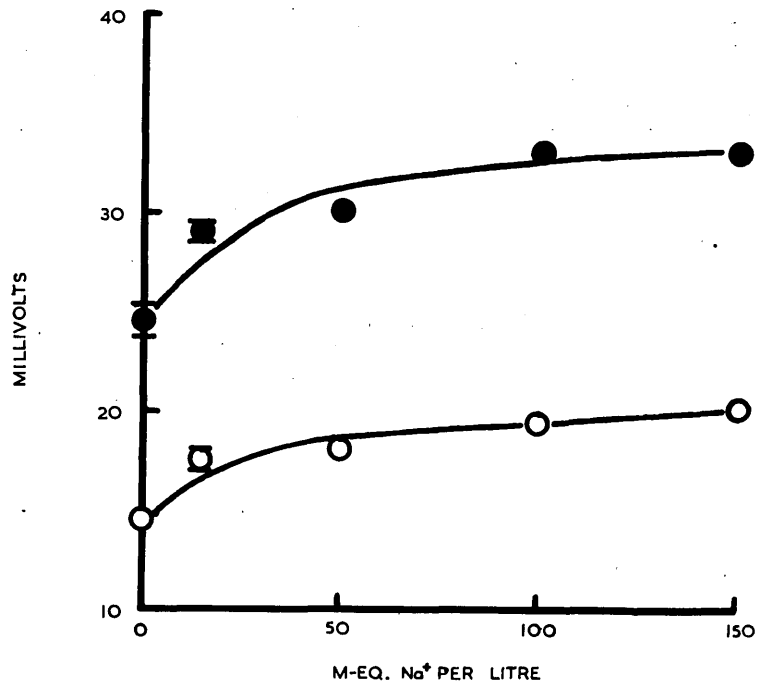


Fig. 25. Relation between size of junctional potential (full circles), active membrane response (hollow circles), and external sodium concentration. Different experiment from Fig. 24.

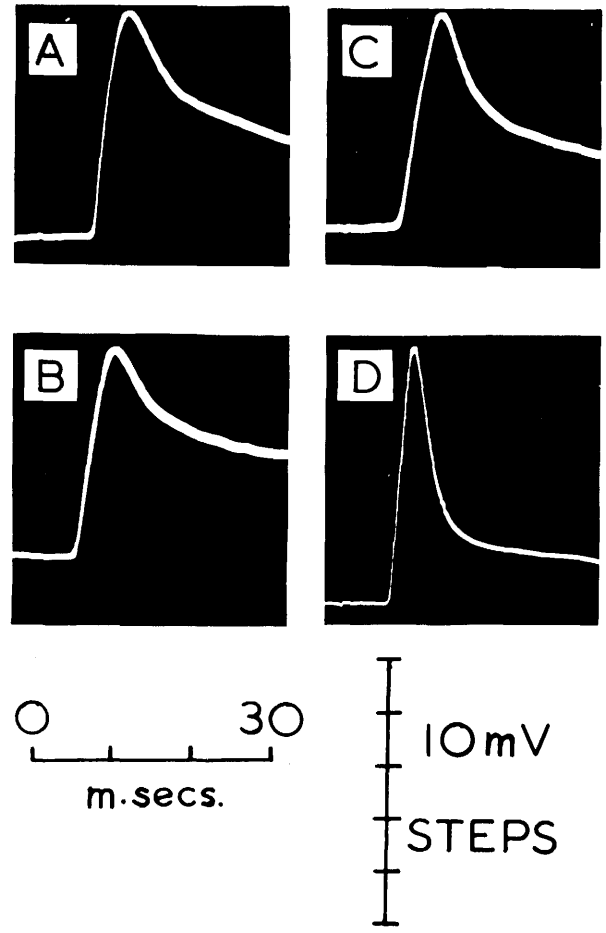


Fig. 26. Records from a fibre transferred from normal saline (A) to zero Na (B); and from another transferred from normal saline (C) to 200 m.equiv. Na (D). Calibration: mV and m-secs.

a similar manner by changes in sodium concentration (see Fig. 29) and it follows that the rate of rise from start to peak of the action potential is a rough measure of the rate of rise of each of its two components. In three separate experiments, the increase in the rate of rise of the action potential between zero and 200m-eq. sodium was 5.8, 6.1 and 7.9 V/sec., giving a mean value of 6.9 V/sec. The corresponding increase in the size of the action potential was 12, 10 and 12.5 mV. respectively, giving a mean of 11.5 mV.

Quaternary ammonium ions.

In an attempt to throw more light upon the role of sodium in the production of the action potential in Carausius, experiments were performed in which the sodium content of the salines was replaced by an equivalent quantity of a quaternary ammonium ion.

The muscle became inexcitable after being bathed for 30 minutes in concentrations of tetrabutylammonium ions as low as 15 m-eq./litre. It is not known whether this was due to a direct effect upon the muscle fibres or to some action upon the nerve endings. Conduction along the crural nerve was not affected. Excitability did not return when the saline was replaced with normal

saline containing sodium.

Tetraethylammonium (TEA) ions tended to produce an irreversible fall in the value of the resting potential, the effect being quicker the higher the concentration. The fall in resting potential was accompanied by a fall in the magnitude of the action potential. Nevertheless, the rate of rise of the action potential increased considerably by comparison with sodium salines (Fig. 27).

TEA salines also caused the muscle fibres to become abnormally excitable. The slightest mechanical stimulus to any part of the muscle, or a slight movement of the tibia, resulted in a vigorous and prolonged contraction of the whole muscle. A similar contraction followed single shock stimulation of the crural nerve. In sodium salines a single nerve stimulus produces only a small movement of the tibia, and the increase in size of the twitch in increased sodium concentrations is slight. In concentrations of TEA as low as 15 m-eq. the tibia showed complete and prolonged flexion as a result of a single nerve stimulus. The vigorous and prolonged nature of the contraction in TEA salines appeared to be associated with tetanic trains of action potentials following each applied single stimulus. It is not known whether this was due to an effect on the nerve, or NM junction, or both.

When tetramethylammonium (TMA) ions were substituted

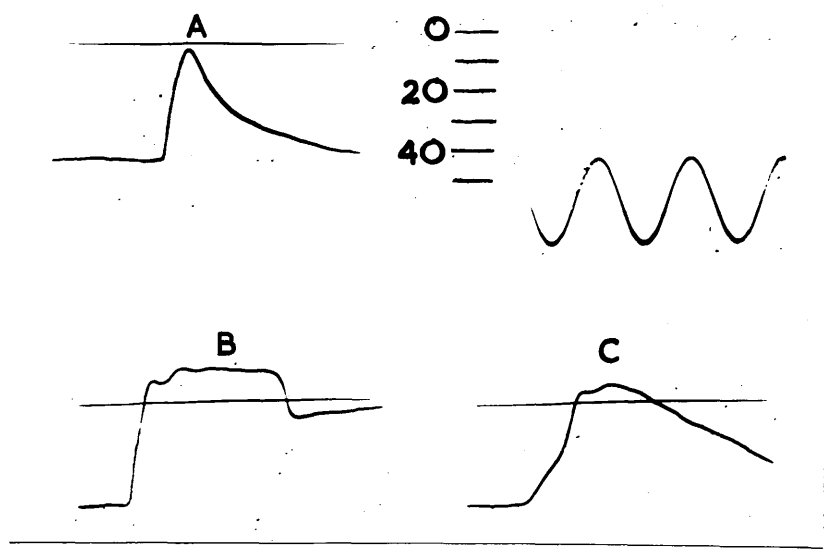


Fig. 27. Effect of TEA ions on F response. A, in normal saline. B, C, in saline containing 18.75 TEA per litre. In B, the electrode was jerked from the fibre by the force of the contraction. The type of response shown in C was observed only occasionally. Calibration: mV and 50 c/ s.

for sodium ions, the values of the resting and action potentials were very similar to those obtained in the equivalent sodium salines (Fig. 28). However, although TMA ions proved a suitable substitute for sodium in this respect, their use resulted in a marked decrease in the rate of rise of both the junctional potential and the active membrane response by comparison with sodium ions (Figs. 29 and 30). This effect could be reversed by bathing in normal saline containing sodium.

The effect of potassium ions.

Potassium ions exerted a depolarising action upon the muscle fibre membrane similar to that observed in other excitable tissues (Hodgkin, 1951; Fatt and Katz, 1953a; Hoyle, 1953b). The largest resting potential occurred in zero potassium: as the external potassium concentration was raised, the magnitude of the resting potential fell (Fig. 31).

The action potential was also affected by alterations in the potassium level. It declined in magnitude as the potassium concentration was raised (Fig. 32). In some records it was possible to distinguish between the junctional potential (j.p.) and the active membrane response (a.m.r.): one example can be seen in Fig. 9D. In this case the action potential fell, after 30 minutes

TABLE 4. Magnitudes of resting and action potentials in different concentrations of sodium and tetramethylammonium (TMA) ions.

M.equiv. per litre	Number of samples	Mean resting potential in millivolts ± S.E.	Mean action potential in millivolts ± S.E.
Sodium			
0	12	36.5 ± 1.6	36.5 ± 1.8
15	11	40.5 ± 0.93	44.5 ± 1.4
50	9	41.5 ± 0.70	45.0 ± 1.6
100	10	40.5 ± 1.2	48.5 ± 0.98
150	10	43.0 ± 1.1	49.5 ± 2.2
TMA			
25	12	42.5 ± 0.76	43.0 ± 1.2
50	9	43.0 ± 0.90	46.5 ± 2.3
100	10	43.5 ± 1.2	47.5 ± 2.0
150	12	44.5 ± 0.60	47.5 ± 1.3

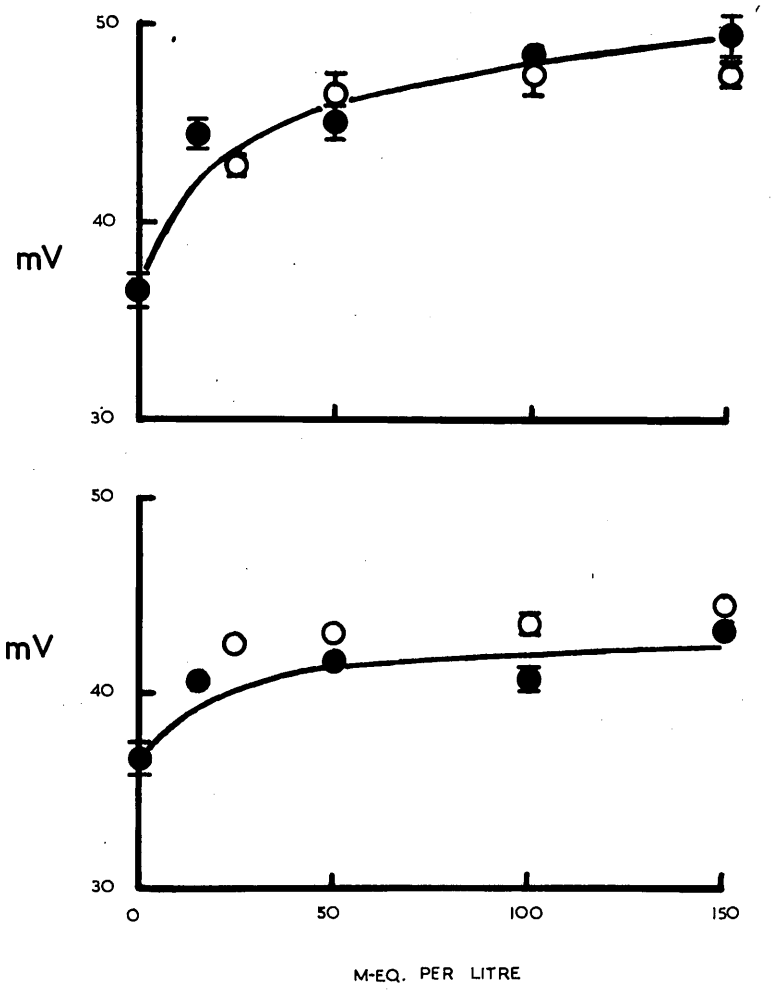


Fig. 28. Relation between size of resting potential (lower graph) action potential (upper graph) and external sodium (full circles) and tetramethylammonium (hollow circles) concentrations.

Table 5. Rate of rise of junctional potential and active membrane response in different concentrations of sodium and tetramethylammonium ions.

M.equiv. per litre	Number of samples	Mean rate of rise of junctional potential in volts/sec. ± S.E.	Mean rate of rise of active membrane response in volts/sec. ± S.E.
Sodium			
0	12	11.5 ± 0.49	6.8 ± 0.53
15	11	13.1 ± 1.2	7.8 ± 0.45
50	9	14.1 ± 1.8	7.8 ± 0.47
100	10	17.3 ± 1.6	10.8 ± 1.3
150	10	18.0 ± 1.6	14.4 ± 1.8
TMA			
25	12	11.5 ± 1.1	7.6 ± 0.64
50	9	12.7 ± 1.49	9.0 ± 0.99
100	10	10.8 ± 0.83	6.7 ± 0.45
150	12	7.1 ± 0.90	5.7 ± 0.57

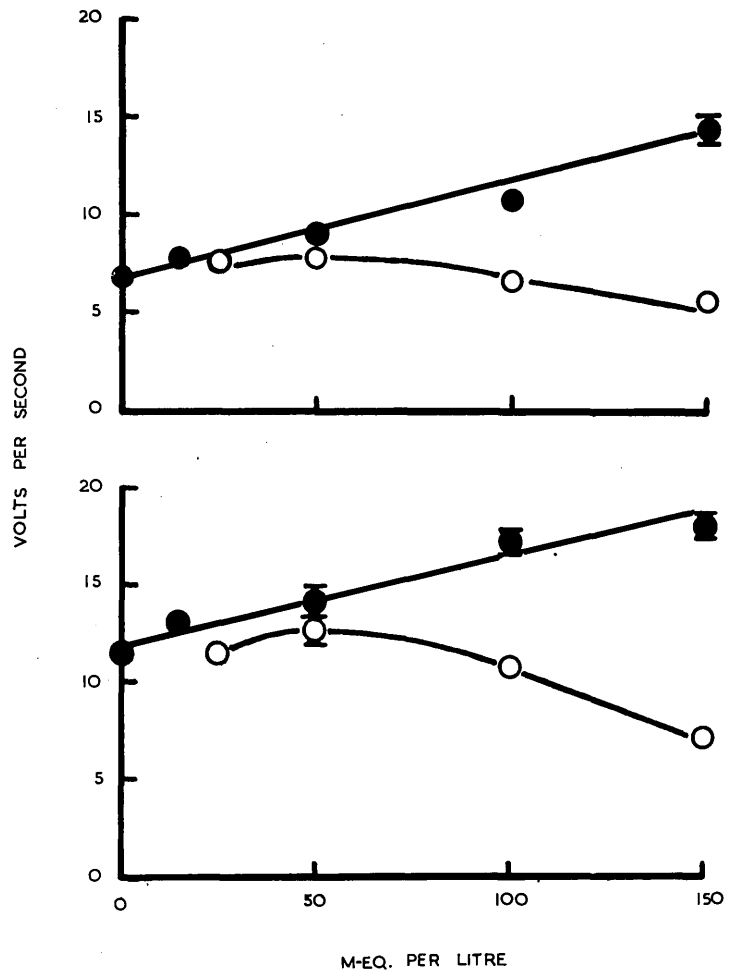


Fig. 29. Relation between rate of rise of junctional potential (lower graph), active membrane response (upper graph) and external sodium (full circles) and tetramethylammonium (hollow circles) concentrations.

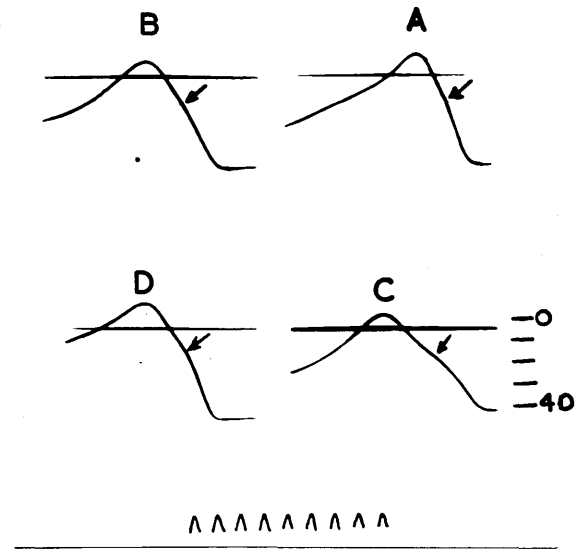


Fig. 30. Effect of TMA ions. A, normal saline, B, 15 TMA; C, 100 TMA; D, zero Na. Calibration mV and 50 c/s.

Table 6. Magnitude of resting potential in different potassium concentrations.

M.equiv. potassium per litre	Number of samples	Mean resting potential in millivolts ± S.E.
0	10	44.4 ± 0.84
10	12	41.9 ± 0.78
18	15	38.0 ± 0.75
25	8	32.1 ± 0.63
50	11	22.5 ± 0.72
100	12	16.1 ± 0.56
150	10	10.6 ± 0.58
200	9	5.6 ± 0.47

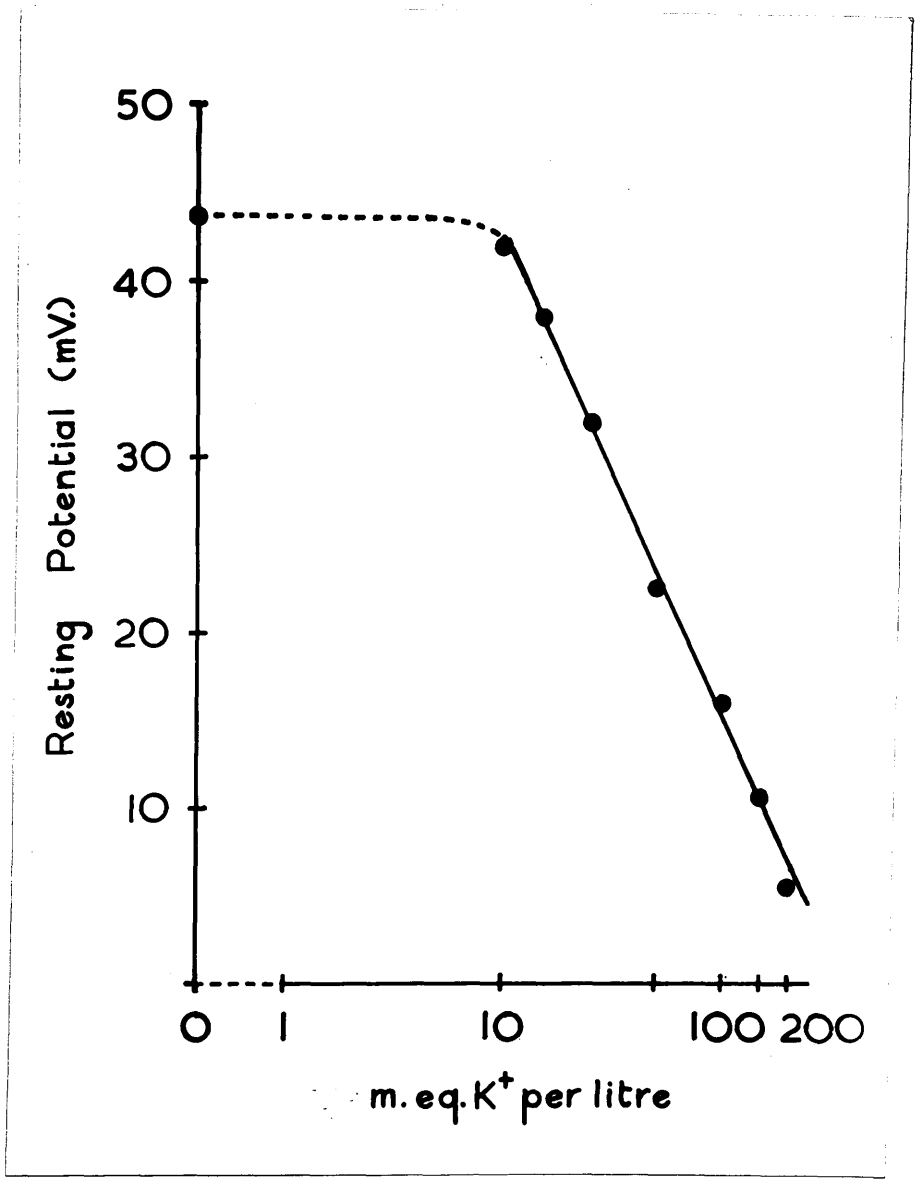


Fig. 31. Relation between size of resting potential and external potassium concentration (logarithmic scale)
(Standard replacement time).

in 100 m-eq. potassium, to about 20 mV., compared with a mean value of 44 $\pm$ 1.2 mV. from fibres of the same muscle in zero potassium saline. The j.p. accounts for about 13 mV. and the a.m.r. for about 7 mV. of the reduced action potential. This and other records are in agreement with the findings of Hoyle (1955a) in locust and cockroach muscle fibres. He found that in raised potassium the a.m.r. showed a progressive decline which was correlated with a decline in the j.p. In fibres of Carausius which show an overshoot in normal saline, the overshoot persists when the potassium concentration is raised up to about 100 m-eq., though above this figure it tends to disappear.

A replacement time of 30 minutes is not sufficient for the complete development of the effect of a change in potassium concentration in superficial fibres. The decline in size of the resting and action potentials in raised potassium begins almost immediately the saline has been changed and is rapid initially, but becomes slower, and the final equilibrium levels may not be reached for an hour or more. Thus, with a longer replacement time, the curve relating resting potential to potassium concentration (Fig. 31) slopes more steeply to cross the baseline at about 150 m-eq. potassium,at

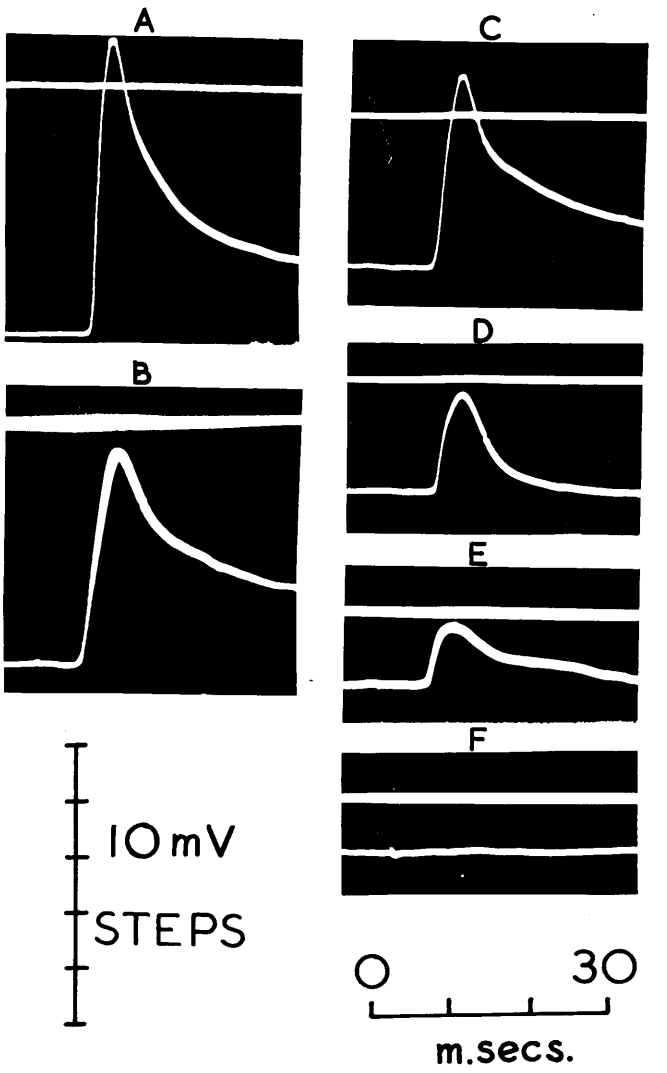


Fig. 32. Samples from a muscle at different potassium concentrations. A, zero; B, 18; C, 50; D, 100; E, 150; F, 200 (m.equiv. per litre). Note the overshoot in C, and the presence of both j.p. and a.m.r. in D. Calibration: m.secs. and mV.

which concentration, if the muscle is allowed to soak in it for a sufficient period, the action potential is abolished. If it is soaked in the saline long enough for all the fibres of the muscle to be affected in this way, the muscle fails to contract in response to either single or repetitive stimulation of its nerve.

The effect of calcium ions.

A rise in the external calcium concentration is accompanied by an increase in the magnitude of the resting and action potentials (Fig. 33). The increase in size of the resting potential from zero to 20 m-eq. calcium was about 5 mV: that of the action potential was much greater, amounting to about 36 mV. The change in size of the action potential was greatest between zero and 10 m-eq. calcium, the part of the curve between these values being approximately linear. Above about 10 m-eq. an increase in calcium concentration has a progressively smaller effect upon the action potential, and it will be seen from Fig. 10 that the slope of the curve relating to the action potential is more comparable to that of the resting potential. In a few cases, the muscle was bathed in 50 m-eq. calcium, but this resulted in an increase in the action potential of only a few millivolts,

Table 7. Magnitudes of resting and action potentials in different calcium concentrations.

M. equiv. calcium per litre	Number of samples	Mean resting potential in millivolts ± S.E.	Mean action potential in millivolts ± S.E.
0	12	35.0 ± 0.65	11.5 ± 0.94
2.5	12	35.5 ± 0.74	18.0 ± 0.75
5.0	8	38.5 ± 0.56	33.0 ± 0.68
6.25	12	35.5 ± 1.0	33.0 ± 0.80
7.5	8	37.5 ± 0.92	37.0 ± 0.72
10	12	38.0 ± 0.72	40.5 ± 0.75
15	9	40.5 ± 0.64	44.5 ± 0.76
20	10	40.0 ± 0.95	46.0 ± 1.5

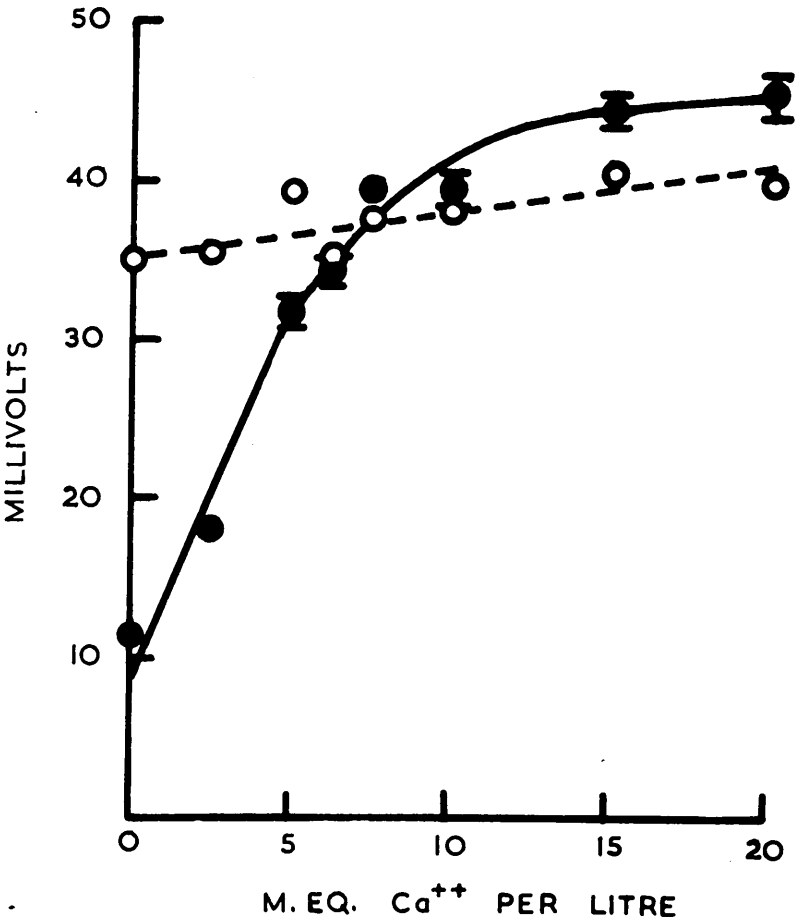


Fig. 33. Relation between size of resting potential (hollow circles) and action potential (full circles) at different calcium concentrations.

indicating that the curve is almost horizontal at concentrations above 20 m-eq. On one occasion, bathing in 50 m-eq. calcium resulted in the appearance of "miniature potentials" about 0.4 mV. in height. Miniature end-plate potentials were observed originally in frog muscle (Fatt and Katz, 1952b) and the only insects in which their occurrence has so far been reported are some of the Acrididae examined by Hagiwara (1953), in which the muscle fibres had been injured. This was the only occasion on which they were observed in Carausius. If miniature potentials are present in healthy fibres, they must produce depolarisations which are below noise level. These might summate abnormally to larger units.

When the muscle was transferred from normal saline to zero calcium, there was a rapid alteration in the shape of the action potential (Fig. 34). The a.m.r. came off the j.p. progressively later, and the time course of the whole response became slower. As the lack of calcium took full effect, both components declined in size, but the decline was more rapid in the case of the a.m.r., and it disappeared while the j.p. was still quite large. The j.p. itself did not fall below about 8 mV. by the end of the standard replacement time of 30 minutes.

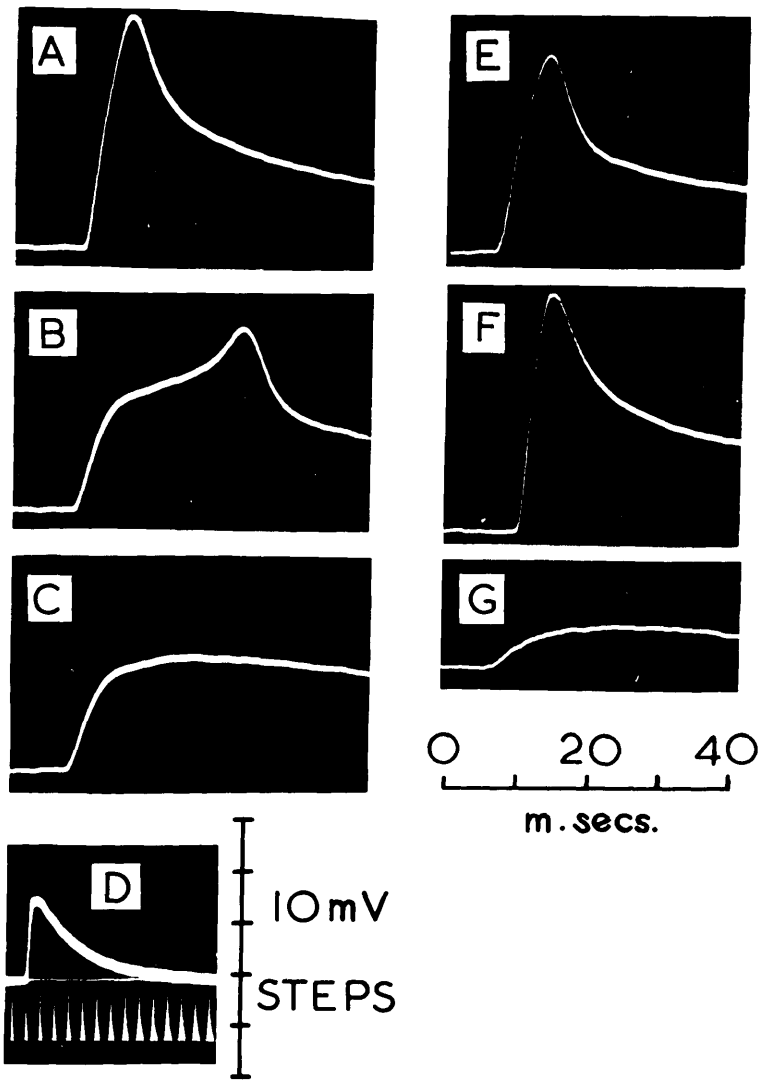


Fig. 34. The effect of calcium. A-D records from the same fibre. A, in normal saline. B, 3 mins. after transference to zero calcium. C, after a further 3 mins. D, a record taken a few minutes after F, on a faster time base to show time-course.
E-G, records from another fibre. E, in normal saline; F, in 20 Ca G, in 0 Ca (after 30 mins.).
Calibration: 50 c/s in D.
Otherwise m.secs. and mV.

The separation of the j.p. from the a.m.r. in reduced calcium allowed the magnitudes and rates of rise of the two components to be examined separately. As in the case of the results obtained in sodium salines described earlier, the bend in the rising phase of the action potential was taken as the junction between the two. To investigate the validity of this criterion, the muscle was transferred from normal to zero calcium saline, and when the supposed a.m.r. had just disappeared a pair of suitably spaced stimuli were applied to the crural nerve. Each stimulus of the pair produced a separate response of the muscle fibre membrane, and the two responses tended to summate, when they were followed by a further membrane response, similar in size and shape to the supposed a.m.r. which had previously disappeared (Fig. 35). The two responses were not followed by a further response when the paired stimuli were sufficiently far apart to prevent summation; nor when further soaking in zero calcium had reduced the size of the two summating responses below a certain level. The summating responses must apparently reach a certain critical height if they are to give rise to the further membrane response. It is evident from these results that the hump in the rising phase seen in reduced calcium

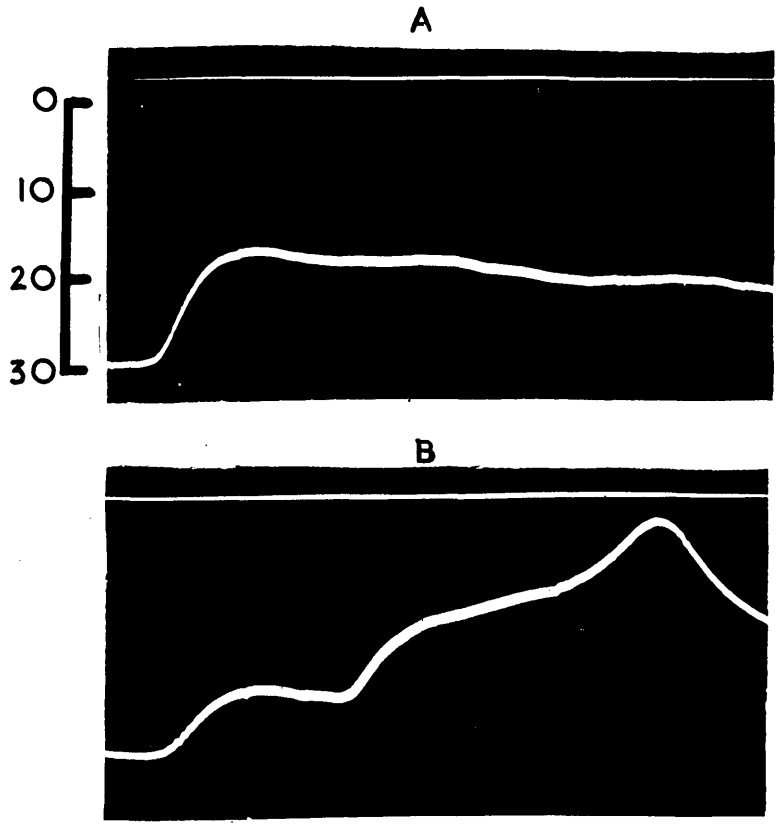


Fig. 35. A, in zero Ca, junctional potential only. B, same fibre a few minutes later: paired stimuli elicited an active membrane response. Calibration: mV.

marks the junction of two distinct components, and it is assumed that the similar, but slighter, hump noted in other salines, is comparable. It appears reasonable to suppose, by analogy with the muscle fibres of other animals, that the two components involved are the end-plate or junctional potential and the active membrane response.

Fig. 36 shows curves which relate the magnitudes of the two components to the calcium concentration. Between about 5 and 10 m.equiv. calcium the relationship between the calcium concentration and the a.m.r. is substantially linear. Above about 10 m.equiv. the curve tends to become horizontal. An a.m.r. is not present below 4 - 5 m. equiv. calcium: above this level it shows only about 3 mV. variation up to 20 m.equiv calcium. A similar degree of variation occurs in the resting potential in the same calcium concentrations (Fig. 33). It is possible that the size of the a.m.r. in different calcium concentrations is related to the value of the resting potential. In any case, the variation involved is very small, and it appears that, although there is a critical level of j.p. below which an a.m.r. is not generated, an increase in the size of the j.p. above this level makes little difference to the size of the a.m.r., when the

Table 8. Magnitudes of junctional potential and active membrane response in different calcium concentrations.

M. equiv. calcium per litre	Number of samples	Mean junctional potential in millivolts ± S.E.	Mean active membrane response in millivolts ± S.E.
0	12	11.6 ± 0.94	-
2.5	12	18.0 ± 0.75	-
5.0	8	21.5 ± 0.70	11.3 ± 0.65
6.25	12	21.8 ± 0.80	11.3 ± 1.0
7.5	8	25.7 ± 0.71	11.5 ± 0.72
10	12	28.0 ± 0.96	12.3 ± 0.50
15	9	30.5 ± 0.68	14.5 ± 0.92

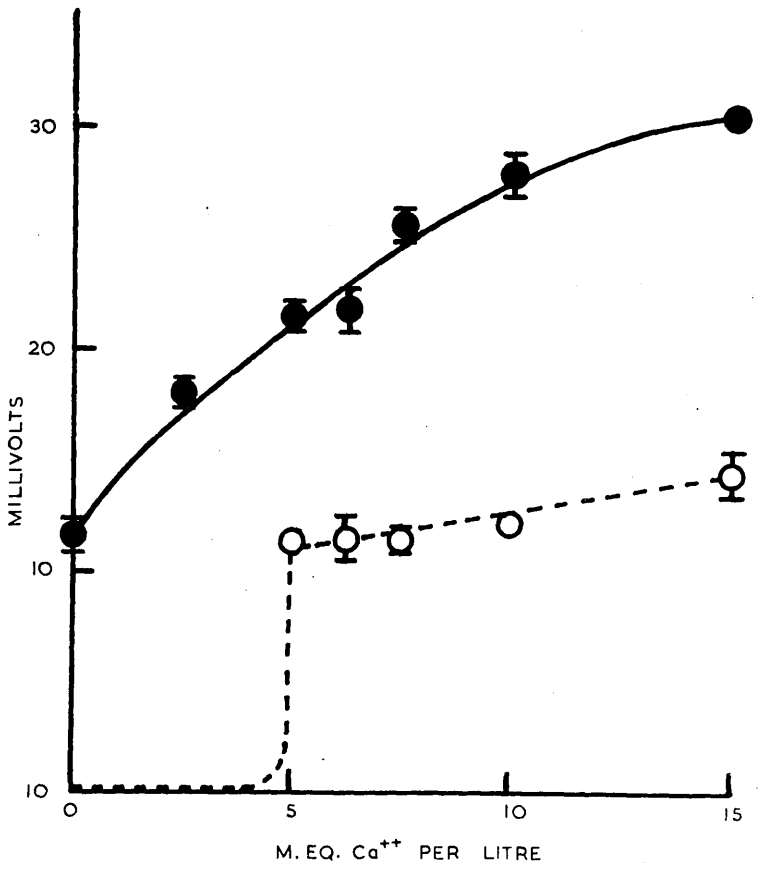


Fig. 36. Relation between size of junctional potential (full circles), active membrane response (hollow circles) and external calcium concentration.

calcium concentration is varied.

A concentration of at least 4 - 5 m.equiv. calcium is necessary for the development of the a.m.r. (Fig. 36). In some cases, the level at which an a.m.r. appeared was not sharply defined. In muscles transferred from normal saline to zero calcium, a small a.m.r. was sometimes seen just before the total disappearance of the a.m.r. (Fig. 34C).

The rates of rise of the j.p. and a.m.r. are affected by alterations in the calcium concentration (Fig. 37). Between zero and 15 m.equiv. calcium the rate of rise of the j.p. is directly proportional to the external calcium concentration. After an initially steep rise the curve for the a.m.r. continues upwards at a slower rate than that for the j.p. The rate of rise of the a.m.r. increases by about 5 V/sec. when the calcium is raised from 5 to 15 m.equiv. As stated, the corresponding increase in size of the a.m.r. is only 3 mV.

The rate of rise and the magnitude of the j.p. are both affected by changes in the calcium concentration (Figs. 36 and 37). The sensitivity of the junctional potential to changes in the calcium level suggests that in Carausius, as in the locust and cockroach (Hoyle, 1955a), calcium exerts an effect upon the release of

Table 9. Relationship between calcium concentration and rate of rise of junctional potential and active membrane response.

M.equiv calcium per litre	Number of samples	Mean rate of rise of junctional potential in volts/sec. ± S.E.	Mean rate of rise, active membrane response in volts/sec. ± S.E.
0	12	0.97 ± 0.05	-
2.5	12	2.4 ± 0.01	-
5.0	8	3.9 ± 0.16	1.6 ± 0.26
6.25	12	4.8 ± 0.22	2.5 ± 0.36
7.5	8	7.0 ± 0.43	3.3 ± 0.18
10	12	7.8 ± 1.2	4.2 ± 0.43
15	9	10.5 ± 1.0	6.9 ± 2.23

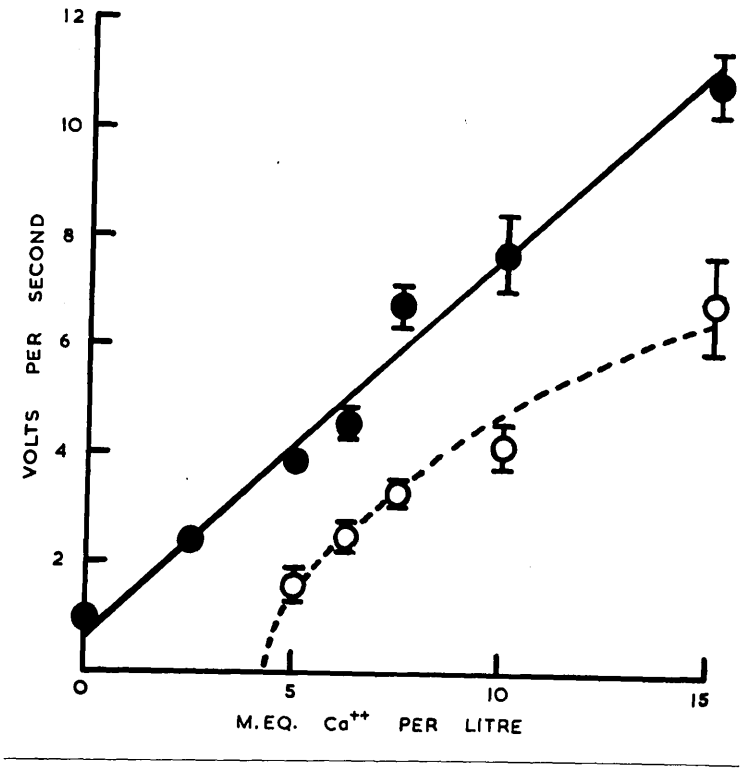


Fig. 37. Relation between rate of rise of junctional potential (full circles) and active membrane response (hollow circles).

some neuromuscular transmitter, similar to that seen in vertebrates (del Castillo and Stark, 1952); Hoyle, (1955a) pointed out that, nevertheless, calcium does not alter the time course of the vertebrate end-plate potential. He found that in the locust and cockroach, as in Carausius, the time course of the junctional potential became slower in low calcium.

The effect of magnesium ions.

The relationship between the magnitudes of the resting and action potentials and the external magnesium concentration is shown in Fig. 38. The resting potential does not alter significantly in size between zero and 400 m.equiv. magnesium. The action potential, however, decreases in size above and below a magnesium level of about 100 to 150 m. equiv. In the locust and cockroach (Hoyle, 1955a) and in vertebrates (del Castillo and Engbaek, 1954) magnesium inhibits the release of the neuromuscular transmitter, and a decrease in magnesium concentration is accompanied by an increase in the height of the end-plate or junctional potential. In Carausius it appears to be the action potential as a whole which declines, as in high potassium.

Above about 150 m.equiv. magnesium, the action

Table 10. Magnitude of resting and action potentials in various magnesium concentrations.

M. equiv. magnesium per litre	Number of samples	Mean resting potential in millivolts ± S.E.	Mean action potential in millivolts ± S.E.
0	10	41.2 ± 1.0	20.6 ± 1.76
50	10	42.4 ± 1.3	34.3 ± 1.3
100	14	41.9 ± 1.0	42.8 ± 0.61
225	10	41.0 ± 0.98	35.6 ± 0.9
300	8	40.8 ± 1.01	18.3 ± 1.36
400	12	41.2 ± 0.7	-

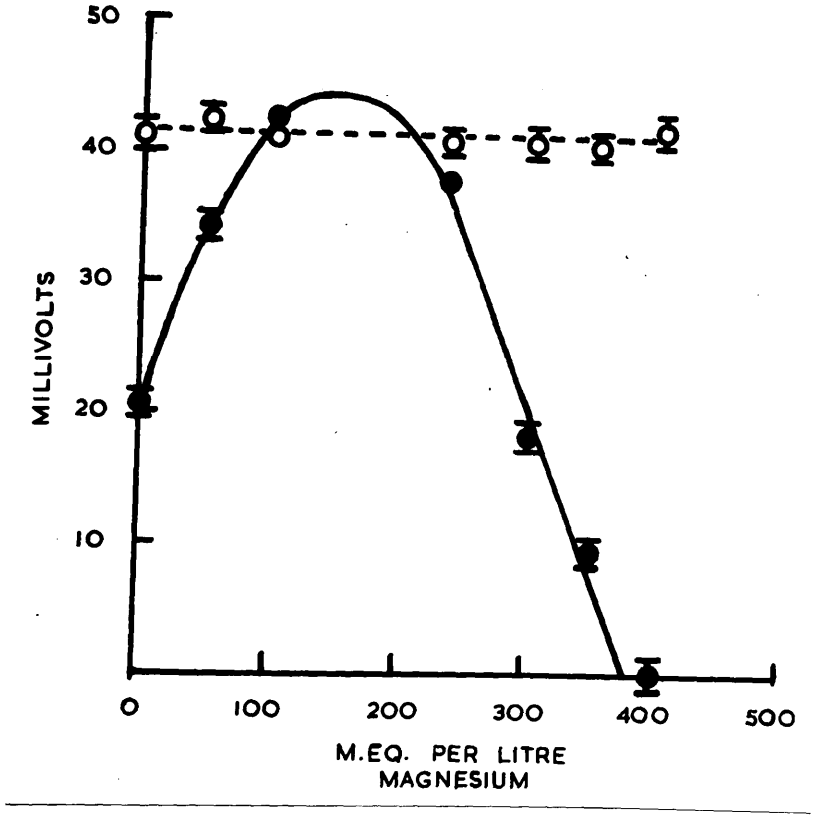


Fig. 38. Relation between size of resting potential (hollow circles), action potential (full circles) and external magnesium concentration.

potential falls in size as the magnesium level is raised. The change in the action potential is similar in some respects to the neuromuscular block which occurs in other muscle fibres subjected to magnesium concentrations several times greater than normal (del Castillo and Engbaek, 1954; Hoyle, 1955a). As the magnesium level is raised, the decline in the action potential increases, and the a.m.r. eventually disappears, the decrease in size of the j.p. being more gradual (Fig. 39). The general effect is therefore similar to that seen in low calcium; and in the locust and cockroach (Hoyle, 1955a) and in vertebrates (del Castillo and Engbaek, 1954) calcium antagonizes the neuromuscular block produced by magnesium. However, the changes produced in these animals by high magnesium are not entirely comparable to those which occur in Carausius, where the a.m.r. and j.p. did not become separated in time, as they did in low calcium; and the effect of high magnesium was not antagonized by calcium ions. The addition to the 400 m.equiv. magnesium saline of sufficient calcium to restore the calcium/magnesium ratio to that of the normal saline neither abolished nor arrested the progressive decline of the action potential. The hypertonicity of the 400 m.equiv. magnesium saline would not be expected

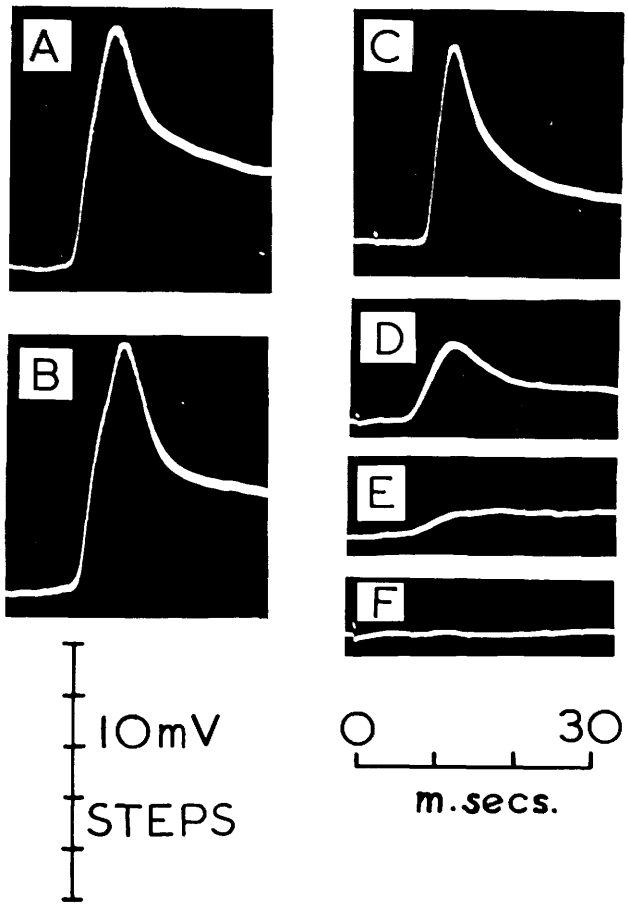


Fig. 39. Effect of low and high magnesium on action potential.
A - C, records from the same fibre. A, in zero Mg; B in 50 Mg; C in 100 Mg.
D - F records from another fibre. D, in 100 Mg; E - F progressive deterioration in 400 Mg; records at 5 minute intervals after transference to this saline.
Calibration: mV and m-secs.

to cause a decline of this magnitude, if at all.

In all cases where the action potential was reduced in size, the twitch tension of the muscle fell. When the muscle was soaked in 400 m.equiv. magnesium long enough for all the muscle fibres to be affected, the twitch tension fell to zero, and no contraction could be elicited either by single or repetitive stimulation. The effect could be reversed by prolonged washing in normal saline.

The action of hyaluronidase.

In order to test the possibility that some kind of barrier surrounds the muscle fibres in Carausius, experiments were carried out in which "hyalase" was added to certain salines. It was hoped that any connective tissue component of such a barrier would be attacked by the enzyme, resulting in a significant alteration in the action of the salines. The hyalase was added to the normal saline at a strength of 1,000 units per 100 ml. and the muscle was soaked in this saline for 3 hours. It was then transferred to zero or 200 m.equiv. sodium salines, also containing hyalase. No change was observed in the electrical response of the muscle fibres as a result of soaking in any of these salines.

DISCUSSION.

As stated, the nerves of the locust (Hoyle, 1953b) and possibly of all insects (Hughes, 1953) are surrounded by a sheath which probably acts as a selective barrier and which provides a more favourable ionic environment for the nerve than the haemolymph. Several of the facts reported here might be considered to indicate the presence of a similar kind of selective barrier around the muscle fibres of Carausius. These are

(i) the relatively small effect of sodium ions upon the size of the action potential;

(ii) the high magnesium concentrations required to produce neuromuscular block;

(iii) the rather long replacement times necessary for changes in the external ionic concentration to exert their full action upon the muscle fibres; and

(iv) the considerable depression of neuromuscular transmission which takes place in hypotonic salines.

The latter could be due to the osmotic passage of water through the barrier, the absence of a compensating passage of ions resulting in dilution of the fluid contained between the barrier and the muscle fibre.

There is much evidence against the possibility that any barrier in Carausius is actively selective. The absence of any change in the action of salines after the application of hyaluronidase is interesting but not at all conclusive. However, there are also a number of facts which suggest that the various ions are, in fact, reaching the muscle fibres and acting directly upon them, but that in some cases their action upon the fibres is different from that expected from a comparison with the muscle fibres of other animals.

Thus, a barrier of the kind under discussion might be expected largely to exclude magnesium and possibly potassium ions, but to concentrate sodium ions around the muscle fibres. In this case, it seems probable that after transference to a low magnesium saline the barrier would either tend, at least for a time, to withdraw even more magnesium from the fluid surrounding the fibre; or, if slow acting, maintain the magnesium level at its normal value for the duration of the experiment. From results obtained with the muscle fibres of other animals (del Castillo and Engbaek, 1954; Hoyle, 1955a) this should result in an enhancement of neuromuscular transmission or in no effect at all. Even if the barrier adjusted so rapidly that its action was

missed, a depression of neuromuscular transmission in low magnesium salines would not be expected. The fact that such a depression is observed in Carausius suggests that the reduced magnesium concentration is acting directly upon the muscle fibres, but the effect is a different one from that seen in other animals so far studied.

The long replacement times are adequately explained by the presence of tracheolated connective tissue around the groups of muscle fibres, and around individual fibres. Hoyle (1953b) found that the tracheolated membrane around locust muscle acted as a partial diffusion barrier to potassium ions. A comparable tracheolated membrane is present in Carausius, and would be expected to exert a similar delaying effect.

The size of the action potential in different sodium salines varies less than might have been expected in view of the results obtained from vertebrate muscles (Fatt and Katz, 1952a). However, similar variations are observed when sodium ions are replaced by TMA ions. It is therefore possible that any "carrier" mechanism which may be involved in the inward current of the action potential (Hodgkin, 1951) is not specific for sodium ions.

Sodium and TMA ions also influence the rate of rise

of the action potential, but here their actions are different. Increased sodium concentrations result in an increased rate of rise, whereas TMA salines have the reverse effect. If sodium ions are not, in fact, specific carriers of the inward current, it could be argued that their major effect is upon the way in which charge is transported across the membrane. If TMA ions could not exert a similar influence, the reason for the different rates of rise in the two salines is explicable. It is necessary to remember, however, that the reason might equally lie in the relative ability of a "carrier" to deal with the two different ions.

It is thus evident that a selective barrier is not the only possible explanation of the alterations in the action potential in sodium and TMA salines. It is interesting to note that Fatt and Katz (1953a) replaced sodium ions by quaternary ammonium ions in the fluids bathing the muscle fibres of certain crustaceans, and found that they continued to function as well as, or better than, in comparable sodium salines. They suggested that the results they obtained with TMA salines could be explained on the assumption that the action potential in these animals involves an influx of calcium or magnesium ions, or an outflux of some internal anion;

and that sodium plays only an indirect role during the production of the action potential, "conditioning" the excitatory reaction of the membrane without being a carrier of the current. A similar explanation could be applied to Carausius.

It must be admitted that such an explanation does not account for the fact that impulses passing down the motor nerve were able to excite the muscle fibres in zero sodium. Histologically, the visible sheath which is regarded as the ion barrier protecting the nerve from adverse environmental conditions (Hoyle, 1953; Roeder and Twarog, 1956) appears to end at the point where the motor nerve ending branches out into the terminal claw, or end-plate. The end-plate is therefore exposed to solutions bathing the muscle fibres. If the observed facts were due merely to the presence of a passive barrier then a steady but slow decline in both nerve and muscle response would be expected with prolonged soaking, since some effect is observable within a few minutes of changing the experimental saline. In this case, soaking in zero sodium extended over three hours, and no change occurred in the muscle response after the expiry of the experimental time of 30 min.; and the nerve was still conducting after 3 hours. If, on the other hand, a selective barrier is

operating, it must be operating only for sodium ions, since calcium and potassium act in a comparable way in Carausius and in vertebrates; and a selective barrier in respect to magnesium ions has already been rejected.

On balance, therefore, the presence of motor nerve activity in zero sodium is best left as a fact which at present cannot be explained.

The question arises whether calcium or magnesium, or both, contribute to the inward current involved in the production of the action potential. While it seems highly probable that calcium ions affect the size and rate of rise of the junctional potential, through their influence upon the release of a neuromuscular transmitter, the fact that the active membrane response varies only 3 mV between .5 and 20 m.eq. calcium, suggests that any contribution of this kind by calcium ions is negligible.

If magnesium contributes to the inward current, an explanation of the decline in size of the action potential at low magnesium concentrations becomes apparent. The tolerance of the muscle fibres of Carausius to high magnesium concentrations is so striking, however, by contrast with other muscle fibres (del Castillo and Engbaek, 1954; Hoyle, 1955a) that it is possible that the decline in low concentrations is bound up with the

mechanism involved in this tolerance. There is no evidence to show what this mechanism may be.

The suggestion of Fatt and Katz (1953a) that in certain crustacean muscle fibres an outward flux of some internal anion might be responsible for the action current must also be considered in regard to Carausius. Direct evidence is lacking, but it is just possible that hypotonic salines depress the muscle response by producing an osmotic inflow of water into the fibre which results in the dilution of such an anion within the muscle fibre. Although both the resting and action potentials are altered in size by hypotonic salines, the reduction in the action potential is greater than the reduction in the resting potential.

There is nothing to show how sodium and TMA ions alter the magnitude of the resting potential. It might be supposed that changes in the resting potential when the concentrations of these ions are altered merely reflect the accompanying change in chloride concentration. However, there is no evidence that the muscle fibre membranes of insects are freely permeable to chloride ions. In Carausius, variation in the magnesium chloride content of the bathing fluid over a wide range of concentrations had no effect upon the magnitude of the resting potential.

The effect of calcium upon the resting potential may be connected with the shift it produces in the curves relating sodium permeability to membrane potential (Frankenhauser and Hodgkin, 1955) in the squid giant axon.

The action of potassium ions upon the resting potential appears to be similar to that observed in other muscle fibres. The time necessary for replacement of these ions may be due to tracheolated tissue surrounding the muscle fibre membrane; or to a slight impermeability to potassium ions. However, replacement does eventually occur, and the normal resting potential is rather low compared with, for example, that found in the locust (Hoyle, 1953b) but not so low that the functioning of the muscle fibres is seriously impared. Hoyle (1954) suggested that high blood potassium might be a factor tending to produce sluggish movement in herbivorous insects. The blood potassium level in Carausius is not as high as it is in many other herbivorous insects (Duchateau et al., 1953). It may be that in insects with a very high blood potassium level mechanisms may be discovered which counteract the apparently harmful effects of potassium, such as a protective sheath surrounding the muscle fibres. Nevertheless, the resting potential in Carausius has been shown to be low compared with that in

many other insects; yet the mechanical performance of the muscle is strikingly high. It is at least arguable that in insects with high blood potassium concentrations the contractile mechanism may be adapted in such a way that a large contraction is evoked by a relatively small depolarisation of the muscle fibre membrane. In our present state of knowledge this is a possibility that cannot be ruled out. In the author's opinion it is a matter of common observation that many herbivorous insects which are stated to have a high blood potassium level and a low or non-existent blood sodium level, such as the honey-bee or many Lepidoptera (Duchateau et al., 1953), perform considerable muscular activity of a kind which cannot be called "sluggish". Some kind of adaptive mechanism will very probably be found in such insects.

The results presented in Sections I and II of this thesis tend to support the view of Hoyle (1955a) that neuromuscular transmission in insects proceeds in a way which is essentially similar to the corresponding vertebrate process. This conclusion rests particularly upon the presence of two components in the action potential, the junctional potential having an exponential decay and giving rise to an active response when it reaches a critical level; and the probability that calcium ions

enhance the action or the release of a neuromuscular transmitter. There are nevertheless several anomalies. One is the apparent decline of the active response with the junctional potential in high potassium concentrations. This occurs in such a way that an a.m.r. is present when the j.p. has declined below a height which is normally critical in other salines for the production of an active response. It may be significant that potassium ions affect the resting potential level. The active response seems to be closely linked with the resting potential level. It could be speculated that the j.p. must reach a certain level relative to zero potential in order to fire off an a.m.r. rather than a particular magnitude. This is a possibility which deserves attention in the future.

The other anomaly is seen in the results obtained with magnesium ions. These do not appear to act in the normal way except at very high concentrations. The fact that magnesium ions do not antagonize the action of calcium ions suggests that the decline of the action potential of Carausius in high magnesium concentrations is due to a different effect from that which magnesium ions are known to produce in vertebrates and the locust. Certainly, it is hard to harmonize the tolerance to

magnesium ions observed in Carausius with the depressive effects exerted by those ions in other animals. It would be interesting to speculate on the changes in neuromuscular transmission involved in the development of this tolerance: but this aspect must await further evidence.

SUMMARY.

1. The anatomy and innervation of the coxal and femoral muscles of the pro- and metathoracic legs have been described. The pattern of nerves and muscles is similar in both legs.

2. Histological examination has shown that the motor nerve endings on the flexor tibialis muscle fibres of the prothoracic leg are of the "Doyère-cone" type, and are spaced at intervals of approximately 60 μ along the length of the fibres.

3. The electrical responses of the fibres of the flexor tibialis muscle have been examined with the aid of glass capillary intracellular microelectrodes. Records have also been made of some of the mechanical responses of this muscle.

4. Two types of electrical responses are present: "fast", non-facilitating responses resembling the action potentials of vertebrate muscles, and associated with quick twitch-type contractions of the muscle fibres; and "slow", readily facilitating responses resembling small end-plate potentials, associated with slow smooth movements and maintenance of the tonus of the muscle.

5. The two responses are produced by two separate types of motor axons innervating the muscle.

6. The muscle is capable of developing a tetanus tension of 800 g./cm^2./unit cross-sectional area of individual fibres. The tetanus/twitch ratio is high.

7. Pharmacological substances which alter vertebrate neuromuscular transmission do not affect the fast response.

8. Progressively lowered temperatures lengthen the time course and to a slight extent the size of the "fast" response, and an inflexion appears in the rising phase which was taken to indicate that the "fast" response consists of two components, a junctional potential and an active membrane response.

9. Refractoriness occurs in the decay phase of the "fast" response. A pair of stimuli sufficiently close together will produce effects which summate to give an enhanced junctional potential. A similar effect was not observed in the case of the active membrane response.

10. Analyses of its blood ionic composition have confirmed that Carausius is a typical herbivorous

insect, with blood sodium/potassium and calcium/magnesium ratios of less than unity.

11. The effects of changing the sodium, potassium, calcium and magnesium concentrations of the bathing fluid on the "fast" response have been studied.

12. Lowering the sodium concentration results in a decrease in the magnitudes of the resting and action potentials, but an active membrane response is present in zero sodium. The rate of rise of the action potential increases as the sodium concentration is raised.

13. When tetramethylammonium ions are substituted for sodium ions in the bathing fluid, variations in the magnitudes of the resting and action potentials similar to those seen in the corresponding sodium salines are observed; but the rate of rise decreases as the TMA level is raised.

14. It is suggested that sodium ions are not essential for the production of an active membrane response, but that they may affect the excitability of the muscle fibre membrane.

15. Calcium ions are necessary for the development of the junctional potential. It is suggested that they influence the liberation of a neuromuscular transmitter substance. They may also affect the sodium permeability of the membrane.

16. When the magnesium concentration is raised above about 150 m.equiv. per litre, the action potential declines in size, and neuromuscular block eventually results. This effect is not antagonized by calcium ions.

17. The action potential also declines in size when the magnesium concentration is lowered below about 100 m.equiv. per litre. The resting potential is not affected by magnesium ions.

18. The resting potential is directly proportional to the log. of the potassium concentration except at low concentrations.

19. These results are discussed in the light of similar work on vertebrates and other arthropods. In particular, it is considered that the fast response is produced by a process similar to vertebrate and locust neuromuscular transmission, but that the

sodium and magnesium results represent adaptations to a herbivorous habitat; and the possibility that a selective ion barrier is present around the muscle fibres of Carausius is considered and rejected, with reservations in the case of sodium ions.

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