Studies on the neuromuscular anatomy and physiology of certain Lepidoptera.

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In the introduction to lepidopteran neuromuscular anatomy I have unfortunately omitted a reference to the work of Nüesch* on Telea polyphemus. This author examined the thoracic anatomy and described three separate ganglia, although the meso- + metathoracic ganglia are practically continuous and almost completely fused. In this thesis, such a large combined ganglion has been called the metathoracic ganglion as there tendency in the group is towards fusion to two ganglia. This follows the terminology of Heywood (1965) in Pieris brassicae

Henry Pendrant

Nüesch, H. (1957). Die Morphologie des Thorax von Telea polyphemus (V. (Lepid.) II. Nervensystem. Zool. Jb (Anat.) 75, 615-642.

STUDIES ON THE NEUROMUSCULAR ANATOMY AND PHYSIOLOGY OF CERTAIN LEPIDOPTERA.

by

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CONTENTS

GENERAL INTRODUCTION

Most of the literature in the field of neuromuscular transmission pertains to work upon the vertebrates and crustacea. However, the assumption that interpreations obtained from vertebrates or even the crustacea can be directly transferred to insects is immediately dispelled on examination of the anatomy of the insect neuromuscular apparatus.

Vertebrate muscles are composed of many thousands of fibres, innervated by many hundreds of motor axons, which form physiological units, the motor units, within the muscle itself. The motor end-plates of vertebrates are of a very complex structure (Couteaux, 1955; Birks,Huxley and Katz 1960; Cole, 1955) and usually there is only one end-plate per fast extrafusal muscle fibre, or if more than one, they are still few in number, occupying distinct regions of the muscle fibre, usually at opposite ends (Alnaes,Jansen, and Rudjord, 1964).

On the other hand, the muscles of insects are composed of smaller numbers of fibres, innervated by relatively few motor axons. Many insect muscle fibres, however, do have the ability to produce different electrical responses under the influence of different innervating axons (Pringle,1939;

Hoyle,1957b; Wood,1958). This is made possible since insect muscle fibres are usually innervated by more than one axon of more than one kind, a phenomenon known as polyneuronal innervation. This latter feature is typical of all the arthropods so far examined. The commonest number of motor axons innervating a single insect muscle fibre is two. These axons were designated 'fast' and 'slow' by Pringle(1939), depending on the time course characteristics of the responses they initiated.

It would thus appear that the neuromuscular systems of insects are much simpler in structure than those of vertebrates. In insects, the central nervous system is much more diffuse than is the case in vertebrates, being present outside the insect head in the form of segmentally arranged nerve ganglia, this being consequent upon the segmental arrangement of the whole insect body. The insect brain is less dominant than is the vertebrate brain, each ganglion being responsible for muscle control in the segment it innervates. Peripheral control is thus of some importance in insect locomotion. This feature of peripheral control allows studies to be carried out on fairly isolated preparations, such as a complete segment, hence minimising the modifying influence of the rest of the body. Although insects can provide excellent preparations for the

study of neuromuscular mechanisms, such studies lag far behind those of crustacea and the vertebrates.

Hoyle (1957a) has pointed out that advances in physiological technique have set new standards for research in this field. He lists the principles required for an ideal programme of research as:-

1. The nature of the muscle under investigation should be known, along with its innervation and end-plate distribution.
2. The individual axons innervating the muscle should be stimulated separately.
3. Recordings of electrical phenomena should be made with intracellular electrodes from single muscle fibres.

In addition to the above points (which are here considered valid), it is important that a chemical investigation of the haemolymph be carried out as part of the work. Not only does this enable accurate artificial salines to be constructed for experimental use, it also indicates the ions which are most likely to be charge carriers in that particular system for the generation of membrane potentials.

The ionic hypothesis, given its classical statement by Hodgkin, 1951, attempts to explain the electrical activity of excitable tissues in terms of the distribution and action of ions present in the tissues, and lays great stress on the quantity and relative abundance of the main cations in blood.

Apart from sodium and potassium with their known effects upon the action and resting potentials, magnesium and calcium are also important. Magnesium has been found to have a depressant action upon neuromuscular transmission in vertebrates (del Castillo and Engback, 1954) while calcium has been found partly to antagonise the effects of high magnesium, and also to increase the action potential amplitude and rate of rise. Any investigation into the processes of neuromuscular transmission should thus be interpreted in the light of our knowledge of the ionic balance in the animal's blood. It is from this viewpoint that herbivorous insects assume a position of great importance. In herbivorous insects the blood ions are very different from those in most animals so far studied, and their physiology may well be different also.

Recently, a certain volume of evidence has been published which is not easily reconciled with the ionic hypothesis. Earlier misgivings over the ionic hypothesis were mainly based upon the observations of Falk and Gerard (1954) and Grundfest, Kao and Altamirano (1954) who performed potassium injection experiments, in which increase in internal potassium was found to have no effect on resting potential in frog muscle. The observation of Tobias (1950) in which frog muscles were depleted of their internal ions, but were found to retain a significant portion of their resting potential also cast doubts on the classical ionic theory. The more recent evidence in

conflict with the classical ionic hypothesis is mainly based upon studies of the exact ionic gradients across excitable tissues, evaulated by means of intracellular ionic analysis. When examined closely, ionic gradients in several animals have been found to differ quite markedly from the ideal state proposed in the ionic hypothesis. The evidence of Belton and Grundfest(1962 a,b), Wood (1963,1965), and Keynes (1962) bearing upon ionic gradients and related membrane potentials will be discussed in detail in section III where they bear closely on the subject matter under discussion. In general, all these investigators have found discrepancies between membrane potentials measured by means of intracellular electrodes.

These findings serve to underline the importance, not only of knowing the concentration of ions in the blood, but also the intracellular ions, so that ion distribution ratios can be calculated. So far there have been few investigations of insect neuromuscular mechanisms, and only one (Wood, 1957b,1958) based upon a herbivorous insect. It is the aim of this present thesis to examine the neuromuscular mechanisms of an advanced herbivorous insect order, the Lepidoptera. The results have fallen generally into three sections. In the first section the anatomy of the myoneural apparatus is considered, along with histological studies of the

thoracic nerve ganglion, the crural nerve, and the flexor tibialis muscle, from which most of the records of electrical activity are taken. In section II, the recording of the normal electrical and mechanical muscle responses from single muscle cells is considered. In section III, analyses of the lepidopteran haemolymph and myoplasm are discussed. The effects of certain ions on muscle membrane potentials are also investigated and discussed in relation to the ionic gradients in muscle. This section is concluded with an investigation into the role which muscle cell metabolism plays in maintaining membrane potentials.

SECTION I

THE ANATOMY AND HISTOLOGY OF THE NEUROMUSCULAR SYSTEM IN LEPIDOPTERA.

INTRODUCTION

The literature on the anatomy and histology of the Lepidoptera is very scanty indeed. The anatomy of the thoracic nerves in Sphinx ligustri has been dealt with by Newport (1832), but this author limited himself to the larval and pupal stages. In addition, the drawings are sketchy and the leg nerves are not really considered. Newport refers to earlier work by Heroldt upon Pieris brassicae but gives no publication references. Two recent papers by Sharplin (1963a,b), concerned with wing base structure in Lepidoptera, cover to a certain extent the anatomy of the tergal plates and some of the muscles which operate them. A recent paper by Heywood (1965), has covered the development and fusion of the central nervous system in Pieris brassicae, but otherwise there is no recent publication on lepidopteran myoneural anatomy.

It was initially intended to centre this investigation upon a single species, Sphinx ligustri(L.), but owing to the rather brief period in the adult stage (April to late July), the work was extended to include Bombyx mori(L.), Telea polyphemus(Cr.), and Actias selene(Huebner), which have adult stages slightly overlapping each other. As

a result, adults were available for experiment from March to late September. All species were obtained in the pupal stage. During the first experimental year a certain amount of difficulty was experienced over emergence from the pupae, especially in Sphinx ligustri. In this species the diapause is fairly lengthy, lasting from early autumn until the following spring. Because of the lengthy diapause, death due to natural causes such as bacterial and fungal attack was fairly high. In addition to this, there was a large pupal mortality almost certainly due to desiccation and failure to complete diapause through culturing the pupae in unnatural temperatures. The second experimental year was much more successful as far as pupal emergence was concerned. By means of adjusting the humidity and temperature of the culture it was possible to reduce pupal mortality in all species examined to about 10%.

The work involved in this section of the investigation has fallen roughly into two parts, study of the neural structure and study of the muscle organisation in the leg. The investigation of the nervous system has involved study of the metathoracic ganglion, with its internal structure and external nerve distribution, the crural nerve with its axon complement, and the end-plates upon the muscle fibres. The second part of the work has involved a study of the muscle structure in the

femur and the relation of the structure to the mechanical efficiency in the operation of the leg. In some cases where large numbers of routine resting potential recordings were taken, followed by muscle excision for ion analysis, the dorso/ventral flight muscle preparation was employed, owing to its large size and remarkably parallel fibre arrangement. This section thus contains a description of the anatomy of this preparation.

The innervation of Insect muscle.

In order to evaluate accurately the electrophysiological results obtained from muscle, it is important that the number of motor axons supplying the muscle be known, and in particular the number of motor axons supplying the individual motor end-plates on the muscle fibres. Much attention has been paid to the former, and a great deal of information has been provided by a number of authors. The picture that emerges is that insect muscles, in contrast to the condition found in vertebrates, are innervated by relatively few motor axons. From single up to multiple motor axon innervation of individual muscles has been reported. The sound muscle of Cicada has single motor axon innervation (Hagiwara, 1953) although double motor axon innervation seems to be most common in the insects (Hoyle, 1957a). Triple motor axon innervation

has been reported in Locusta (Hoyle, 1955b), and Hydrophilus (Montalenti, 1928). Innervation by four or more motor axons has been reported in several cases, mainly in association with the flexor tibialis muscle, particularly so in Schistocerca (Hoyle, 1957a) and Romalea (Ripley, 1954). Wood (1958) found that individual units of the flexor tibialis muscle in Carausius morosus were dually innervated each probably by a separate pair of axons and it is possible that this is the case in other insects. It is not of fundamental significance exactly how many axons supply a muscle as a whole, the important point being the number of motor axons which terminate at the end point of the neural system, the individual motor end-plates. As yet there is no evidence of multiple motor axon innervation of single end-plates. On the contrary, most of the evidence points to innervation of end-plates by two axons (Hoyle, 1955b,1957a; Wood, 1957b). This implies that when there are 3 or more axons to a muscle, not all fibres receive the same kind of innervation.

There is evidence that carefully graded increases in stimulation intensity produce a step-wise increase in tension. The tension increases have only been measured over the whole muscle, hence it is not necessary to postulate that each muscle fibre or unit (where present) is innervated by many motor axons. Such tension increases could be brought about by varying the number of muscle fibres or

units in operation by a process of recruitment in a manner analagous to the vertebrate motor unit mechanism.

Such an explanation is complicated however, due to the property possessed by insect muscle fibres of responding differently to separate innervating axons (see Section II). 'Slow' axons are characterised by the production in the muscle fibre of slow, readily facilitating responses, concerned mainly in maintainance of tonus and sustained contractions. 'Fast' axons produce fast, non-facilitating responses in the muscle fibres and are responsible for most of the rapid body responses. Several fast axons may supply a muscle as a whole, but any individual unit or fibre need only be supplied by one of these axons. Evidence is presented later in this section to show that only two axons innervate the motor end-plates in the Lepidoptera, even in fibres where both fast and slow responses are present. This is evidence that at least only one fast axon is necessary in some, if not all fibres in the flexor tibialis muscle, even though the muscle as a whole receives probably three fast motor axons. A histological study of the innervation of the flexor tibialis muscle down to the end-plates by means of serial sections and muscle squash techniques is thus important in helping to solve the confusion about innervation in such polyneuronally innervated muscles.

Motor nerve endings

Although much literature exists upon the topic of motor nerve endings, there seems little in the way of concrete histological observation. The most important facts we need to know about motor nerve endings are their shape and structure, their size, and the distance between them. Nerve endings are very difficult to distinguish from other structures on the surface of insect muscle fibres, and most metallic deposit methods of staining affect to some extent these other structures, in particular the dense tracheolor network in insect muscle. Silver tends to deposit upon nerve endings, the muscle fibres themselves, and the tracheae, but gold usually stains the nerve endings and the muscle fibres only. Information upon distance between end-plates is fairly complete. Foettinger (1880), working upon Hydrophilus and Chrysomela found endings along the muscle fibres about every 100μ, and Hoyle (1955b) found endings every 60μ on the muscle fibres of Locusta. Marcu (1929) observed nerve endings every 80μ in Geotrupes muscle and every 50μ in the muscles of Musca, and Weiant observed nerve endings in Periplaneta muscle

every 40μ (Unpublished, quoted in Roeder, 1953) while Wood found end-plates every 60μ in Carausius muscle. Morrison (1927) however, working on Apis, and Tiegs (1955) working on Erythroneura claimed to observe only one end-plate per fibre. The latter two authors seem to be the only investigators to claim single point innervation in insect muscle. It would appear that the insects in general, like the Crustacea, have multiterminal innervation.

The morphology of the motor nerve ending has also received much attention. Two main types of ending have been described, the Doyère-cone type and the filiform type. Morrison (1927) working on Apis, Mangold (1905) working on Dytiscus, Hoyle (1957a) working on Locusta, Edwards, Ruska and De Harven (1958a,b) working on Vespula and Cicada, and Wood (1957a) working on Carausius all describe motor nerve endings of the Doyère-cone type. Marcu (1929) working on Geotrupes, Montalenti (1928) working on Dytiscus, and Hilton (1925) working on Dendroides all claim filiform endings. In addition both Hilton and Marcu claim that the nerve endings they observed penetrated the substance

of the muscle fibre, a claim made also by Teigs (1955). The claim that the nerve endings penetrate the muscle fibres seems very unlikely on physiological grounds, since as Katz (1949) has pointed out, the very high potassium content of the myoplasm would very quickly depolarise the unprotected axon terminals permanently, preventing neuromuscular transmission.

Insect muscle is however, pervaded with a dense network of tracheae which do have very obvious filiform endings, many of which can be seen to penetrate the muscle fibres, and it is possible that some earlier authors may have confused these tracheal endings with nerve endings, especially if silver stains had been employed. This is very probably the case where endings have been described as filiform and also penetrating the muscle fibres. This latter argument would seem to make the whole case in favour of filiform endings rather dubious, in insects at least.

The most complete description so far given of insect motor end-plates is the electron microscope study by Edwards et al (1958a,b) upon the femoral muscles of Vespula and the flight and tymbal muscles of Cicada. The end-plate in these insects is seen to be of the Doyère-cone type, and in many ways is as complex as the vertebrate end-plate (Birks,Huxley, and Katz, 1960), being composed of a thick basement membrane lemnoblast,

with axon, folded mesaxon,nuclei,mitochondria, and neuro-filaments inside the axon itself. It differs from the vertebrate condition in not possessing a secondary synaptic cleft. The end-plate is fitted into a wedge shaped groove on the muscle fibre surface, being overfolded by the basement membrane of the lemnoblast and the tracheal membrane. At the end-plate, the plasma membranes of the axon and the muscle fibre are only 12mμ apart. At this point the axoplasm contains numerous synaptic vesicles. The mitochondria of the muscle fibre are strongly concentrated and orientated towards the region of the axon terminal. This large complex of both nervous and muscular tissues composes the end-plate proper, and closely parallels the condition in vertebrates. It is reasonable to suppose that other insect end-plates of the Doyère-cone type approximate to this description in most details.

Methods

For this investigation it is essential to know the nerve distribution to the femoral muscles. This was determined by dissection of the nerve ganglion in the thorax, then by tracing the path of the emergent crural nerve in the leg by means of serial transverse sections. Freshly killed animals were pinned out dorsal surface uppermost, and the wings were removed. The dense clothing hairs were removed from the thorax as far as possible, then the tergites were carefully removed to expose the thoracic

box.

Owing to the highly developed faculty of flight, the thoracic segments of Lepidoptera are highly specialised, most of the thoracic space being filled with the enormously enlarged dorso/ventral, lateral, and oblique flight muscles. Most of these muscles were removed, the preparation was flooded with 0.5% methylene blue for 30 minutes, and then fixed with saturated ammonium molybdate. Under these conditions, the nerves appeared a translucent blue against the white fat body and tracheae which intimately surround the ganglion. Dissection under 50% alcohol was also found very useful, the nerves appearing an opaque white, the only drawback of this method being the hardening effect the alcohol had upon the muscle fibres which tended to fragment during the dissection. In the Lepidoptera, the meso- and methathoracic coxae are fused to the thoracic box, the coxal muscles being contained in the thorax. In such cases it is very difficult to determine the function and homology of these muscles.

The most convenient way to study the muscle arrangement in the femur is to cut serial sections, and this has been done in all four species. The femora were severed at the trochanteral and tibial ends. Owing to the long thin nature of the material, Carnoy's fixative, which was rapidly acting and very penetrating, was initially employed. Although this fixer has the advantage of sufficiently fixing the centre of the femur, it was found to cause a fair amount

of shrinkage. Carnoy's fixative was later abandoned in favour of Heidenhain's 'susa' (Carleton and Drury, 1957) which was found to cause less shrinkage. Some femora were stained in bulk, but most were first sectioned at 10μ and then stained. Several staining methods were employed, De Castro silver and Ranvier-Loewit gold chloride (Carleton and Drury, 1957), and the gold toned silver methods of Willis (1954) and Frazer Rowell (1963). For general purpose histological work, Enrlich's haematoxylin and eosin, and Heidenhain's iron haematoxylin were employed.

All material was double-embedded in celliodin in methyl benzoate, and vacuum-embedded in paraffin wax. Excess hardening of the cuticle was counteracted by either placing in phenol for one hour after fixing (Gray, 1954), or by employing tert-butyl alcohol for dehydration instead of the normal ethyl alcohol (Gray, 1954). Attempts to scrape off the dense clothing hairs on the femur usually resulted in cuticular damage, so the scales and hairs were left on, even though they tended to fragment when sectioned. Serial sections of the femur were cut on a rotary microtome at 10μ thickness.

The fixative usually employed for the ganglion and crural nerves was 5% neutral formol saline. The sections of nervous material were cut at 5μ thickness and stained in Heidenhain's iron haematoxylin. For end-plates, the muscles were bulk stained in gold chloride and then squashed direct on to the slide. The results for this section will be considered under five separate headings.

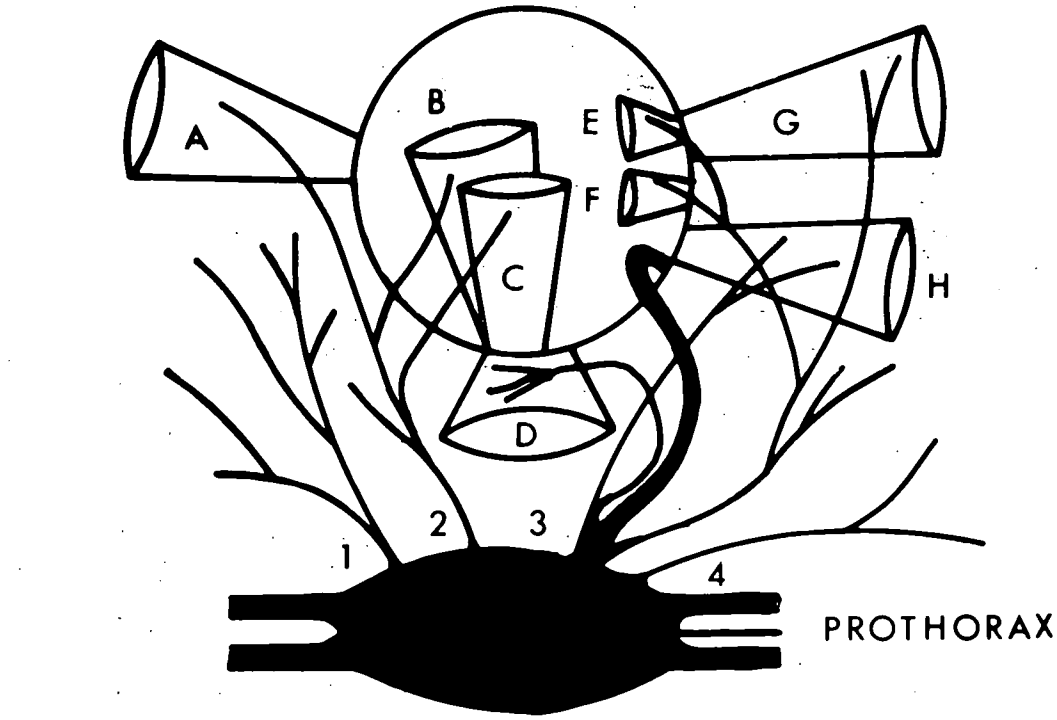
RESULTS

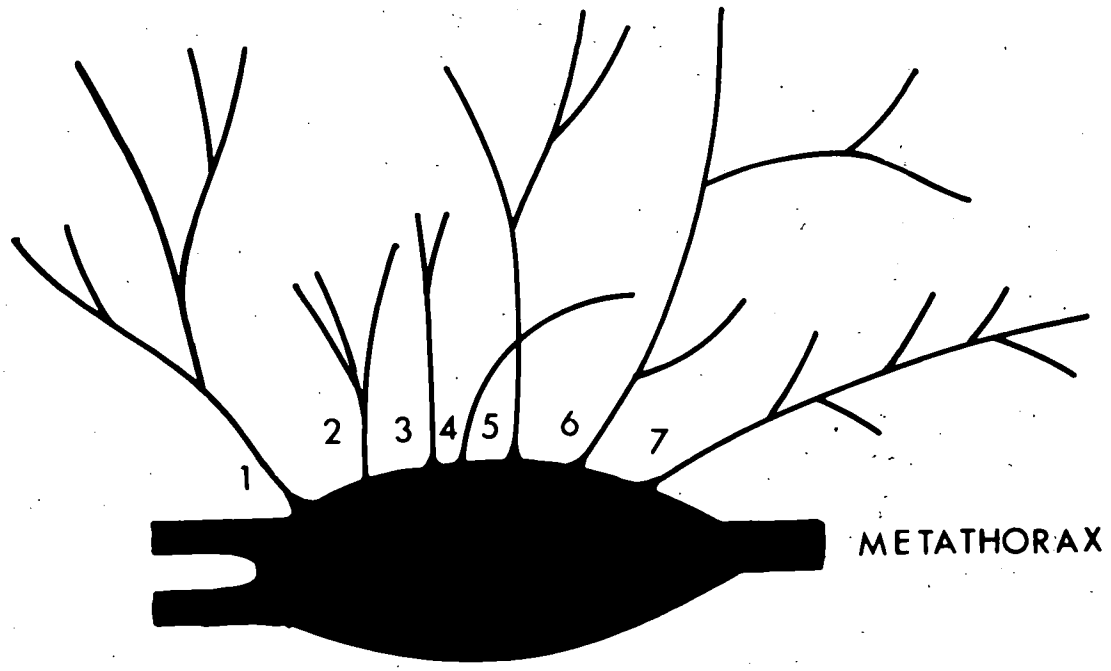
A. The thoracic ganglion and nerve distribution.

With few exceptions, advanced Lepidoptera possess only two thoracic and four abdominal nerve ganglia (Imms, 1957; Heywood, 1965). The Lepidoptera thus show a great degree of concentration of the nervous system, only exceeded among insects - by the Diptera. The first of the thoracic ganglia is the prothoracic, but the second is multiple in origin, being composed of the fused meso- and metathoraic ganglia, along with the first and possibly the second of the abdominal ganglia. The resulting ganglion is of large size (up to 3m.m. long in Sphinx ligustri), and it innervates the meso- and metathorax, as well as the anterior part of the abdomen. For convenience, this ganglion will be referred to as the metathoracic ganglion.

The metathoracic ganglia have been examined in all four species under investigation, as well as the prothoracic ganglion in Actias selene, the latter to check the possibility of double neural innervation in the legs from two separate ganglia. These ganglia are shown in Figures 1 & 2. In all species, the muscles of the femur, which will be described later, appear to be innervated by only one nerve from the ganglion, in the case of all legs. In Actias selene, the prothoracic leg is supplied by nerve III, while nerve I supplies muscles of the collar region, nerve II and branches of nerve III supply the main coxal muscles, and

ACTIAS FIG.1





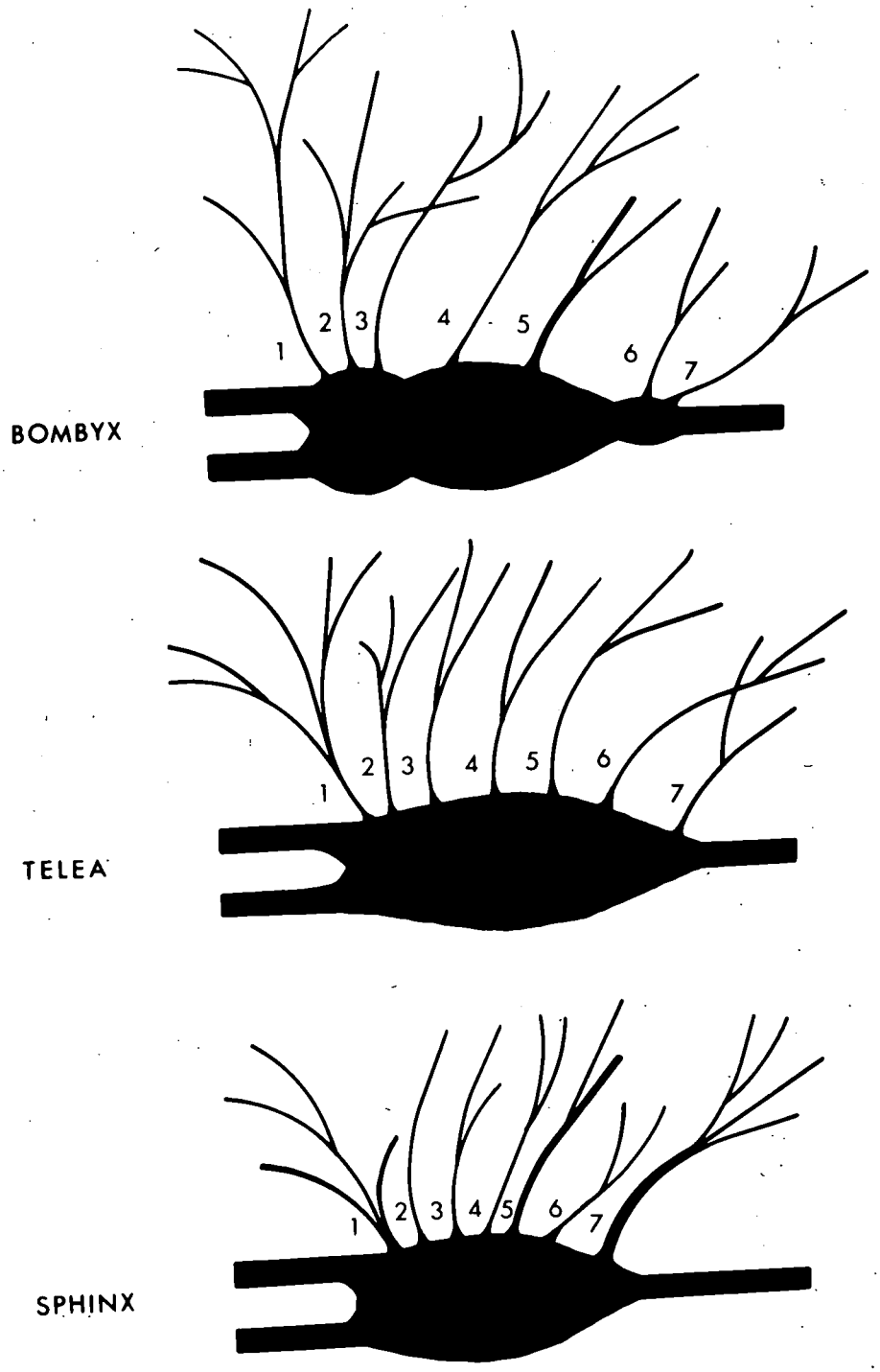


FIG. 2 The metathoracic ganglia

nerve IV supplies the posterior of the prothoracic segment.

The meso and metathoracic legs are supplied from the metathoracic ganglion. Nerve I from this ganglion innervates the fore-wing base as well as the tergosternal and epipleural muscles. Nerves 2 and 3 innervate the coxal and also the epipleural muscles. Nerve 4 innervates the mesothoracic leg, passing down the edge of the episternum and posterior coxal margin to enter the femur without division. Nerves 5 and 6 innervate the coxal and epipleural muscles of the metathorax, but the main branch of nerve 6 supplies the hind wing base. Nerve 7 is large and innervates the metathoracic leg, the side divisions supplying the dorso/ventral flight muscles. One branch of this latter nerve passes into the abdomen to innervate the muscles of the anterior abdominal wall.

The metathoracic ganglion of Telea polyphemus and its nerves are similar to the above, but in Bombyx mori and in Sphinx ligustri nerves 5 and 7 supply respectively the meso- and metathoracic legs. This difference is probably of no real significance since the metathoracic ganglion is fused to differing extents in all these species. The numbered nerves are thus not necessarily similar in origin in all species. The nerve innervating the metathoracic leg has been designated the 'crural' nerve in all cases since it has the function of the nerve designated 'crural' in other insects, but this does not imply homology.

The fine internal histology of the metathoracic ganglion in Sphinx ligustri is seen in Figures 3,4, and 5, the transverse sections in Figures 3 and 4 showing the emergence of the crural nerve. The body of the ganglion and the crural nerve is seen to be invested in a stout neural lamella (the neurilemma of vertebrate literature). This consists of two parts, the lamella and the sheath cells directly under it, the latter having large, deeply staining muclei. The sheath cells form a continous layer under the lamella itself, similar to the condition described recently in the locust (Ashhurst and Chapman,1961,1962). Large fibre tracks of internuncial neurones are seen coursing outwards and upwards in the ganglion. To either side of the centre are seen four groups of longitudinally orientated neurones. The latter are most probably tracts of through conducting neurones from the brain, presumably to the abdomen (Guthrie, 1964). The dorsal and lateral parts of the ganglion are seen to contain non-nucleated spaces which can only be regarded as large vacuoles, but their function is uncertain.

By far the largest cells present in the ganglion sections are the central motor neurone cell bodies which supply the axons to the lateral emergent nerves. These cells average 20 to 30μ in diameter. An enlargement of a group of these cells from a transverse section of Sphinx metathoracic ganglion is shown in Figure 5. The cells are

Figure 4. L.S. Crural nerve, Sphinx ligustri.

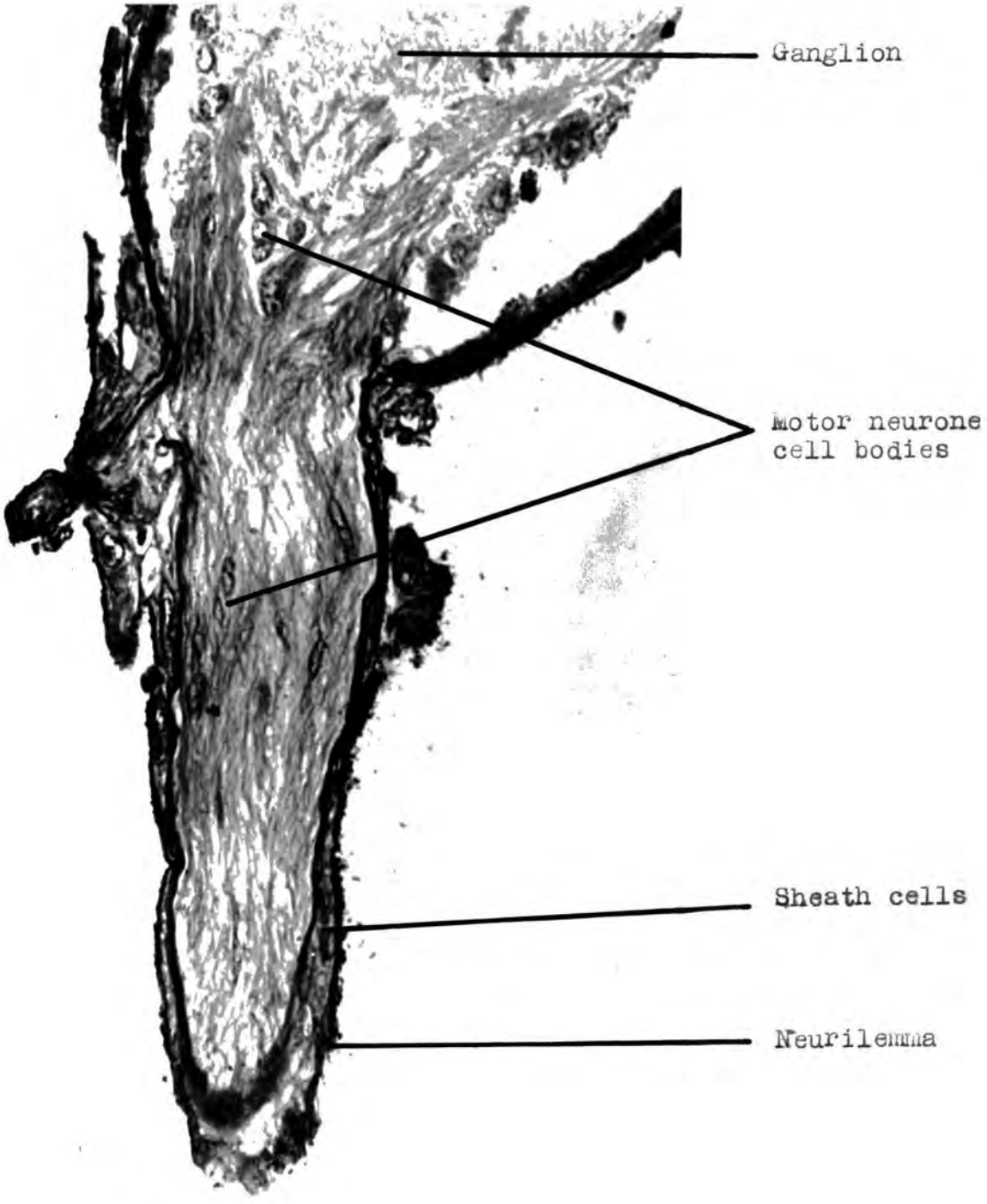


Figure 5. Transverse section of Sphinx ligustri metathoracic ganglion showing a group of large central motor neurone cell bodies at the edge near the emergence of the crural nerve root. Stained in Haidenhain's iron haematoxylin.

seen to be conical in shape with very large nuclei, and are typical of the general shape of vertebrate central motor-neurone cell bodies. These cells are present on either side of the ganglion, both above and below the emergent nerve root, into which their axons are channelled.

Figure 4 shows a section through the root of the crural nerve as it leaves the edge of the ganglion. Prominently placed are the central motor neurone cell bodies, which are not confined to the edge of the ganglion, some being found in the proximal part of the crural nerve trunk itself. The crural nerve is seen to have a neurilemma continous with that of the ganglion, and to contain a large number of axons. Osborne (1963) claimed that in the nerve supply to the abdominal stretch receptor in Periplaneta, each individual axon was invested with a sheath which he called the Schwann cell sheath. Such nerves he called 'tunicated'. This does not appear to be the case in lepidopteran nerves (see Figure 6).

In transverse section (Figure 6), the crural nerve appears to have two distinct neuronal regions. The ventrally placed region is composed of large neurones from 5 to 20μ in diameter, there being about 40 of these neurones. The eight most ventrally placed of these are very large, with an average diameter of 20μ. The dorsally placed region is composed of about 150 to 200 neurones with a fairly uniform small diameter, in the region of about 3μ.

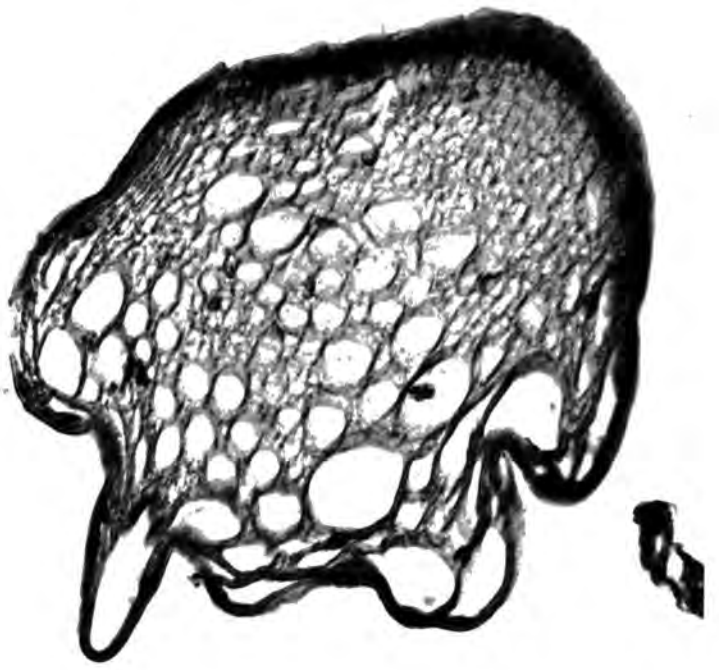


Figure 6. Transverse section through the crural nerve of Sphinx ligustri showing the division of the nerve into two neuronal types, of about 20u diameter, and of about 3u diameter.

The crural neve is a mixed nerve, giving motor supplies to the femur,tibia, and tarus, and also containing sensory axons connecting to the receptors in the tarsus. It is probable that the large neurones in the crural nerve are the motroneurones supplying the muscles, and the small neurones the sensory neurones from the tarsal receptors.

The crural nerve divides on entering the femur to give separate supplies to the flexor and extensor tibialis muscles. Once again the division of the axons into large and small is present, but the number of large motor axons in each seems to be reduced to about six. Figures 7 and 8 show transverse sections of the separate nerve supplies to the flexor and extensor tibialis muscles.

In comparison to the nervous innervations of vertebrate muscles, these insect muscles would appear to be innervated by few axons, a feature fairly typical of insects (Pringle, 1939; Hoyle, 1957a; Wood,1958). Although Wood (1958) described a relatively large number of axons in the flexor tibialis branch of the crural nerve in Carausius, the ultimate terminals upon the muscle fibre contained only two axons. Although there are about seven large motor axons in the flexor tibialis branch of the crural nerve in Sphinx ligustri, evidence is presented later to show that the nerve terminals on the muscle fibre also contain only two axons.

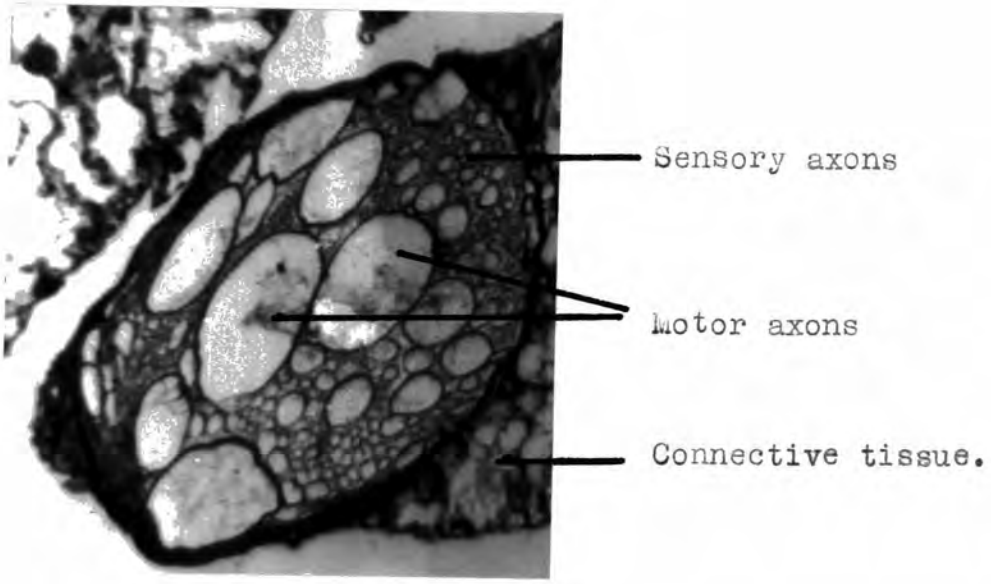


Figure 7. Transverse section of the crural nerve branch to the flexor tibialis muscle, Sphinx ligustri.

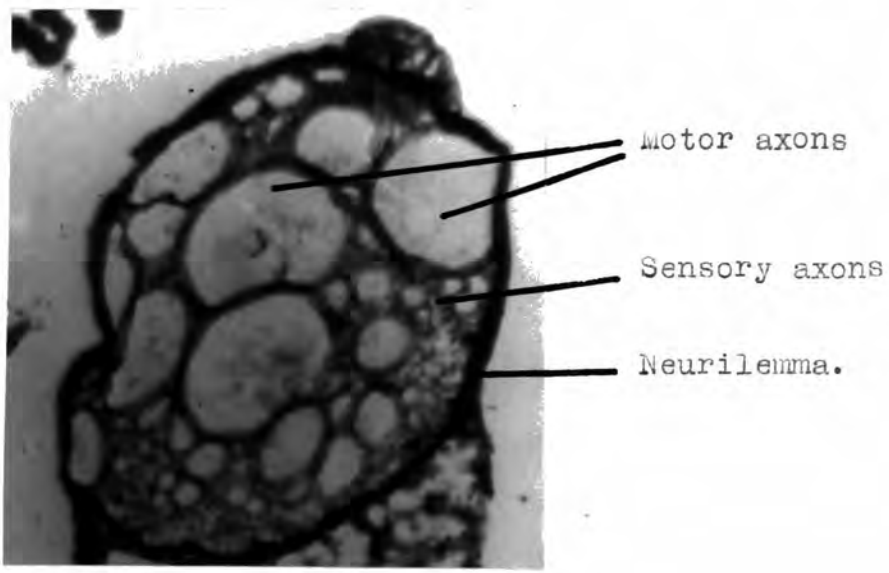


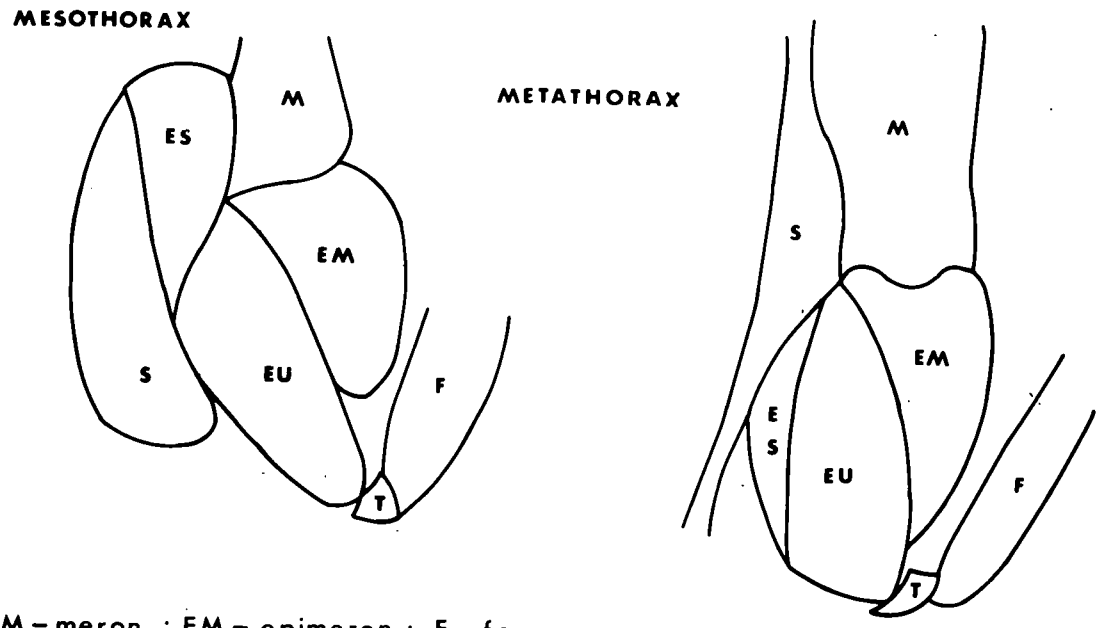
Figure 8. Transverse section of the crural nerve branch to the extensor tibialis muscle, Sphinx ligustri.

The number of axons in the crural nerve is of no real significance since it is not postulated that all the muscle fibres in the flexor tibialis muscle are innervated by exactly the same two axons. Although the muscle is not divided up into separate motor units, as are the muscles of those insects which have separate supplies for each unit (or group of units), it is quite possible that in Sphinx ligustri, separate regions of the flexor muscle have their own individual supplies from central motor neurones in the ganglion. This would explain the disparity in the number of motor neurones in the crural nerve, and the number of motor neurones at the individual end-plates.

B. The morphology of the leg.

In all four species the leg morphology was similar. The coxae are more or less completely fused to the thoracic sclerites, the subcoxal plates being particularly prominent. Figure 9 shows the meso- and metathoracic coxae of Telea polyphemus. A meron,epimeron, and episternum are quite large and distinct, the eucoxa being fused along its anterior rim to the episternum, and along its posterior rim to the epimeron. This allows practically no movement of the coxae, the femoral muscles being largely responsible for movements of the leg. In the prothoracic leg, the coxae are relatively independent and mobile. The meso and

COXAL SCLERITES IN T. POLYPHEMUS



M – meron ; EM – epimeron ; F – femur ;
T – trochanter ; EU – eucoxa ; S – sternum ; ES – episternum

FIG. 9

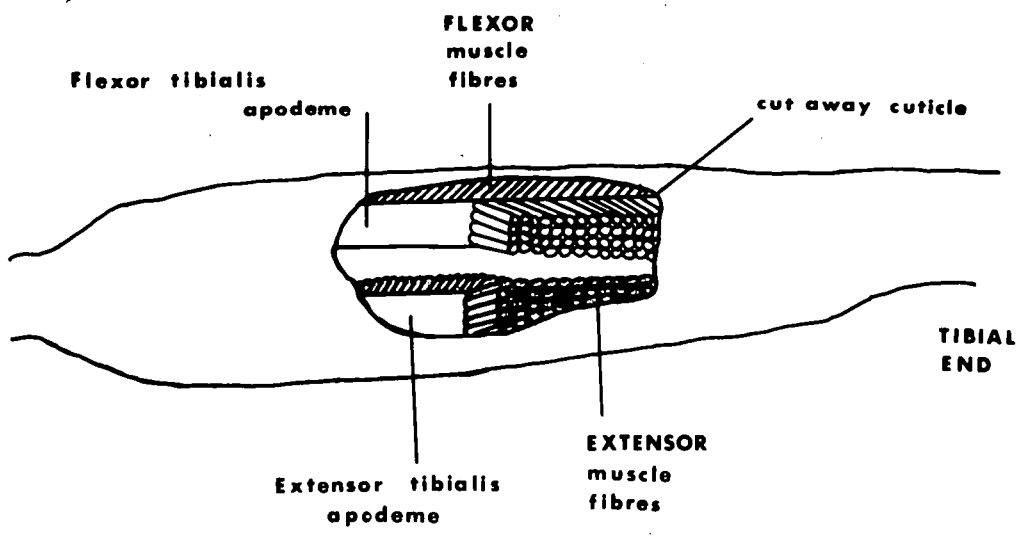
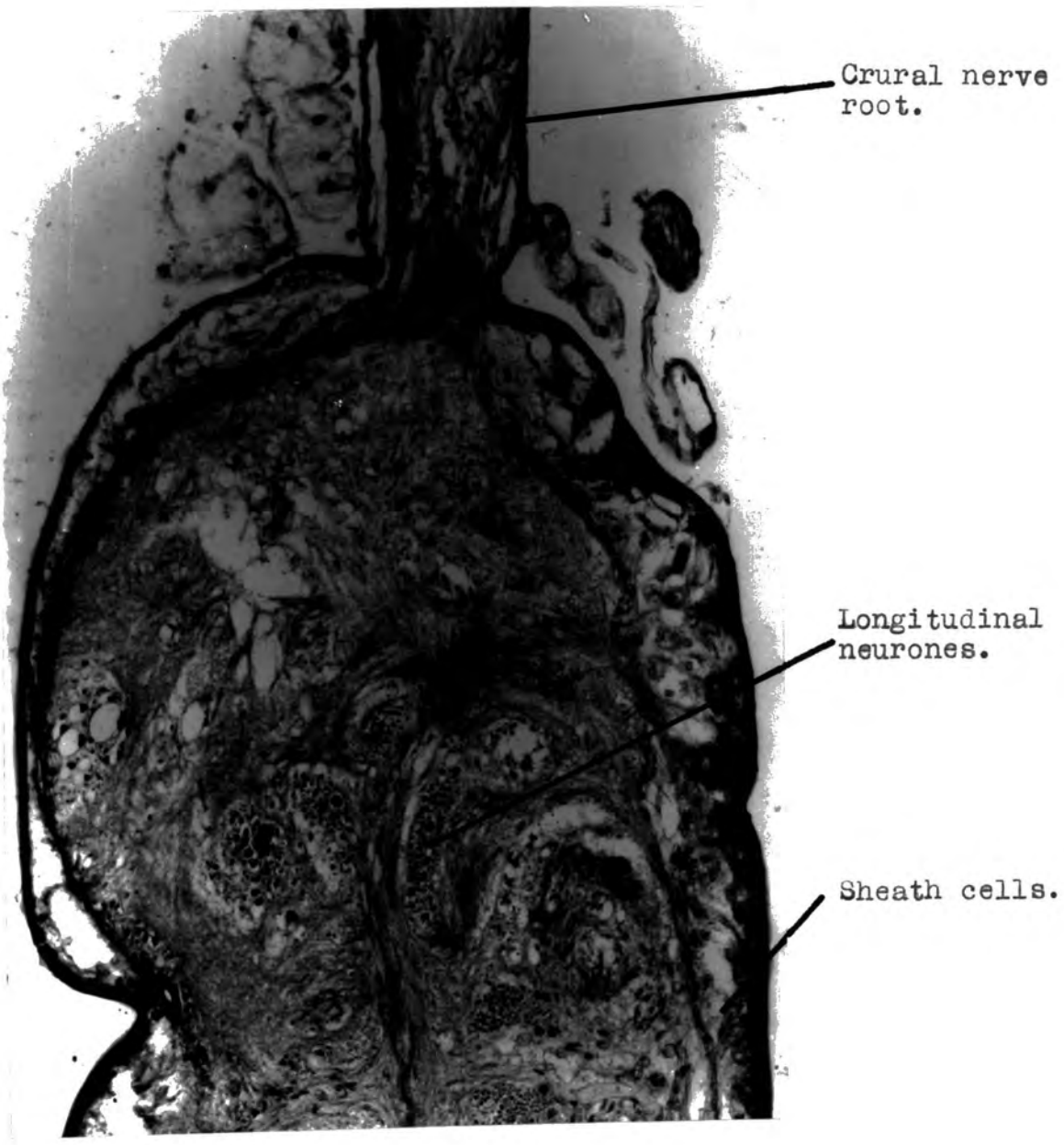
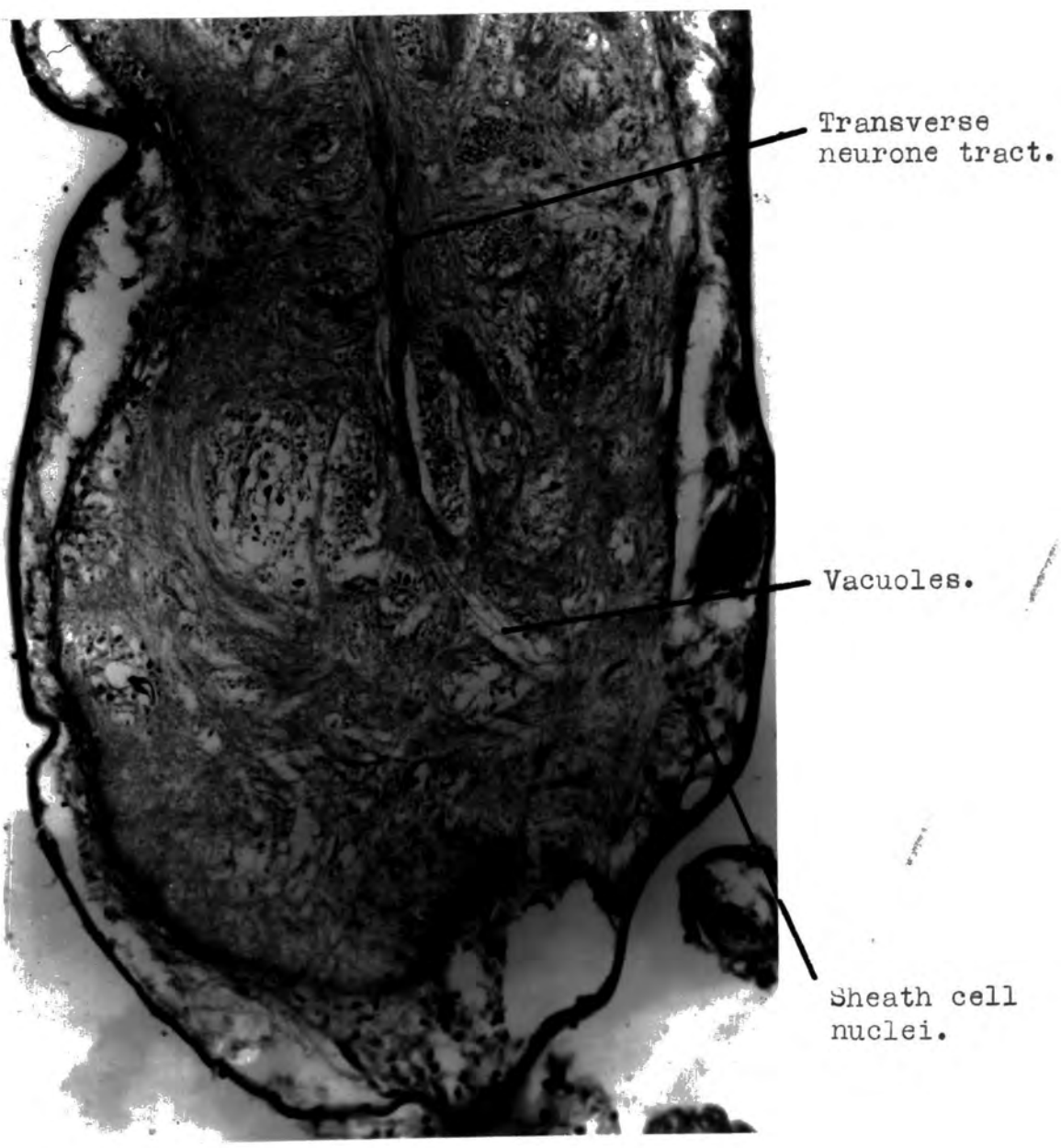


FIG. 10

Figure 3. T.S. Metathoracic ganglion, Sphinx ligustri.





metathoracic coxae point directly inwards to such an extent that the trochanters almost touch in the ventral midline.

The trochanter is a small boat-shaped segment, being fused to the femur and moving against the coxae by a dicondylic articulation. This allows levation and depression but little in the way of rotation. The femur itself is the largest segment in the leg, being very stout and held horizontally at rest. The femur has a dicondylic articulation with the tibia, which has a prominent spur fitting into a groove in the anterior cuticle on the inside. The tarsus has five joints and a large claw, and is the only part of the leg capable of free movement in a lateral plane and capable of any degree of rotation. From the viewpoint of muscular control of locomotion, the femur is thus by far the most important leg segment, and its two muscles, the flexor and extensor tibialis are particularly important.

C. Anatomy and histology of the femoral muscles.

The muscle arrangement in the femur is similar in all four species studied, there being however, variations in size and orientation of the muscle fibres. The sketch in Figure 10 shows the general muscle arrangement. This arrangement consists of a dorsally placed flexor tibialis muscle, and a ventrally placed extensor tibialis muscle.

Both muscles are generally similar in construction, the flexor being usually the smaller of the two. The fibres of these muscles do not run the length of the long axis of the femur, but are typically 'pinnate' muscles of the kind already described in the locust (Hoyle, 1955b), and in the stick insect (Wood, 1958). Both flexor and extensor muscles have a central, vertically orientated apodeme (Figure 11) with laterally placed fibres running out from the apodeme with insertions upon the lateral cuticle of the femur. The histological details of the apodemal origins of the flexor tibialis muscle fibres are seen in Actias selene in Figure 12c, and in Sphinx ligustri in Figure 13 a and b. The individual muscle fibres in any one species showed a fair degree of diameter variation but the mean diameter of twenty randomly selected fibres was not found to vary greatly between species, being the range 25 to 28μ for the flexor muscle.

Although the general muscle arrangement is similar to that of the stick insect (Wood, 1958), all four lepidopteran species differed to some extent as far as internal divisions of the muscle were concerned. The flexor tibialis muscle of the stick insect is divided up into approximately 17 pairs of motor units, but no such division of the lepidopteran flexor or extensor tibialis is seen. In both these muscles,

the muscle fibres simply run in banks from their origin on the apodeme to their insertions on the lateral cuticle (see Figure 14). The apodemes do not run the whole length of the femur, hence, beyond the apodemal ends there are banks of fibres running laterally with no separating central apodeme. Sections cut at this point of the femur may thus give the impression of the existance of two extensors or flexors, even though the muscles involved are single entities (Figure 13b).

Various transverse sections of femora are shown in Figures 11,12, and 13. Although the muscle arrangement is similar in all species, differences in relative size and fibre orientation are seen depending upon the position along the femur at which the sections were cut. This is particularly so in Actias selene since the muscles in this species do not run the whole length of the femur. Figure 15 shows the femur of Actias selene cut in longitudinal section. The flexor and extensor tibialis muscles are separated by a large group of central tracheae, often equal in size to the muscles themselves.

In Figure 12c, the femur of Actias selene is seen cut in transverse section near the tibial end at the point where the flexor muscle first begins. The fibres are seen to radiate out over a wide angle to insert on a large area of the lateral cuticle. This diffuse spread of muscle fibres is typical of pinnate muscles, particularly insect leg

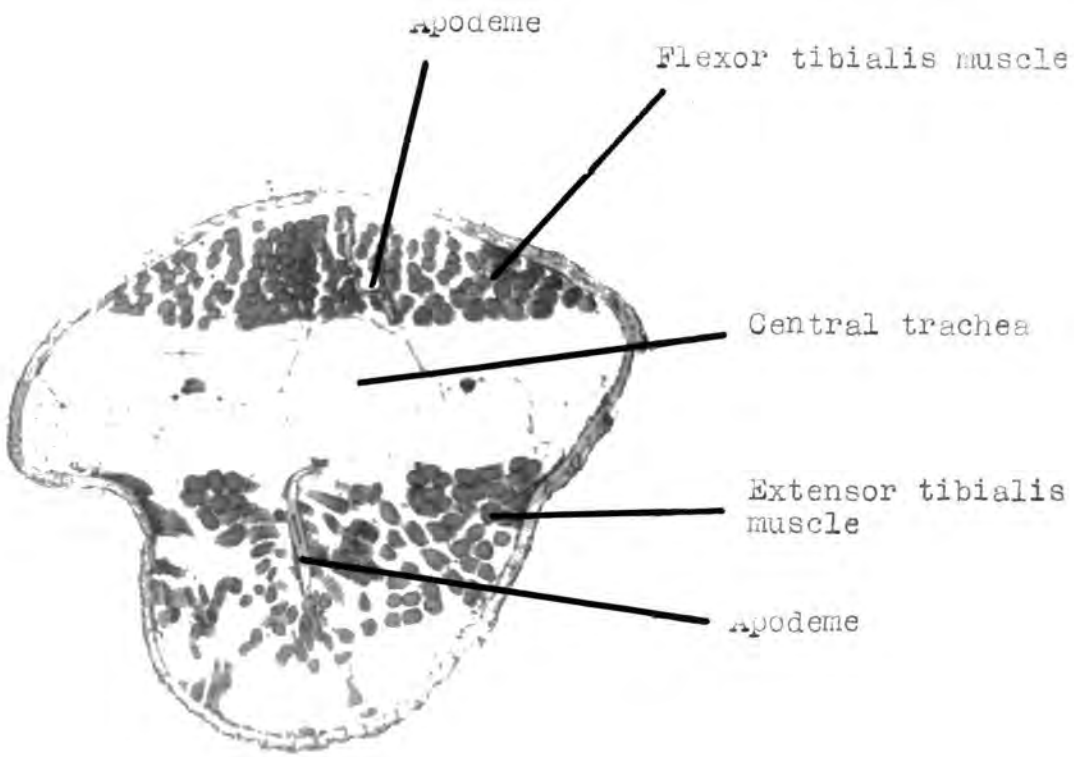


Figure 11. Transverse section of the femur of Telea polyphemus.

Figure 12.

A.

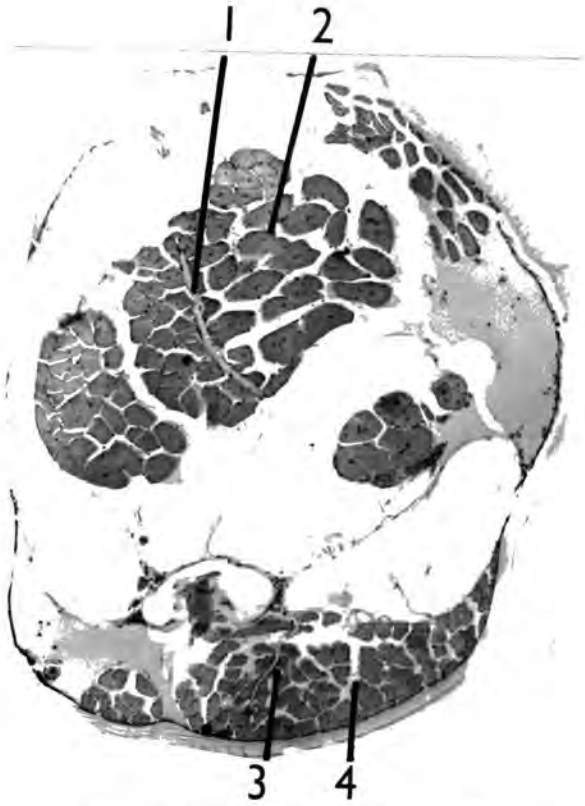
1. Apodeme
2. Flexor tibialis muscle
3. Apodeme
4. Extensor tibialis muscle.

B.

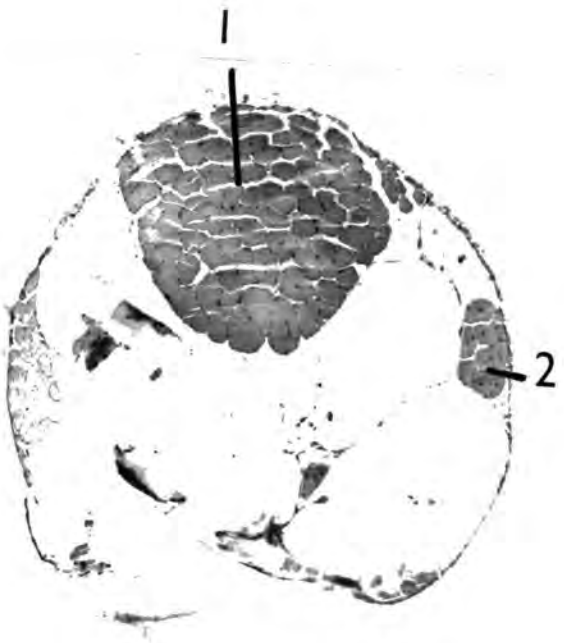
1. Flexor tibialis muscle
2. Insertions of extensor tibialis muscle

C.

1. Parallel fibres of the flexor tibialis muscle near beginning of apodeme.
2. Apodeme at point of attachment to the femoro/tibial articulation.



A. Transverse section through the central part of the femur.



B. Transverse section at the trochanteral end of the femur showing the side insertions of the extensor tibialis muscle.

C. Transverse section through the tibial end of the femur showing the origin of the flexor tibialis muscle.

Figure 12. Muscle arrangement in the femur of Actias selene.

Figure 13.

A.

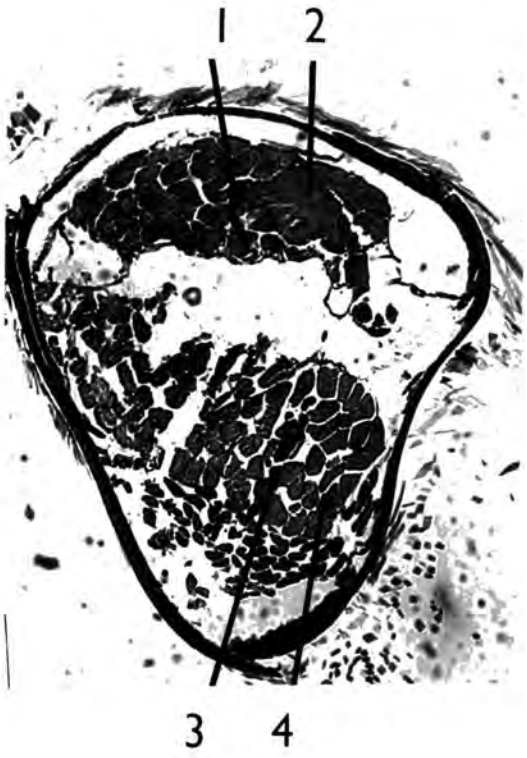
1. Apodeme
2. Flexor tibialis muscle
3. Apodeme
4. Extensor tibialis muscle

B.

1. The two separate parts of the flexor tibialis muscle at the proximal end.
2. Apodeme
3. Extensor tibialis muscle

C.

1. Apodeme
2. Flexor tibialis muscle
3. Apodeme
4. Extensor tibialis muscle.



A. Section through central part of femur.

B. Section through proximal end of femur. No apodeme visible in the flexor tibialis muscle.

C. Section through the distal end of the femur. Both apodemes visible.

Figure 13. Muscle arrangement in the femur of Sphinx ligustri. All stained in Haidenhain's iron haematoxylin.

muscles. In this particular section, the muscle fibres are seen to be fairly straight, but in the main body of the muscle, as seen in other sections, the fibres slope at an angle, hence cross sections cut the fibres at various angles. In Figure 14, from a section cut in the main body of the flexor tibialis of Sphinx ligustri, all the fibres are seen to slope at an angle away from the central apodeme.

D. The flight muscle preparation.

For the routine measurement of large numbers of resting potentials of muscle fibres, followed by excision of the muscle for intracellular ion analysis, a larger preparation than the flexor tibialis muscle was sought for both convenience and accuracy, since chloride analyses can become inaccurate when small quantities of muscle tissue are used. The preparation finally selected was the dorso/ventral flight muscle mass. The main advantages of this preparation were ease of dissection, large size, convenient position for excision, and close similarities in electrical characteristics with the flexor tibialis muscle of the leg. In all species, the electrical characteristics of the fibres in this preparation were found to be similar to those of the fibres of the flexor tibialis muscles. A diagram of the preparation is shown in Figure 16. The mesoscutellum was slit sideways slightly in front of the median ridge and raised, while the edges were cut forwards and sideways. The insertions of

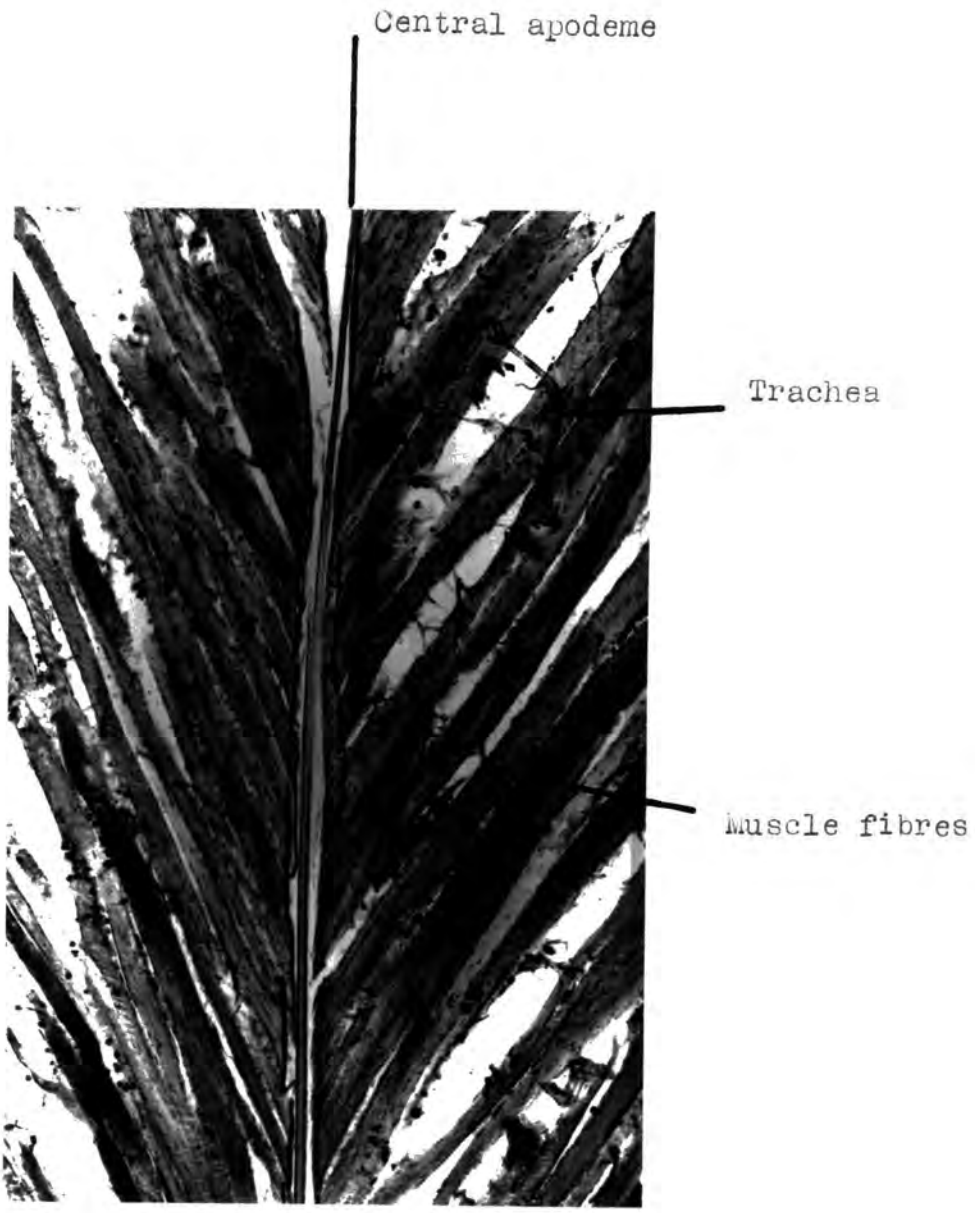


Figure 14. A longitudinal section through the flexor tibialis muscle of Actias selene showing the centrally placed apodeme and the tracheal network in the muscle. Stained in Haidenhain's iron haematoxylin.

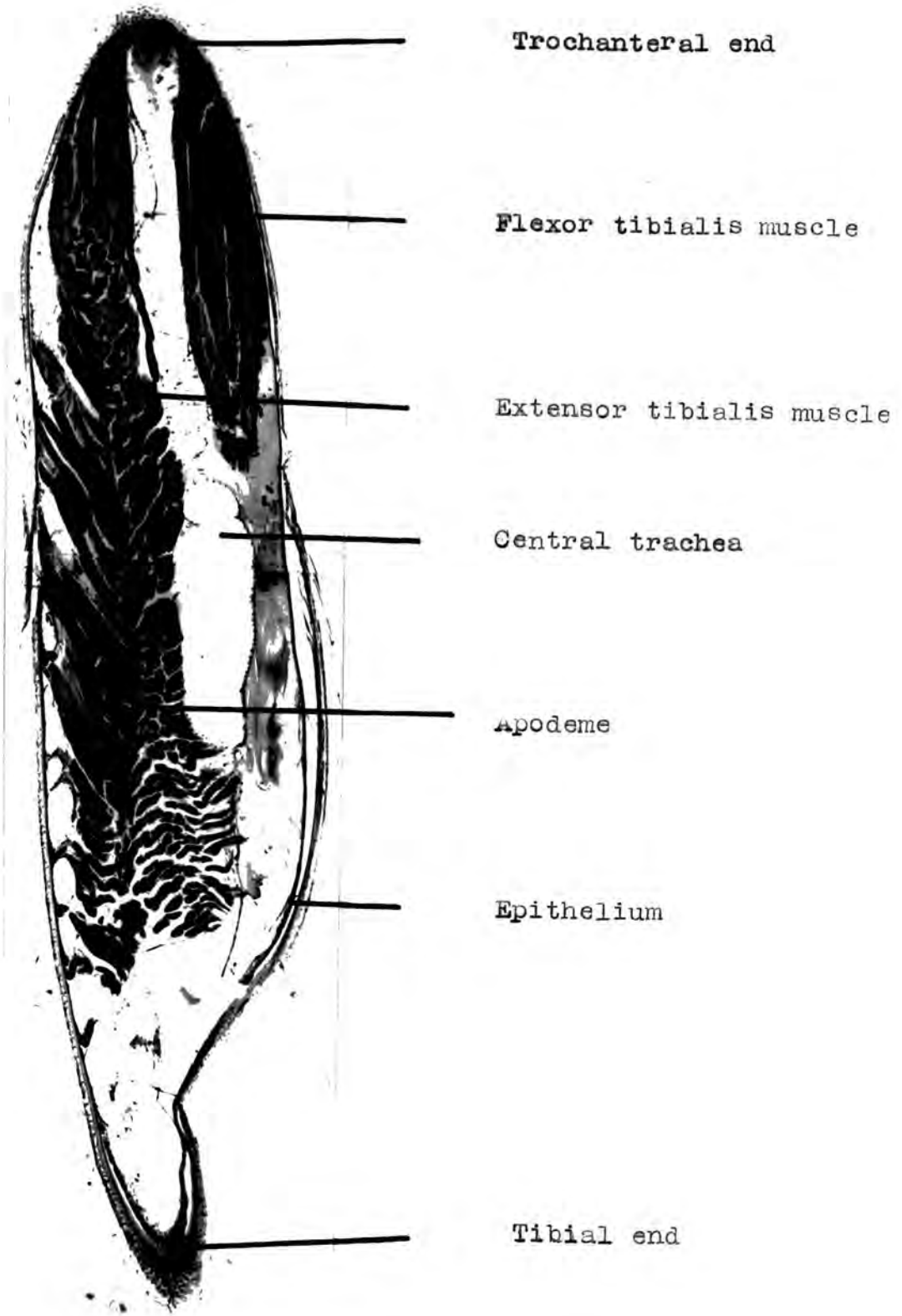
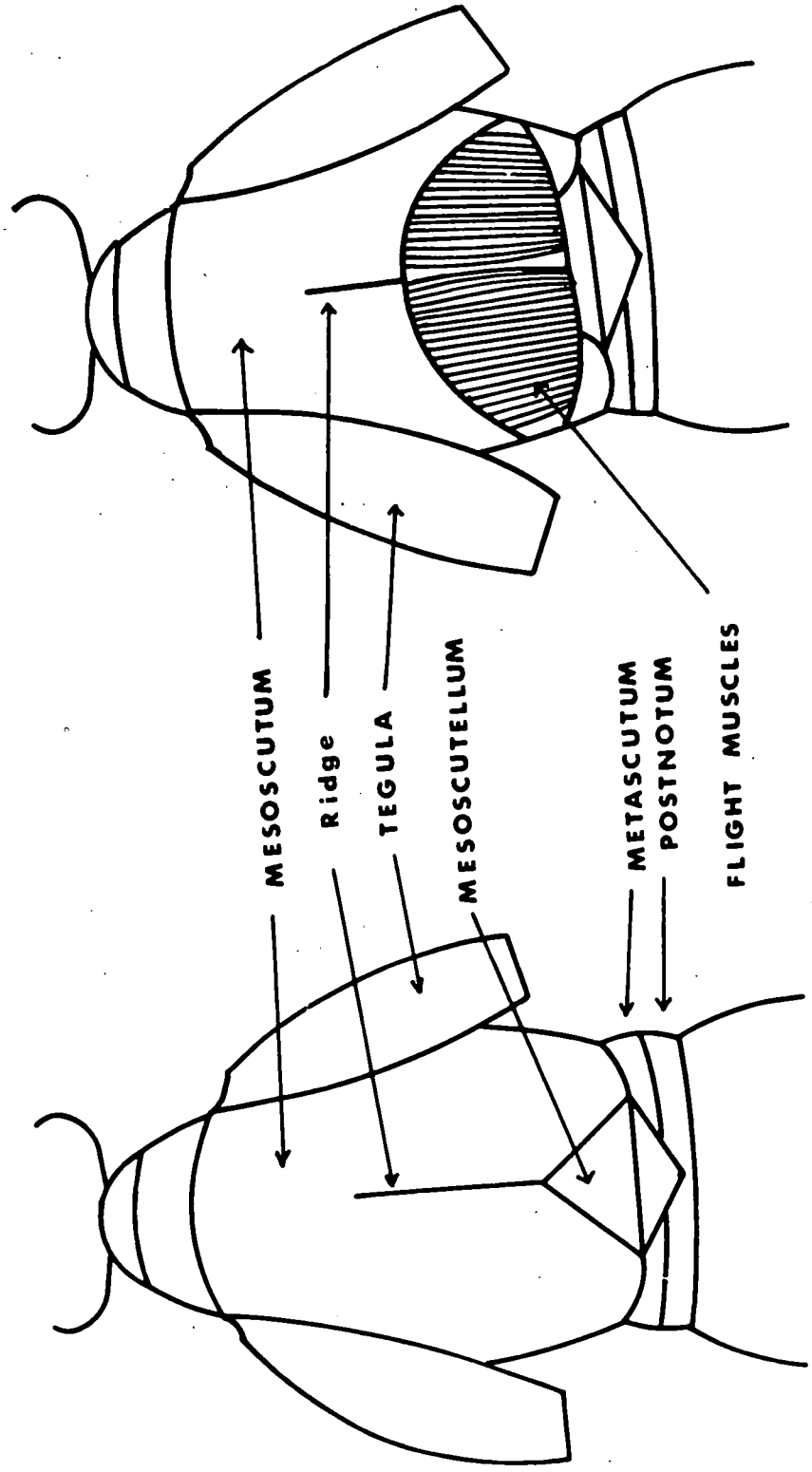


Figure 15.

Longitudinal section of the femur of *Actias selene*, showing the small flexor muscle mainly at the trochanteral end, and the large extensor with central apodeme. Original length 6 mm. Stained in haematoxylin and eosin.

Figure 16


MESOSCUTUM
Ridge
TEGULA
MESOSCUTELLUM
METASCUTUM
POSTNOTUM
FLIGHT MUSCLES

the dorso/ventral muscles are clearly visible on the cuticle of the mesoscutum, and the forward cuts were carried up until just in front of the insertions. The flap of cuticle so formed was cut off, exposing the bank of parallel arranged fibres of the underlying muscle.

In some cases, the space between the mesoscutellum and the underlying muscle was filled with a white fatty connective tissue. This was dissected away to reveal the surface of the muscle. Using this method of dissection, no damage was done to the tracheal supply, which enters the muscle from its lower (inside) surface. This preparation could be kept in good condition for periods of up to 15 hours, allowing extended investigations to be carried out. Since action potentials were not recorded from this preparation, no dissection of the nervous supply to the muscle from the last two ganglionic nerves was necessary.

E. Motor nerve endings.

The motor nerve endings were found to be very refractory to staining. Best results were obtained when the muscle was teased, then bulk stained in gold chloride as described earlier, and then left in glycerol for two days prior to examination. Such treatment left the muscle fibres fairly supple, so that on subsequent squashing under a cover slip the fibres did not fragment.

The motor nerve was found to branch ultimately upon

the muscle fibres fairly frequently, the nerve endings appearing in groups at rather irregular intervals. However, within the groups the endings were fairly regularly placed. In Sphinx ligustri the individual end-plates were placed about 60μ apart, but due to the expanded nature of the individual end-plates, the edge of one end-plate was found to almost touch the edge of the next one in line. In figure 17 an end-plate from the flexor tibialis muscle of Sphinx ligustri is shown in some detail, but although the axon terminals have stained well, the nuclei have not stained at all. Figure 18 shows three end-plates in a row from Telea polyphemus, but all have been damaged in the teasing process.

The motor nerve ending is seen to have the general shape of a spatulate claw with irregular outline, each ending covering an area of about 25μ, and being close to the edge of the next ending. The lepidopteran motor nerve ending can thus be regarded as typical Doyere-cone type. The individual axons can often be seen right up to the end-plate in a sufficiently stained preparation. In Figure 17 the axons to the end plate are fairly obvious, and are seen to be two in number. Hoyle (1957a) has reported two axons to the individual end-plates of the locust, and Wood (1958) reported two axons also in the stick insect. The axons to the end-plate are covered by a fibre sheath, which is presumably a continuation of the neural lamella from

Figure 17. Single end plate from the flexor tibialis muscle of Sphinx ligustri. Stained in gold chloride.

Figure 18. Three end plates in a row from the flexor tibialis muscle of Telea polyphemus. Teased preparation bulk stained in gold chloride.

the metathoracic ganglion. The sheath appears to stop at the end-plate, and at this point it is no longer possible to distinguish any separation of the two axons in the end-plate ground cytoplasm. There also appears to be no division of the end-plate into separate regions related to the two innervating axons. This may mean that the substance of the end-plate may be non-specific to either axon.

The muscle fibre in insects and in other arthropods is capable of giving different types of responses, such as 'fast' and 'slow', and these responses must both have their origin at the end plate. Since the end-plate seems to be non-specific to either axon, it may be that the specificity of response lies in the muscle fibre rather than in the end-plate, although an alternative hypothesis postulating specific neurosecretion down the axon body is feasible. If the former hypothesis were to be correct, then the muscle fibre could possible respond in different ways to different quantities of the same transmitter substance liberated by the end-plate under the influence of different axons.

The end-plate itself does not appear to be covered by the neurilemma extension which sheaths the nerves, and it may be that the end-plate is capable of operating in an ionic medium in which the nerve can not. Hoyle (1952)

showed that injection of high potassium salines under the sheath of Locusta nerves rapdily blocked conduction. Recently, however, Treherne (1965a) showed that conduction could be maintained in Carausius desheathed nerves even in salines with high potassium concentration. This last finding, and the observation that end-plates which have no sheath at all, can operate in high potassium salines strongly suggests that Hoyle's earlier finding was spurious, possibly resulting from damage to the axons during the micro-injection. — or that desheathing does not remove the inner sheath glial cells.

The muscle fibres so far seen with intact endings indicate that the endings are all placed on one side of the fibre. In squash preparations it is difficult to get any idea of the size or nature of the primary synaptic cleft between the nerve and muscle parts of the end-plate. For this purpose a transverse section right through the end plate is needed. Electron microscope work upon vertebrates by Eccles and Jaeger (1958) showed the synaptic cleft to be in the region of 400 to 500 ^{o}A. This distance, which is only about 0.5μ is just at the limit of resolution of the light microscope, hence light microscope work here will be of little use.

In the introduction to the literature it was suggested that some earlier authors who described filiform nerve

endings in insects may have been confusing nerve endings with the tracheal endings which are strongly filiform. A group of such tracheal endings is shown in Figure 19, and they are seen to be very different indeed from the true nerve endings seen in Figures 17 and 18. However, the tracheal endings follow quite closely the sort of descriptions attributed to nerve endings by both Marcu(1929) and Hilton (1925), and this strongly suggests that these authors were in error. Apart from having an annulated trunk(quite unlike an axon sheath), the tracheal endings are very small, less than one tenth the size of a Doyère-cone nerve ending.

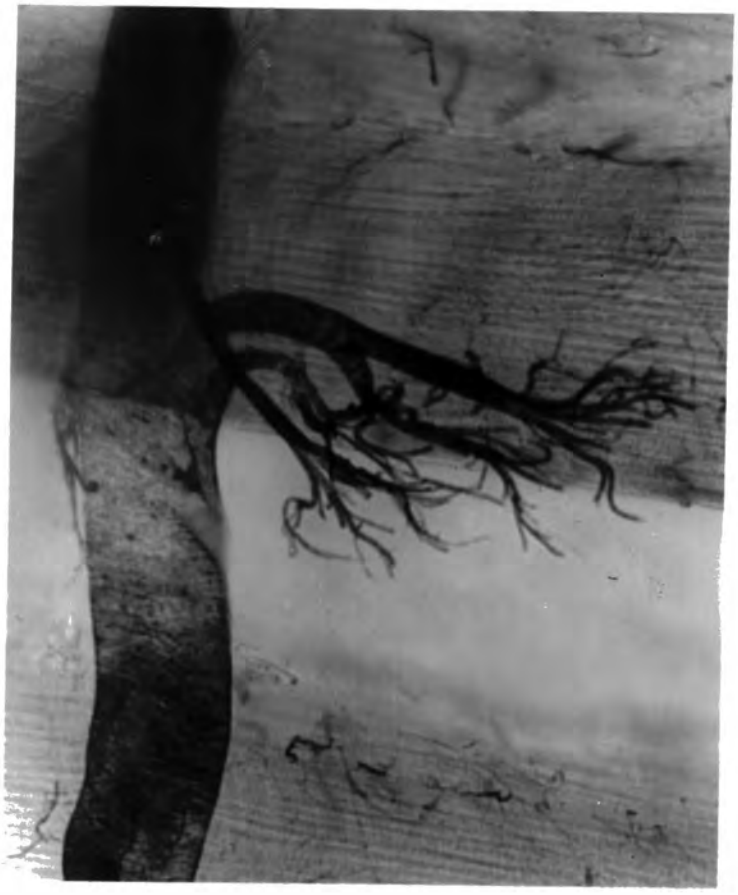


Figure 19. Typical tracheal endings in the flexor tibialis muscle of Sphinx ligustri stained in silver.

SECTION II

RECORDING OF NEUROMUSCULAR MECHANISMS IN THE MUSCLES OF LEPIDOPTERA.

INTRODUCTION

A potential difference is found to exist across the membrane of a muscle fibre. In the resting fibre this potential difference is such that the outside of the fibre is positive to the inside. An impulse arriving at a motor nerve ending results in a partial reversal, or depolarisation of this resting potential. This easily measurable depolarisation is usually termed the end plate potential. If of a sufficient size, this primary depolarisation evokes a further secondary depolarisation in the muscle fibre membrane. In the 'fast' vertebrate extrafusal muscle fibres, the additional depolarisation is referred to as the spike potential and this usually overshoots the zero potential level to a considerable degree. By this means, adjacent regions of the fibre are excited so that the process of depolarisation travels the whole length of the fibre. It is this spike potential which initiates the contractile process. We are thus presented with a mechanism in which the primary depolarisation is local, but the

secondary response it produces is subsequently independent of the end plate potential, and causes the contraction of the whole fibre. Since there is usually only one end plate per fibre in vertebrate skeletal extrafusal fibres of the fast type, a mechanism involving a fast propagated depolarisation is necessary to explain the efficient fast contraction of the individual fibres.

The response of any one single fibre of this type is an all or nothing event. If the end plate potential is sufficiently large to evoke a spike potential, then the whole fibre will contract. If the end plate potential is not large enough then there will be no contraction of the whole fibre since the primary depolarisation is not propagated, but simply decays away exponentially, only causing very local contractions in the end plate region. A graded contraction of a whole fibre is thus not possible with this mechanism. A muscle as a whole may exhibit graded contractions but these are brought about by varying the numbers of fibres contracting at any one time. Since vertebrate muscles are innervated by large numbers of motor axons each with its complement of innervated muscle fibres forming motor units within the muscle, graded contractions enabling very delicate muscle control can

be brought about by progressive recruitment or elimination of active motor units.

The possession of a fast propagated depolarisation is one method of obtaining an efficient contraction of a single muscle fibre, but certainly not the only method. An alternative method is one in which the muscle fibre could have many focal points along its length, each focal point initiating a local contraction. If these foci were innervated from the same source then synchronisation would result in the virtually simultaneous contraction of the whole fibre.

Insect muscles, like those of other arthropods, are innervated by only a small number of motor axons, and each axon often supplies most of the fibres in the muscle. There are also a large number of motor nerve endings along the length of the individual muscle fibres (see section I). These facts strongly imply that the mechanism of muscle fibre control in insects is likely to be very different from that found in the vertebrate fast extrafusal fibre, and is likely to approximate more to the alternative mechanism referred to above. The possession in insects of only small numbers of muscle fibres and very few motor axons does, however, pose great problems relating to the method of fine control of such muscles.

This simple picture has been complicated by the

discovery of Pringle (1939) that two separate types of electrical response exist in the flexor tibialis muscles of the cockroach. This finding has now been well substantiated, and two types of response are now known to exist in many insects (Hagiwara, 1953; Wilson 1954; Hagiwara and Watanabe, 1954; Hoyle, 1955a,b,1957a; Wood 1958). These two types of response seem to be linked to the activity of two separate motor axons. As early as 1932, Rijlant showed that two types of nerve impulse passed to the muscles of Musca domestica. Further evidence of the widespread probability of two separate muscle responses in single muscle fibres is given by the general picture of dual innervation in insect muscle fibres (see section I for discussion).

Although the type of myoneural anatomy so typical of insects was described fairly early (see section I), it was not until Pringle published the results of his investigations upon the cockroach in 1939 that other workers in the field of insect physiology began to consider insect neuromuscular transmission as different in fundamental character from that in vertebrates. As late as 1933 Friedrich considered the neuromuscular physiology of Carausius to be similar in character to the vertebrate system. Pringle (1939) named the two motor axons innervating the cockroach flexor tibialis muscle 'fast' and 'slow'. Subsequent workers have followed this terminology which is also

used in this work.

A 'fast' axon, when stimulated by a single pulse, produces a fast twitch contraction in the muscle. Repetitive stimulation of this axon results in the development of a powerful tetanus in the muscle. Single impulse stimulation of the 'slow' axon usually produces no or very little observable movement. Reptitive stimulation at high frequency results in a slow smooth contraction in the muscle. The greater the frequency of the stimulation, the greater is the tension produced. The two types of motor axons thus produce very different results in the muscle, even though they differ little histologically. In some cases, diameter differences can be seen, such as in Geotrupes (Marcu, 1929) and in Schistocerca and Locusta (Hoyle, 1957b) where the fast axon is slightly larger than the slow axon, but this is not always the case. In the locust there is the added complication in the extensor tibialis muscle of triple innervation, there being one fast axon, one slow axon, and one axon designated a 'hyperpolariser' by Hoyle (1955c), and now thought to be inhibitory by Usherwood and Grundfest (1965).

Thus, although the number of motor axons innervating insect muscles is small, at least two different types of response can be obtained from most fibres. This allows a mechanism for the delicate control of the whole muscle by modulation and superimposition of

one type of response upon the other. Such a mechanism can produce the fineness of control so characteristic of insects, even though some functionally important muscles in very small insects can only be composed of a few fibres. Insects can have a graded type of response which is equal in delicacy to that of the vertebrates, but it is simply achieved by a different mechanism to compensate for the small number of motor axons present.

As was outlined in the general introduction, any complete investigation of the process of neuromuscular transmission should try to relate the physiology of transmission to the myoneural structure present, and should also relate the effects of ions to the generation of the membrane potentials observed. As will be seen from the haemolymph and myoplasm analysis results in section III of this work, the Lepidoptera certainly form an 'interesting' group in the light of accepted ionic theory. The aim of the present investigations in the Lepidoptera is to elucidate the neuromuscular mechanisms in this advanced herbivorous group, and to relate such mechanisms to the myoneural structure. In section III, attempts will be made to relate the observed effects of ions upon the membrane potentials to the generation of these potentials.

METHODS

The experimental animals used in this investigation were placed with their ventral surface uppermost upon a 'Plasticene' bed inside a shallow dish. The animals were fastened to this bed by strips of 'Plasticene' passing over the legs proximal to the femoro/tibial joint. Such a procedure, while imobilising the animal, allowed the tibiae to move freely, the latter being needed in experiments where mechanical performance of the legs was being measured. The legs to be examined were surrounded with 'Plasticene' which was worked to form a well around the femur. The most convenient access to the flexor tibialis muscle was provided by removing part or all of the ventral cuticle. If this cuticle was pulled off from near the trochanteral joint to near the tibial joint in one single strip, the muscle could usually be exposed with little damage to the tracheal supply or the lateral muscle attachments to the cuticle. In certain cases, when pulling off the ventral strip, the lateral cuticle became fracfured. Such preparations could no longer be used to measure mechanical performance, but could still be used for the measurement of electrical responses. When dissecting preparations for experimental use, it was found to be very important that the large group of central tracheae should remain undamaged as far as possible. In cases where this tracheal

group was damaged due to cuticle fracture, a distinct depression in the activity of the muscle fibres was seen, usually accompanied by a fall in the resting potential of the individual fibres. When the preparation was ready for use the whole dish could be mounted on the stage of the manipulator for the recording of responses in the manner to be described.

THE MANUFACTURE AND TESTING OF ELECTRODES

To measure the real values of potential differences across cell membranes, it is necessary to insert an electrode inside the cell itself, causing as little damage as possible to the cell membrane. The invention of the glass capillary intracellular microelectrode by Graham and Gerard (1946), and its subsequent development by Ling and Gerard (1949) has made this possible. The original electrodes used by Graham and Gerard (1946) were drawn from 1 m.m. 'pyrex' glass, and when filled they had to be cemented into a much wider glass shank for final connection to the recording apparatus. The electrodes used in this present work were initially drawn from 7 m.m. 'pyrex' glass. This was pulled out in an oxy-gas flame to about 0.5 m.m. diameter. This was further drawn out by gentle heating in a small coal gas flame issuing from a capillary tube. The glass was then pulled sharply when it was sufficiently hot. Successful electrodes pulled in this way had a tip diameter of about 0.5 but were large enough to permit direct

connection to the recording apparatus without cementing. When drawn, electrodes were placed in a pile of possibly acceptable ones or immediately rejected for redrawing. Examination under the microscope (Ling and Gerard, 1949) revealed electrodes with broken, closed, or over-wide tips, and such electrodes were rejected. Electrodes with a long, even, tapering tip were accepted, and even though 0.5 μ is at the limit of resolution of the light microscope, the appearance of a good tip could generally be recognised. At a later stage in the work, some electrodes were pulled by machine, on a Palmer microelectrode puller, type H 101. By careful adjustment of the rate of applied heat, electrodes could be pulled with tips similar in electrical characteristics to those pulled by hand. Electrodes pulled by this method had a shank diameter of 2.0 m.m. This demanded a separate electrode holder, but in all other respects the two types of electrodes were similar.

Acceptable electrodes were filled with 3 molar potassium chloride as electrolyte, using the filling method of Tasaki, Polley, and Orego (1954). The electrodes were suspended in a beaker of methanol which stood in a vacuum dessicator connected to a water pump (Figure 20). The electrodes were filled with methanol under reduced pressure, then the methanol was replaced at normal pressure with distilled water. The water was subsequently replaced with the

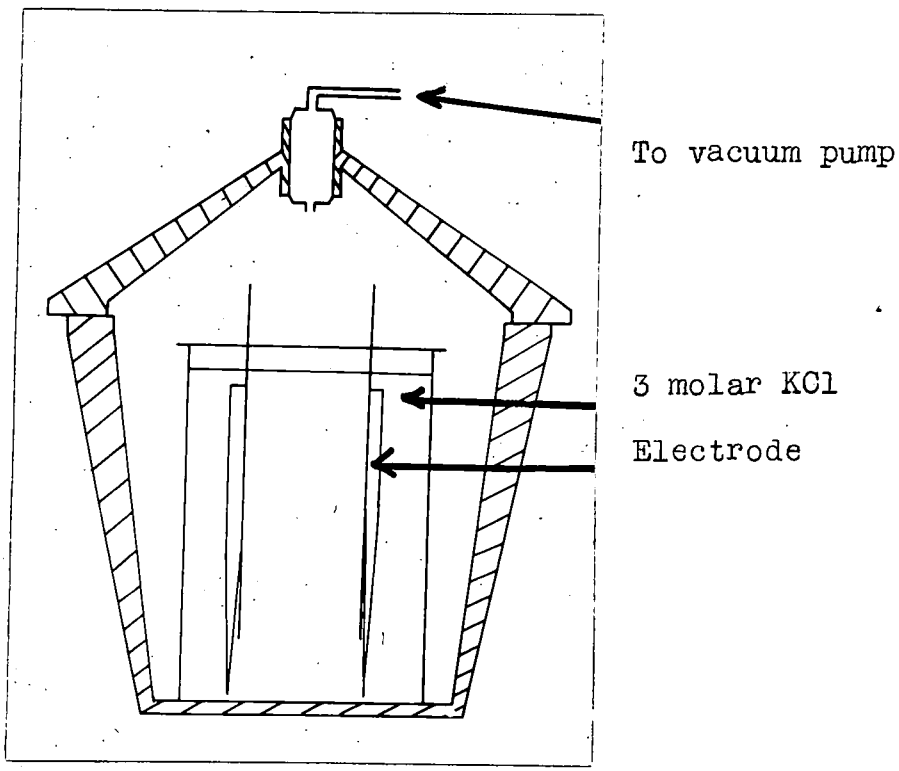
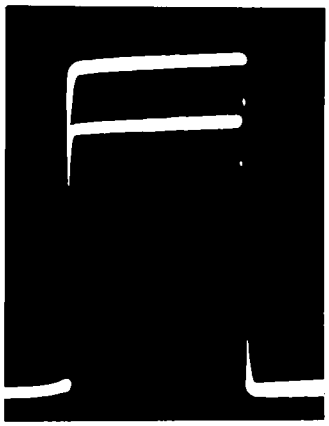


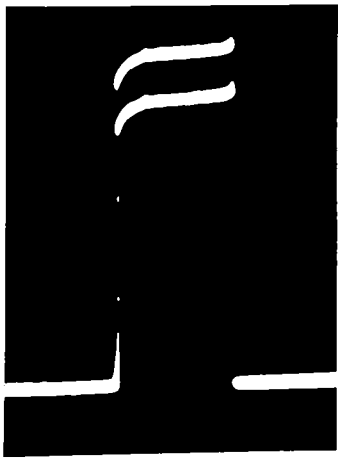
Figure 20. Method of filling electrodes at reduced pressure. The electrodes are held on the central cylinder by elastic bands.

final electrolyte, 3 molar potassium chloride,again at reduced pressure. This method allowed electrodes to be filled at room temperature, and avoided the breakage which occurred in the earlier methods of boiling in potassium chloride directly. The second stage of replacing the methanol with distilled water avoided the development of potassium chloride crystals in the electrode tip, which tended to occur when electrodes were taken from methanol to potassium chloride directly.

The final criterion for the acceptance of electrodes depended upon their electrical characteristics. Nastuk and Hodgkin (1950) have shown that for the best results the electrode should have a tip of about 0.5 μ, and a resistance of between 10 to 30 megohms. The electrical properties of the electrodes used in this investigation were tested by the application of a square pulse to the tip in the presence and absence of a 20 megohm shunt, noting the percentage drop in trace height each time. As a comparison standard, a 10 megohm resistor was used in place of an electrode, the percentage drop on switching in a 20 megohm shunt across the inputs being taken as the standard for electrode acceptance. In figure 21, the results of such a test are shown against a test with an electrode. To measure the absolute impedence



A. Trace of a square pulse recorded through a 10 megohm resistor in place of an electrode. Superimposed is a pulse recorded through the same when a 20 megohm shunt is switched across the inputs.



B. Trace of a square pulse recorded through an acceptable electrode. Superimposed is a pulse recorded through the same when a 20 megohm shunt is switched across the inputs.

Both sets of traces on same scale.

Figure 21.

of an electrode, the voltage effect upon a square pulse can be measured before and after switching the 20 megohm shunt across the inputs. If the initial voltage was V, and the shunt R, and the resultant voltage V_i, then the electrode impedence (Re) will be given by the relation :

$$Re = \frac{R\,(V - V_i)}{V_i}$$

(Donaldson, 1958)

If an electrode showed an excessive drop in trace height with a 20 megohm resistor across the inputs, then its total impedence was too high, probably having a restricted tip. Similarly, a very small drop in trace height in such circumstances would indicate an electrode with low resistance. Electrodes with electrical characteristics like that shown in figure 2 were accepted for use. It sometimes happened that electrodes contained small air bubbles near the tip, producing a distorted trace on the oscilloscope screen. If such electrodes were of the correct resistance characteristics they were returned to the potassium chloride bath and retested at a later date. In many cases these electrodes lost the distortion, presumably by diffusion of the air bubble in the electrode bath.

APPARATUS

The microelectrodes which were used to impale the single muscle fibres were fixed into the modified holder assembly of a Towers grease plate micro-manipulator. By means of the controls on the holder column, the electrode could be carefully manouvred a few microns at a time over the surface of the muscle fibres of the muscle under investigation. The preparation and the electrode tip were viewed during this process by means of a bench mounted Beck binocular microscope. The membrane potentials picked up by the electrode had to be amplified and displayed for viewing and quantitative measurement.

The apparatus which was employed in recording responses from single muscle fibres is shown schematically in Figure 22. The complete assembled equipment is seen in general view in Figure 23. The shielded recording leads from the microelectrode and the indifferent electrode in the muscle bath were taken directly to a Cathode follower input stage. An input stage of this type was necessary because of the very high impedence of the signal source. The cathode follower used was modified from the circuit designed by Bishop (1949) using EF 37A pentodes instead of the original 954 acorn valves, and matched with 22 megohm resistors to earth to suit the electrodes employed. This modified circuit is shown in Figure 24. The 20 megohm

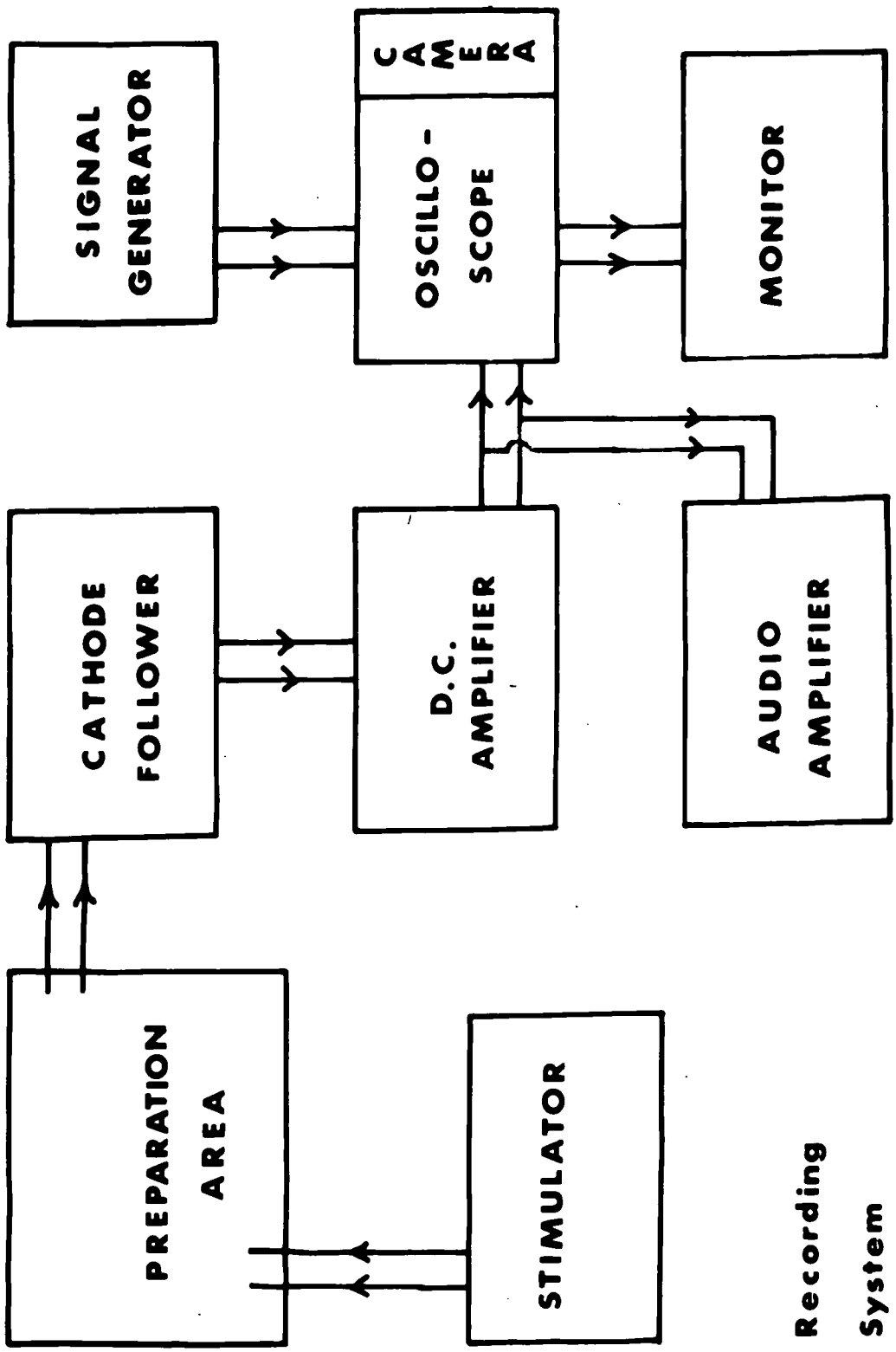

SIGNAL GENERATOR
CAMERA
OSCILLO-SCOPE
MONITOR
CATHODE FOLLOWER
D.C. AMPLIFIER
AUDIO AMPLIFIER
PREPARATION AREA
STIMULATOR
Recording System

Figure 22.

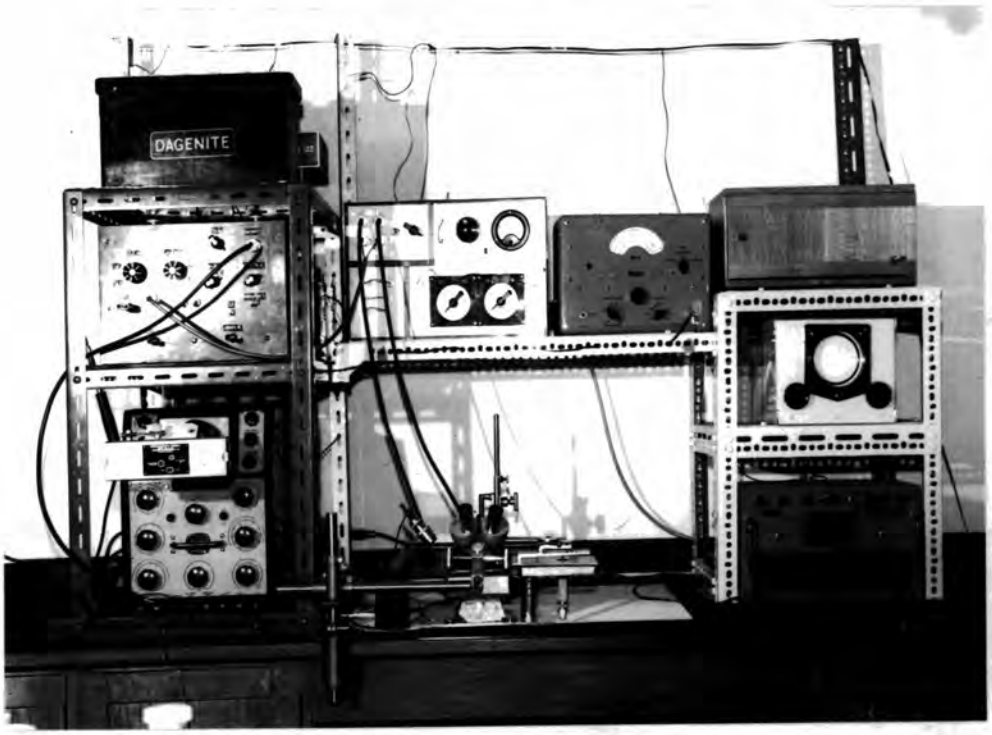


Figure 23. The complete recording system.

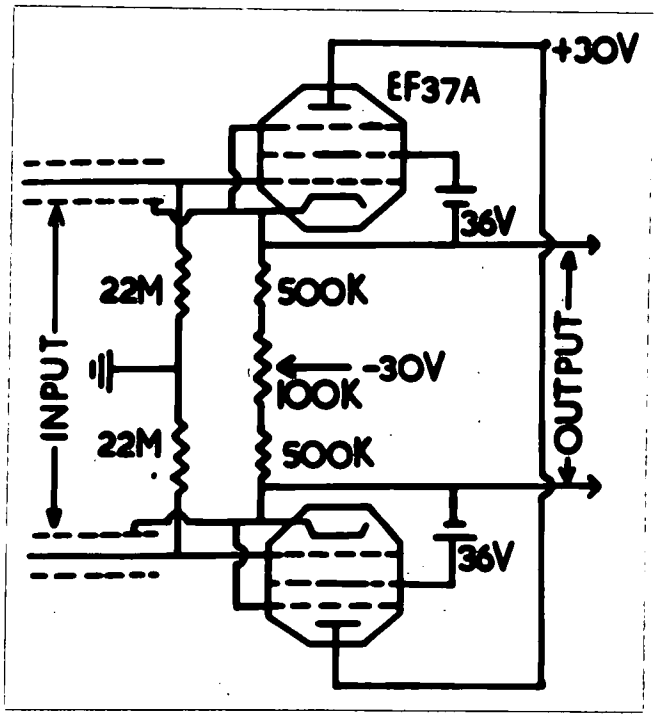


Figure 24. Cathode follower input stage.

shunt for the testing of electrodes was built into the cathode follower along with a simple calibration unit. The calibration unit was a simple circuit based on Ohm's law, and is illustrated in Figure 25. This unit provided ten and one millivolt steps by switching in units on the 10's and 100's decade resistor boxes.

From the cathode follower, the inputs were led to a D.C. coupled preamplifier with wide range D.C. balance control (Copeland, 1952), and then into a Cossor 1948 Mk. III oscilloscope. The oscilloscope was fitted with a Cossor 1428 Mk 2 camera with a differential drive unit for stationary spot recording on moving film. Ilford 5G 91 film was used for recording purposes throughout the work. A loudspeaker amplifier (Dickinson, 1951) worked in conjunction with the oscilloscope input so that signals could be heard as well as seen. This facility was particularly useful when undivided attention was needed during a difficult penetration.

Nerve stimulation was effected by means of a Palmer square wave stimulator which was also used to trigger the oscilloscope sweeps. Stimulating pulses were applied to the prepraration by means of silver wire electrodes made by tapering 0.oo7 in. diameter silver by electrolysis in silver nitrate solution. These electrodes were insulated almost to the tip by means of paraffin wax. The bared

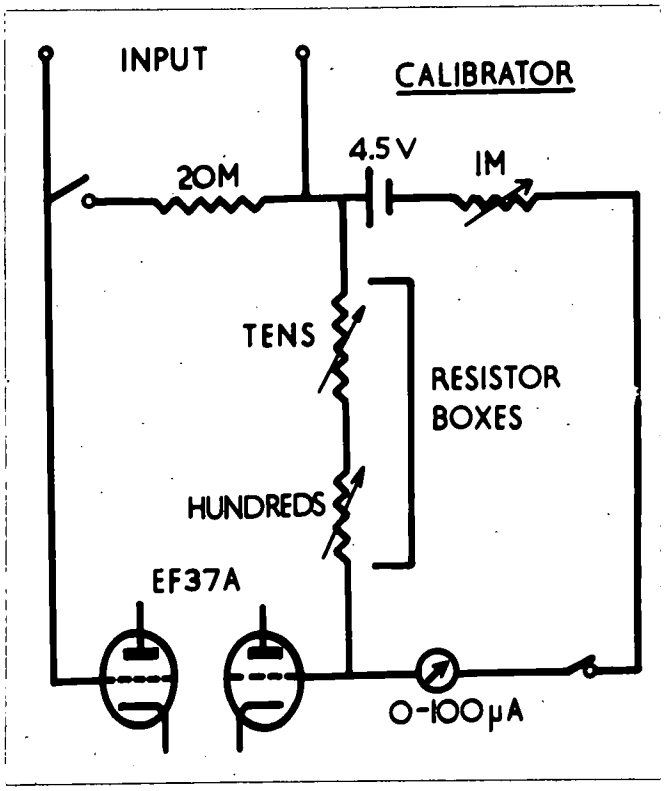


Figure 25. Calibrator circuit.

ends were hooked so that they could be inserted under the nerve and used to raise the nerve from the surrounding saline for stimulation. Manipulation of the stimulating electrodes was achieved by means of Palmer rack-work, placed behind the mounting stage of the Towers manipulator.

When the oscilloscope was in use for actual recording, the screen could not be viewed due to the presence of the camera. To overcome this, a monitor (Figure 26) was built to work in conunction with the main oscilloscope. This monitor had a long after glow screen which was useful for the inspection of transient responses.

The mechanical responses of the flexor tibialis muscle were recorded by means of a simple electro-mechanical transducer. This consisted of a glycerine resistance bath with a small potential difference across it, into which dipped a piano wire. The tip of the tibia was attached to the piano wire with a small piece of cotton, and movements of the wire were fed into a simple circuit (Figure 27) for amplification and subsequent display on the A2 input of the oscilloscope. For accurate time base records in action potential recordings, an Advance signal generator, type H 1 was employed. This was fed to the A2 input of the oscilloscope. The stimulator,oscilloscope, and signal generator were purchased commefcially, but all the other apparatus was built by the author for the work

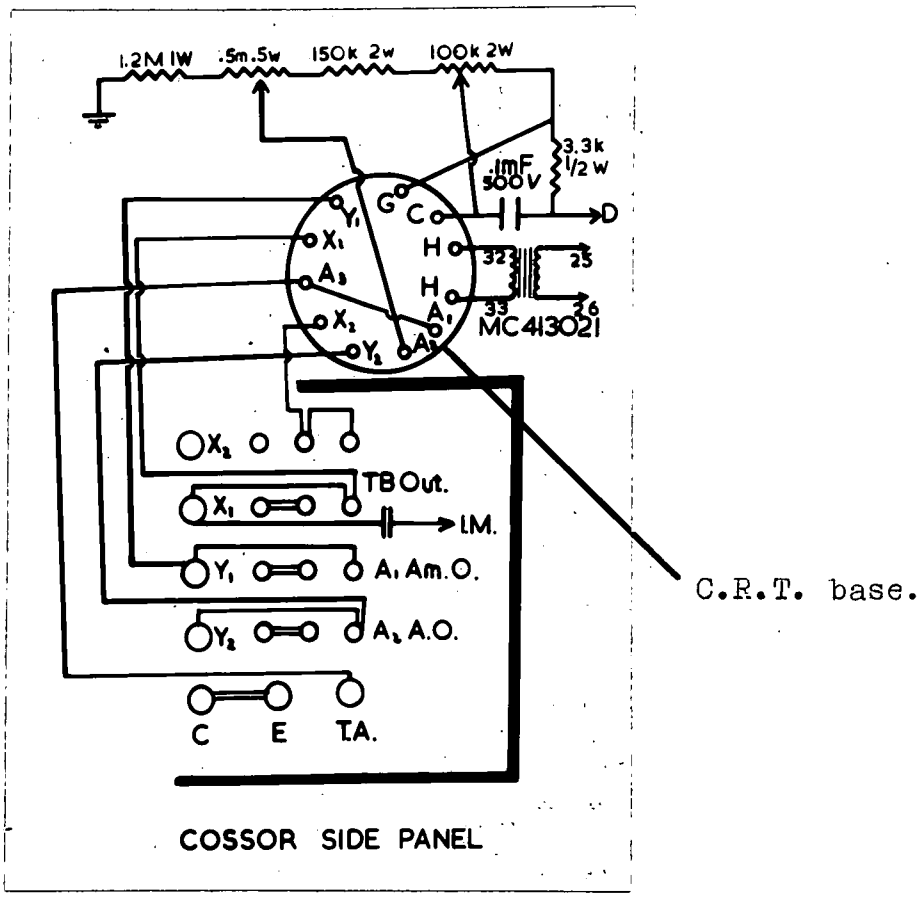


Figure 26. Oscilloscope monitor.

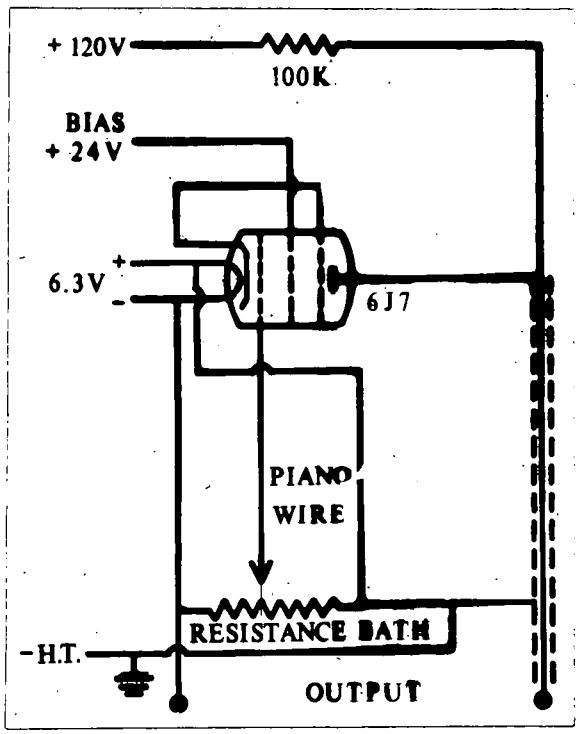


Figure 27. Circuit diagram of the electro-mechanical transducer.

contained in this thesis.

CRITERIA FOR ELECTRODE PENETRATION

When the microelectrode and the reference electrode were both in the saline bath surrounding the preparation, the Y1 trace on the oscilloscope screen represented zero potential. The Y1 trace was then superimposed upon the Y2 trace. By means of the micromanipulator, the electrode tip was manouvred until just above the muscle fibre membrane. The electrode was slowly lowered, and at one point, the Y1 trace was suddenly dropped, just as the microelectrode tip crossed the muscle fibre membrane. The resulting drop in potential on the oscilloscope screen was the negative resting potential of the muscle fibre. In some cases, slight fluctuations were obvious in the potential trace probably caused by blockage of the electrode tip during the penetration, especially if the electrode had been used many times before. Often the blockage of the electrode tip was a temporary affair, being relieved as the tip was withdrawn from the muscle fibre. Any electrode which continued to show erratic potential lines was discarded.

In normal circumstances, the plasma membrane would seal around the electrode tip, insulating the interior of the fibre from the external saline. When action potentials were being recorded, it was necessary to use the Y2 trace as a time base. The potential drop on entering the fibre

could still be easily seen, however, on the long after-glow of the monitor. Although muscle action potentials could be picked up by the microelectrode tip from the outer surface of the muscle fibre, once the tip had entered the fibre such potentials increased greatly in magnitude and became monophasic. This afforded another indication of correct penetration.

Sometimes a fibre became damaged either during the penetration or in the initial dissection of the preparation. This caused a gradual steady fall in the value of the action and resting potentials, the fibre becoming depolarised in a short time. In most experiments, the external saline bathed the preparation for between two to six hours before records were taken. By this time, any damaged fibres invariably showed membrane potential levels quite distinctive by their very low value, and records from such fibres were discarded. The probable reason for the fall in resting potential in these damaged fibres could be traced to two sources, damage to the trachea, with the resultant depression of the muscle fibre metabolism, and the damage to the muscle fibres themselves. The latter would cause a release of the very high concentration of internal muscle potassium in the immediate vicinity of the other muscle fibres, causing a further depression in their resting potential.

METHODS OF STIMULATION

Earlier investigations of insect neuromuscular mechanisms, upon the locust (Hoyle, 1955a), the cockroach (Pringle, 1939), and the stick insect (Wood, 1957b), involved direct stimulation of the crural nerve in the thorax just after the nerve had left the ganglion. Such a method allowed the study of one part of the animal without any disturbance from movements of the rest of the body. In the Lepidoptera, however, such a simple method of indirect muscle fibre stimulation was not possible in the meso- and metathoracic segments of the body owing to the highly specialised development of the thorax. In the meso- and metathoracic segments of these animals the coxae are fused along their length to the underside of the thoracic box in such a way that the trochanters meet in the mid ventral line. Any dissection of the thorax to reveal the metathoracic ganglion would thus involve dissecting off the coxae. To overcome this problem, two methods of indirect stimulation were developed. If a small strip of the ventral cuticle was removed from the first abdominal segment, the ventral nerve cord commisures from the metathoracic ganglion could be easily exposed. With experience it became possible to place the stimulating electrodes only about two m.m. away from the actual ganglion. Another method used was to remove a small strip of the ventral cuticle from the mesothoracic segment,

exposing the ventral nerve cord as it entered the metathoracic ganglion. The stimulating electrodes could then be placed under the nerve cord. Using these methods it was possible to stimulate the nerves emerging from the metathoracic ganglion, including the crural nerve which innervates the leg muscles. However, it was not possible to study contraction of leg muscles without causing contraction of the other muscles innervated from the metathoracic ganglion. A certain amount of body movement could not be avoided, especially movements of the legs on the other side of the body from that being studied, since all of the nerves emerging from the ganglion on both sides were being stimulated. To check that these methods of stimulation were not leading to spurious recordings due to activity of the other muscles of the leg and thorax, some recordings were made from the prothoracic leg. In this leg, the coxae are more or less free, so that conventional direct stimulation of the emergent ganglionic nerves was possible. No significant differences were noticed in recordings by this method, and in recordings by other methods used for the metathoracic leg.

DIFFERENTIAL STIMULATION OF SLOW AND FAST AXONS

Separate stimulation of the slow and fast axons required direct access to the crural nerve, hence experiments to obtain the slow responses in Lepidoptera were performed

of necessity on the prothoracic leg. The two motor axons in the equivalent of the crural nerve in the prothoracic segment could be stimulated separately by means of the anodal blocking technique developed by Kuffler and Vaughan Williams (1953) in which the distance between the electrodes is varied, and the anode of the stimulating electrodes is placed nearest the muscle and the cathode nearest the ganglion. The method relies upon the existence of small differences in the diameter of the slow and fast axons, which is usually the case found in insects, even though the differences may not appear great when examined in section (see section 1).

When the separate axons in a nerve are of different diameter, the impulses which initiate at the cathode propagate along the individual axons at different speeds. By choosing a suitable gap between the stimulating electrodes and by varying the width of the stimulating square pulse, it can be brought about that one impulse will just be passing the leading anode as the anodal pulse developes, and will thus be obliterated by the anodal pulse. The other nerve impulse, which is travelling at a different speed, and which is either following or has preceeded the supressed impulse will continue on its way to the muscle since it has not coincided with

the anodal pulse. By suitable arrangement, it is possible to suppress the fast nerve impulse leaving only the slow, thus permitting separate study of the slow muscle responses.

The salines used for this section of the investigation were made up from the analytical results contained in section III. In general they contained from 40 to 50 mM potassium, 5 mM calcium and sodium, and from 30 to 40 mM magnesium.

RESULTS

A. MECHANICAL RESPONSES

A single supraliminal stimulus applied to the 'fast' axon resulted in a fast twitch of the flexor tibialis muscle. This was accompanied by a twitch in the tibia which was, however, rather small compared with the very visible muscle twitch. A series of such tibial twitches are shown in Figure 28(A). These twitches are similar in magnitude to those reported in the stick insect (Wood, 1958), but are smaller than those reported in the locust (Hoyle, 1955b).

A graded increase in stimulus intensity applied to the fast axon, starting at the point just required for a mechanical response caused a stepwise increase in the amplitude of the twitch contraction. This suggests a gradual recruitment of different groups of muscle fibres,

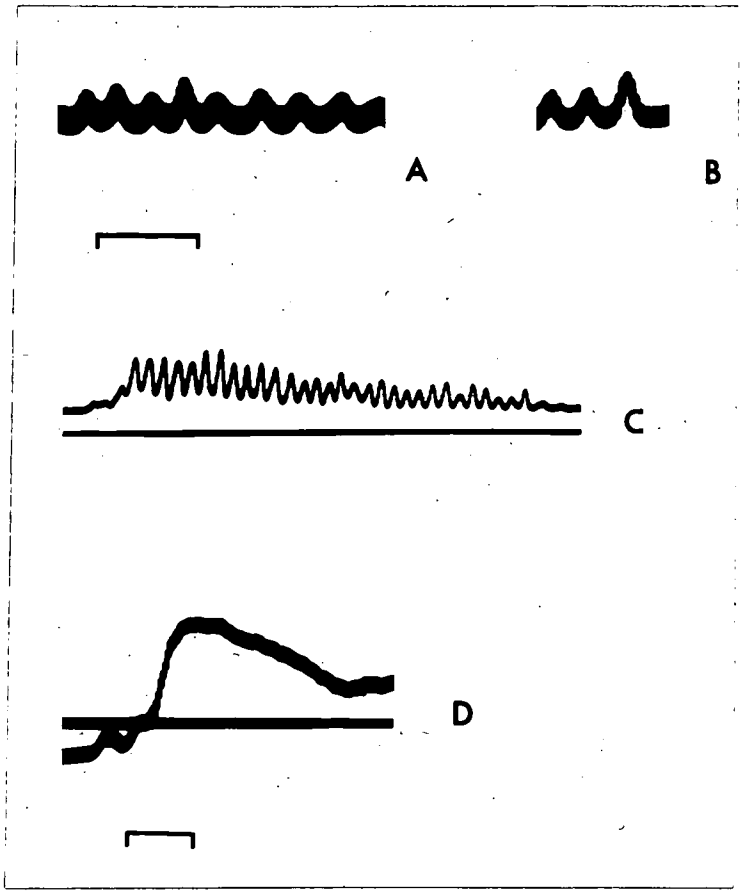


Figure 28. Mechanical responses from fast axon stimulation.

A. Series of single muscle twitches.

B. Effect of stimulus intensity increase on twitch height.

C. Twitches from repetitive stimulation at 10/second.

D. Tetanus after stimulation at 25/second. Note fairly rapid loss of tension in both C and D.

and is illustrated in successive twitches in Figure 28 (B).

Del Castillo ,Hoyle, and Machne (1953) reported that high intensity shocks applied to its 'fast' axon were followed by a tetanus in the extensor tibialis muscle of the locust. Wood (1958) was unable to confirm this in the stick insect, nor has it been observed in the Lepidoptera here examined.

Repetitive stimulation of the 'fast' axon was found to result in a fusion of the mechanical responses into a tetanus at about 20 stimuli per second (Figure 28(C+D)). In many cases however, the muscle quickly fatigued, and the peak tension developed was soon lost (Figure 28(D)).

B. MEMBRANE POTENTIALS

The resting membrane potentials of individual muscle fibres were measured by impaling the fibres with micro-electrodes. In salines approximating to the ionic composition of the insect's own haemolymph, the membrane potentials recorded from the four separate species of Lepidoptera under investigation are shown in table I,. In each species, the range of resting potentials was relatively wide, being from 38 to 64 mV. in Telea polyphemus, 34 to 48 mV. in Bombyx mori, 34 to 58 mV. in Actias selene, and 39 to 60 mV. in Sphinx ligustri. The small differences

Table 1.

Species	Resting potential (mV) Mean ± S.E.	Action potential (mV) Mean ± S.E.
Bombyx mori.	41.8 ± 1.4	40.1 ± 2.1
Telea polyphemus	48.2 ± 1.8	44.0 ± 1.2
Sphinx ligustri.	44.5 ± 2.2	42.3 ± 1.8
Actias selene.	46.4 ± 1.3	45.0 ± 2.3

between the mean values for resting potentials in these species are therefore not really significant, considering the range of values for each species. It has been argued (Kernan,1960: 1963) that recordings taken in artificial salines may differ from those actually found in the plasma of the animal. However, at the very beginning of an experiment the deeper muscle fibres were still bathed in the animal's own haemolymph, and one can thus compare results for surface and for deeper fibres. In one experiment in Bombyx mori, ten of the deeper fibres were examined and found to have a mean resting potential of 42.3 mV, with a range of 36 to 49 mV, values differing very little from those of the surface fibres in the same experiment, which are given in table I.

When the fast axon was stimulated, the muscle fibre resting potential was abolished by the production of the action potential, and the muscle fibres contracted in the form of a quick twitch. The threshold seemed to vary for different fibres in the muscle, since not all of the fibres could be guaranteed to contract unless the stimulus intensity was well above the minimum required for a contraction in certain fibres. This is probably linked with the increased contraction noticed with increases in stimulation intensity. (see above).

The normal action potentials in Lepidoptera are shown

in Figure 29. The action potentials of Telea polyphemus, Figure 29(B) was recorded on a fast time base to show the compound nature of the rising phase. The action potentials generally rose to a peak in about 5 milliseconds, being followed by a fairly long decay phase of 20 milliseconds or so. The decay phase time course was mainly taken up in the form of a prolonged negative after potential.

The actual values of the action potentials are given in Table I. Once again, a fairly wide degree of variation in value in any one muscle is seen in the case of each species. In Telea polyphemus the range was 40 to 50 MV, in Sphinx ligustri, 36 to 52 mV, in Bombyx mori 30 to 50 mV, and 32 to 54 mV in Actias selene. Not only did the action potentials vary in amplitude from fibre to fibre in the same muscle, but successive action potentials in the same fibre were subject to some variation also as a result of variation in the active membrane response. This can be seen in groups of action potentials recorded from the same fibre in Sphinx ligustri, shown in Figure 30.

As can be seen in Table I, very little in the way of overshooting of the zero potential level was present in these insects. In most fibres there was no overshoot, but in the few cases where an overshoot did occur it was noticed that the resting potential of the fibres involved was near the high end of the resting potential range for

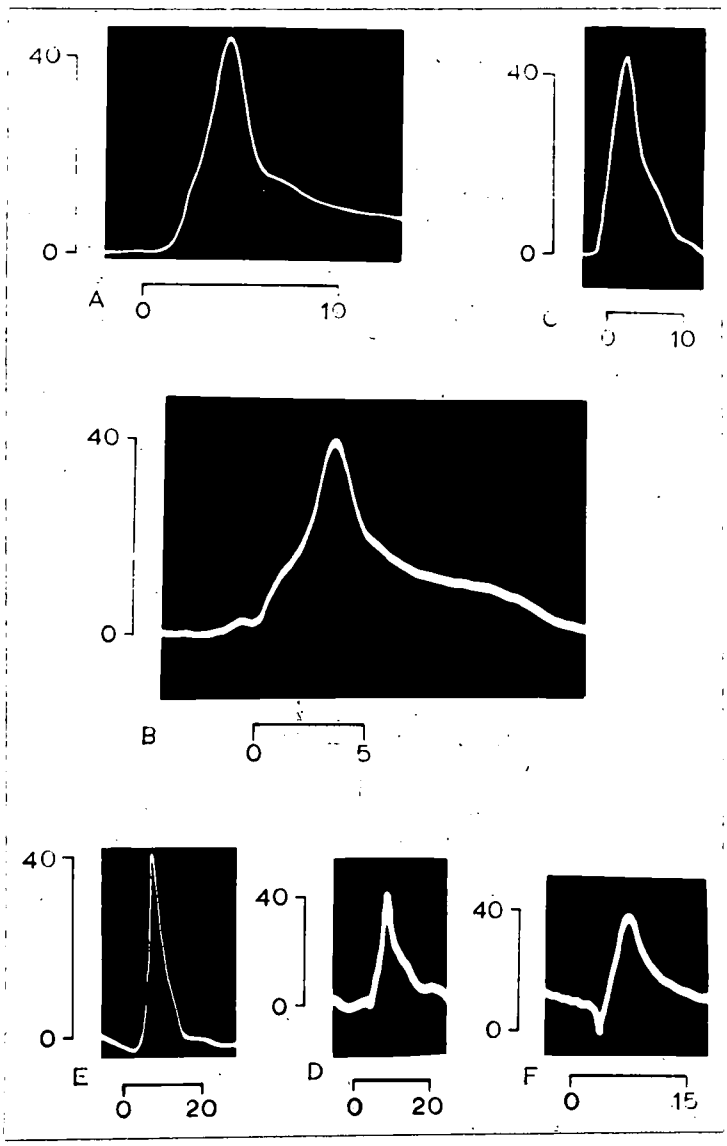


Figure 29. Lepidopteran action potentials. A & B, Telea polyphemus ;C & D, Actias selene ;E & F, Bombyx mori. Calibrations in milliseconds and millivolts.

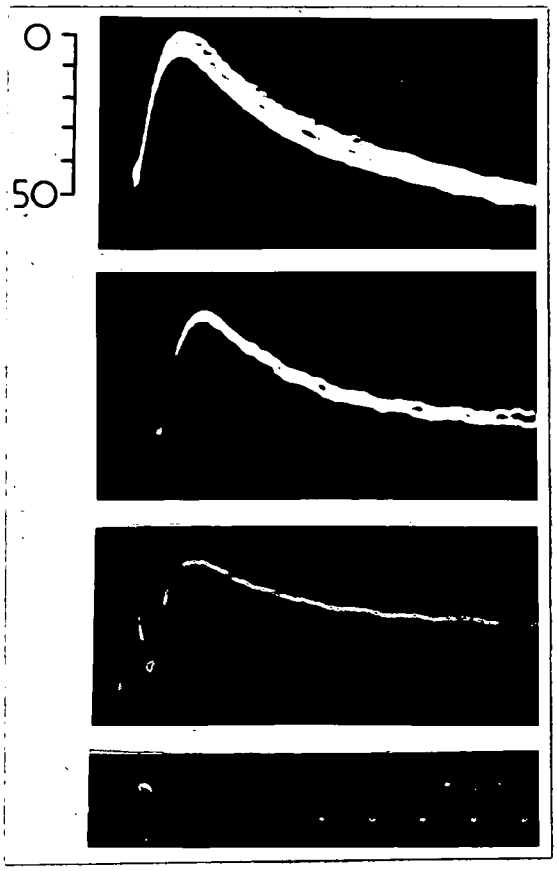


Figure 30. Successive action potentials recorded from the same fibre in the flexor tibialis muscle of Sphinx ligustri over a period of five minutes. Calibrations in millivolts, time scale 200 cycles/sec.

the animal. The mean amplitude of an action potential in a fibre appeared to be related to the value of the resting potential of that fibre. This tended to result in fibres with low resting potentials having low action potentials. However, when the resting potential of muscle fibres was artificially lowered by application of increased external potassium chloride, it was noticed that most fibres lost the overshoot completely, even those which had shown an overshoot in normal saline. In Bombyx mori, a few fibres with high resting potentials (45 mV. plus) were found to have overshoots of zero potential as high as 6 to 8 Mv, but as in Telea polyphemus most fibres were found to be without overshoot, and the action potential seemed to be related to the resting potential value.

Del Castillo ,Hoyle, and Machne (1953) linked the absence of an overshoot with the decay of their locust preparations. In the Lepidoptera however, even fibres from fresh preparations (ten to fifteen minutes after dissection) showed little or no overshoot.

Belton (1958) reported resting potentials in the range of 40 to 65 mV., and action potentials in the range 4^0 to 70 mV. in several species of Lepidoptera, while Van der Kloot (1963) reported resting potentials in the range 42 to 73 mV., and action potentials as high 56 mV. in

the intersegmental muscles of Hyalophora and Telea. The results detailed above seem well in the range reported by these earlier workers. One recent report however, is in contrast with these results. Carrington and Tenney (1959) working upon the late pupae of Telea polyphemus, reported resting potentials of only 15.4 mV. Since the haemolymph concentrations of pupae vary little from those of the adult (see Duchateau et al, 1953), it is difficult to reconcile such low results with those of other authors and those given above. It is possible that these authors may only have recorded from the embryonic membranes around the muscle in the pupae, producing spurious resting potential values from externally recorded muscle action potentials. This is however, difficult to check since the authors reproduced no photographs of actual responses in their paper.

In normal saline the action potential almost always showed a rising phase with a slight inflexion, indicating that more than one phenomenon was associated in the rise. The inflexion became particularly obvious when the action potential was observed on a fast time base (Figure 29,b, d,f). The rising phase of the action potential was seen to be composed of two component events, the first being the junctional potential, the second the active membrane response. The term 'junctional potential' has been employed

for many years in relation to crustacean and insect material, since it was originally thought that such material did not have the complex motor end organs of the type present in the vertebrates (see Hoyle, 1957b). Recent electron microscope work by Edwards et al (1958a,b) has shown that the wasp and cicada at least do have end-plates rivalling the vertebrate type in both size and complexity, and there seems no reason why other insects may not be similar. The results of detailed light microscope work are very scanty, but the drawings of insect end-plates shown by Hoyle (1957a) and by Wood (1957a), and the evidence presented in section I of this investigation indicate that insects do have Doyère cone endings of large size and complexity. As yet the 'en ligne' type endings so typical of crustacea and mammalian intrafusal fibres are unknown in insects, hence it is the opinion of this author that no real objection can be raised to the term 'end-plate potential' in insects, and the term will be used throughout the work. On the other hand although the active membrane response is in some ways analagous with the vertebrate 'spike potential', it differs in the important respect of being non-propagated in all insects so far studied, and so the former term will be used here.

In Telea polyphemus the end-plate potential has a value of about 15 mV., while the active membrane response

is about 29 mV. The overall rate of rise of the action potential was 9.2 Volts per second, that of the end-plate potential being 6.4 V/second, and that of the active membrane response 12 V/second. In Bombyx mori the rate of rise of the action potential was 10.7 volts per second. The end-plate potential was 14 mV, the active membrane response was 26 mV. The two components had slightly differing rates of rise, being 8.5 and 12 volts per second respectively.

C. THE NEGATIVE AFTER POTENTIAL

An interesting and persistant feature of the lepidopteran action potential was the part usually referred to as the negative after potential (see Brazier, 1960). The negative after potential could extend the decay phase of the action potential by as much as 20 to 30 milliseconds (Figure 29 a,b,d,f). The negative after potential in Telea polyphemus was about 15 mV and in Bombyx mori it was 13 mV. Generally the decay rate was negative all the way except in a few cases (Figure 29 d,f) where a small positive region was present. Quite prolonged negative after potentials were present in recordings of action potentials in the stick insect (Wood, 1957 b), the cockroach (Wood, 1961), the locust (Hoyle, 1955a ; Hagiwara and Watanabe, 1954 ; Wood, 1961), the cicada (Hagiwara 1953), and several Lepidoptera (Belton, 1958). After potentials

are also present in both the muscle fibres and axons of a wide range of vertebrates. Persson (1963) elicited action potentials in quick succession so that subsequent action potentials fell within the after potential of the first, and noticed that no summation or alteration of any kind took place in the value of the negative after potential. Frank (1957) however, found that the decay of the after potential was faster after several impulses in the fibre in quick succession. In addition, Frankenhauser and Hodgkin (1956) working upon squid giant axons, and Narahashi and Yamasaki (1960) working upon the cockroach giant axons found that successive negative after potentials added in a linear manner. The evidence from the Lepidoptera, on the other hand, supports Persson's findings. In Bombyx Mori muscle fibres no addition was seen during successive after potentials (Figure 31). It seems possible that apparent axons and muscle fibres behave quite differently in this respect.

D. THE EFFECT OF PHARMACOLOGICAL PREPARATIONS UPON MEMBRANE POTENTIALS

Several pharmacological substances, known to have quite definite effects upon the process of neuromuscular transmission in vertebrate muscle fibres were applied to muscle fibre preparations of Bombyx mori and Sphinx ligustri. The substance used were acetylcholine (10^{-3} to 10^{-5}),

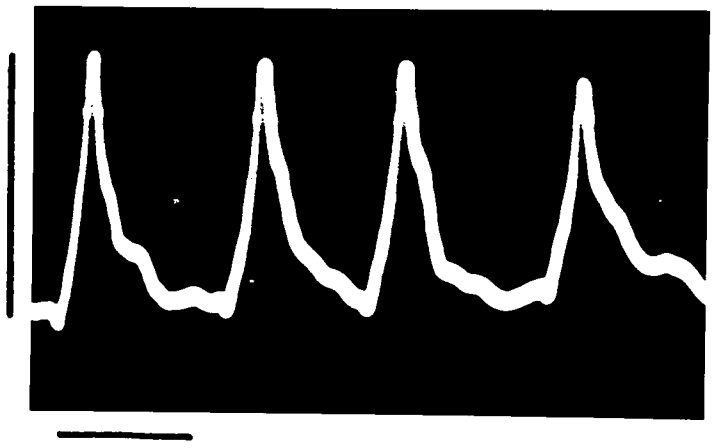


Figure 31. Successive action potentials from the flexor tibialis muscle of Bombyx mori showing no potentiation of the negative after potential. Calibrations 20 msec., and 40 millivolts.

atropine (10^{-3} to 10^{-5}), adrelalin hydrochloride (10^{-4} to 10^{-5}), and chlorpromazine hydrochloride (10^{-4} to 10^{-5}). These substances were added to the normal salines of the animals concerned. The test salines were allowed to bath the preparations for several hours, membrane potential recordings being checked at intervals. In no case was any alteration in amplitude and time course of the action potential seen, nor was any alteration of the resting potential seen with any of these substances.

E. THE SLOW RESPONSE.

During many experiments, slow spontaneous movements of the tibia were observed. These mechanical responses were not of the twitch type associated with the fast axon, but were due to a separate mechanism. Because of the very slow time relations of the mechanical changes observed, this type of response has been called the 'slow' response. Intracellular electrical recordings of some of these responses elicited by nerve stimulation (see section on stimulation) are shown in Figure 32. The slow response is much smaller in amplitude than the fast response, being only about ten millivolts. Unlike the fast response, there is no obvious inflexion in the rising phase of the slow responses so the latter may be considered to be a phenomenon which is similar in origin to the end plate potential, differing only in the feature of time course, in which respect the slow response has only one quarter of the rate of rise of

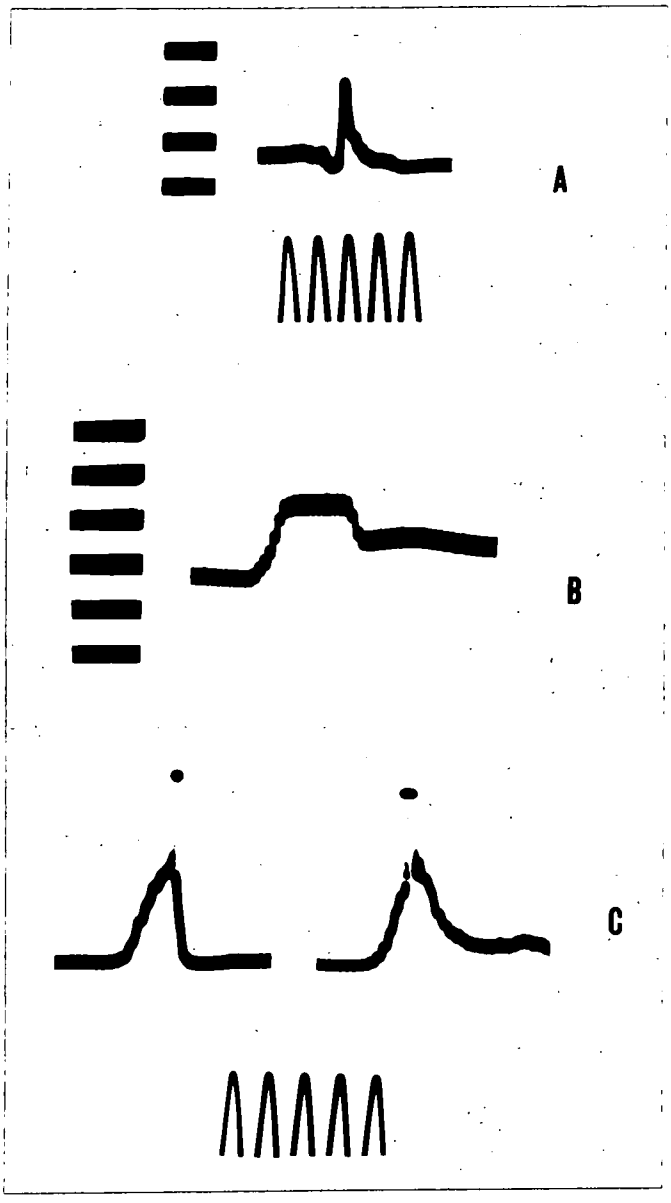


Figure 32. Slow responses. A, single response ; B, a train of responses ; C, fast and slow responses combined. Calibrations : Time, 20 cycles per second, Voltages, A- 5 mV. stages, B and C 10 mV stages.

the end plate potential. The decay phase is of the normal exponential shape found also in the end plate potential, but being about four times slower.

REPETITIVE STIMULATION OF THE SLOW AXON

At a stimulation frequency of 20/second, the slow responses are seen to fuse together (Figure 32b). At a stimulation frequency of 30/second the membrane depolarisation was further increased (Figure 32 c), and it would seem that membrane depolarisation level depended on stimulation frequency. A single slow response was found to produce no observable mechanical response in the tibia, and it would appear that any movements of value to the animal executed by the slow responses would have to be in the form of tetanic responses produced by long trains of impulses passing down the slow axon. This would certainly have to be the case in the very long protracted movements associated with maintainance of posture, not only in the leg muscles but in the body wall muscles also.

In several experiments using the anodal blocking technique (Kuffler and Vaughan Williams, 1953) in order to obtain slow responses by themselves, the fast axon was accidentally stimulated during the development of the slow responses. The result was that in certain cases, both fast and slow responses were obtained together from the fibre (Figure 32c). In the cases where this did happen, the fast responses were seen to add on to the

depolarisation already produced by development of the slow responses. Since the time courses of the fast and slow responses are very different, a single slow response having a full time course of 30 to 50 milliseconds, and a fast response having a time course of about 10 milliseconds, the components of the fast response were impossible to follow. The total depolarisation however, of these combined responses was greater than that of the fast response alone, overshooting the zero potential level to the extent of about 10 millivolts. This large overshoot is similar in most respects to the increased overshoot found by Wood (1958) in the stick insect when closely paired shocks produced combined action potentials greater in amplitude than a single response. The overshoot of 10 mV. in Bombyx mori (Figure 32c) is much greater than the sort of overshoot found in certain fibres with single responses of the fast type alone, and is by far the highest recorded overshoot in Lepidoptera so far.

Fast responses have been found in all fibres impaled but slow responses do not seem to be so evenly distributed since they have only been found in about half of the fibres in which they have been looked for. This does not necessarily mean that they were not present in other cases, since their detection depends on correct application of the anodal blocking technique. Hoyle (1957a) reports slow axon innervation varying between 30 to 50% of muscle fibres,

depending upon which leg was examined in Locusta, but Wood (1957a) found slow responses in all fibres examined in Carausius. It is possible that in this respect the flexor tibialis muscle of Carausius is simply unspecialised. The relatively unspecialised protharacic extensor tibialis muscle in Locust contained 50% slow fibre innervation, while the highly specialised metathoracic extensor had a corresponding figure of only 30% (Hoyle, 1957a). It would seem that specialisation of muscles for extra activity is correlated with a reducation in the number of individual fibres having an innervation by slow axons. In this scheme, the Lepidoptera would appear to be relatively specialised, but not to the extent of the locust.

DISCUSSION

The resting potentials of the four species of Lepidoptera given in Table I of this section can be seen to be in good agreement with the earlier results of Belton (1958) and Van der Kloot (1963). This serves to strengthen the argument that the very low results of Carrington and Tenney (1959) are probably spurious. The resting potential values of between 40 to 50 mV. for Lepidoptera are however, rather low compared to the usual values given for insects. Hoyle (1955a) quotes values of 60 mV for Locusta and Periplaneta, but a more recent investigation by Wood (1963) gives values of 63 mV and 53 mV respectively. Hoyle (1957a) also gives a value of 60 mV for the resting potential in Schistocerca, Calliphora, and Dytiscus, while values of 50 to 70 mV have been recorded in the locust Oxya (Hagiwara, 1953), Romalea (Cerf, Grundfest, Hoyle, and McCann, 1957) and Tenebrio (Belton and Grundfest, 1962a & b). The resting potentials of Lepidoptera are not however the lowest so far recorded among the insects. Hagiwara and Watanabe (1954) found only 41 mV in the locust Mecopoda and 42 mV in the Cicada Graptopsaltria while Wood (1957b) found only 41 mV in Carausius. Very low resting potentials in the region of only 35 mV have recently been reported in the muscle fibres of Ascaris by Del Castillo Mello, and Morales (1963).

Belton and Grundfest (1962b) have shown however, that

Tenebrio muscle fibres can easily be set to a wide range of resting potential values depending upon the concentration of potassium ions in the external saline (from 40 to 90 mV in Tenebrio for only a change of 1.5 to 7.5 mM potassium). Wilson (1954) obtained a value of only 45 mV for the resting potential in Periplaneta in a saline containing only 2.7 mM potassium ions. These conflicting results serve to underline the necessity of measuring electrical potentials only in salines which approximate closely in ionic composition to the haemolymph of the insect concerned, particularly in view of the results of Wood (1957b), and the results in Section III of this investigation which show the effect, not only of potassium but also of sodium ions upon insect resting potentials.

The comparable figures of resting potential for Crustacea are much higher than those in insects. Fatt and Katz (1953) reported 70 mV in muscle fibres of Potunus and Carcinus, while Atwood (1964) reported 71 mV in Cancer magister. Values of about 70 mV were obtained by Furshpan (in Hoyle, 1957b) in muscle fibres of Cambarus and Panulirus. The highest resting potential values of all are those reported in vertebrates, these usually being in the region of 90 mV. Such a value was reported in Rana (Nastuk, 1953 ; Kuffler and Vaughan Williams, 1953) and in teleost fast fibres (Barets, 1961). Mammalian muscle fibres also have high resting potentials. In the rat, Kernan (1963) found 91 mV, Hoffman

and Suckling (1953) reported 85 mV in the dog, while West (1955) reported 78 mV in the rabbit. The fast fibres of the hagfish however have resting potentials ranging from 60 mV to 77 mV, the mean being 66 mV (Alnaes et al 1964). This value is considerably lower than that normally met with in vertebrates.

An interesting feature of vertebrate fast and slow fibres is the difference they exhibit in resting potential value. While the hagfish fast fibres have a mean resting potential of 66 mV, the slow fibres have a mean value of only 40 mV (Alnaes et al 1964). The comparable results for teleosts are 55 mV for the slow fibres and 90 mV for the fast fibres (Barets, 1961). Kuffler and Vaughan Williams, (1953), found a resting potential of only 60 mV in the frog slow muscle fibres, whereas the fast fibres show 90 to 95 mV. Recently, Atwood (1964) reported two distinct types of muscle fibre, phasic and tonic, within the same muscle in Cancer magister. These fibres have resting potentials of 71 and 57 mV respectively. This author reports that such resting potential differences are present in other crab muscles, and it may be that such systems are widespread in the Crustacea as well as the vertebrates.

In addition to possessing fast and slow muscle fibres with differing resting potentials, vertebrate muscles are further complicated by the possession of intrafusal fibres which form the muscle spindle receptor organs. Eyzaguirre

and Du Vial (1956), working on the toad showed that the resting potentials of the intrafusal muscle fibres ranged from 61 to 88 mV. Koketsu and Nishi (1957) found an average value of 40 mV for the intrafusal muscle fibres of the frog. These values are well below the normal resting potential of extrafusal fast fibres, and in the case of the frog, the intrafusal fibres have even lower resting potentials than the extrafusal slow fibres.

The differences found in resting potential between muscle fibres in the same muscle, such as fast and slow in the Crustacea, and intrafusal in addition in the vertebrates are very remarkable and show a great contrast to the condition found in insects. In an insect muscle, probably all the fibres receive a fast motor innervation, and many in addition also receive a slow motor innervation, depending upon the level of specialisation of the muscle. No significant differences are found however, in the published values of resting potential between the individual muscle fibres in any one muscle, although a case may recently have been found in the locust (Usherwood, 1965). Until the position of the latter is clarified, the bulk of the evidence is still in favour of the existence of only one type of insect skeletal muscle fibre in any one muscle. It seems remarkable that the crustacea, which have an innervation pattern similar in most respects to the insect type should show resting potential difference trends rather like the vertebrates

to which they show no similarities in innervation pattern. In the case of the frog slow system (Kuffler and Vaughan Williams, 1953), there is no overlap between the fast and the slow innervation. The slow muscle fibres in this case have a very different innervation from the fast muscle fibres which are in the majority, and this could possibly explain the differences in resting potential between the two types of muscle fibre. In the muscle spindles of the toad (Eyzaguirre, 1957), an overlap of the fast and slow innervation to the intrafusal muscle fibres is present, and this may well be the case in other muscle spindles. The crustacea also show an overlap of fast and slow innervation to any one muscle fibre, and in this respect at least, the toad muscle spindle and the crustacea are similar in innervation to the insects. Considering these similarities in innervation it is very difficult to explain why crustacea and vertebrates show such resting potential differences within a single muscle while the insects show no such differences

The action potentials of the Lepidoptera which result from fast motor axon stimulation are similar in most respects to those recorded from other insect muscles, and from the Crustacea. The values of 9.2 and 10.7 Volts/second for rates of rise of the Lepidopteran action potentials, although higher than the 5 to 7 volts/second of Carausius

(Wood, 1958) are very low in terms of the vertebrates, and rather low in terms of other insects. The frog action potential has a reported rate of rise of 670 volts/second (Nastuk, 1953), while the cockroach has a rate of rise for the action potential of 36 Volts/second (Hoyle, 1955a). The locust (Oxya) wing muscle has a rate of rise of about 33 Volts/second (estimated from Hagiwara, 1953). The locust (Schistocerca) extensor tibialis muscle has a rate of rise for the action potential of 17 Volts/second (Hoyle, 1955b).

The slowness of the rising phase of action potentials in Arthropods is not correlated with particularly weak muscles. The closer muscles of the claws of the crustaceans Portunus and Carcinus have action potentials with a rate of rise of only 20.5 Volts/second (Fatt and Katz, 1953) but these muscles are very powerful indeed. What does seem to stand out is that the muscles of herbivorous insects have the slowest rising action potentials so far reported. It is interesting to note that Lepidopteran muscle fibres have been found to be rather impermeable to most of the normal ions found in physiological salines (see section 3) compared with the corresponding permeabilities in the vertebrates (Hodgkin, 1951). This could well be the cause of the slowness of the time course of the action potential in herbivorous insects, since any ion fluxes which may take place will be considerably slowed down.

By means of techniques such as recording at low temperatures, and also by recording on suitable time bases, the insect action potential has been shown to be a compound phenomenon composed of several parts (Hagiwara, 1953 : Hoyle, 1954,1957a ; Wood, 1957b). This can also be seen to be the case in the Lepidoptera. The concept of a compound rising phase is based upon the evidence of an inflexion in this phase, and the effect of ions such as calcium upon this inflexion (see Wood, 1957b), which is quite distinctive in most insects. The two components of the rising phase of the insect action potential are considered to correspond to the end plate and spike potentials of vertebrates (Hoyle, 1957b). It has been argued earlier in this section that the term 'end plate potential' should be applied to insect muscle responses, although Hoyle's (1957b) term 'active membrane response' is used instead of the vertebrate term 'spike'.

In both Telea polyphemus and Bombyx mori the end plate potential and the active membrane response have differing rates or rise. Wood (1958) found no such differences in the stick insect, and from the figures of Hoyle (1955a) in the cockroach and locust, any differences in the two components were slight. In the Lepidoptera, the differing time courses of these two components are obvious and remain so even during alteration of the amplitude of the action potential under the influence

of potassium ions (see section III). In the frog (Nastuk, 1953), the end plate potential had a rate of rise of 220 volts/second, while the spike response had a rate of rise of 670 volts/second. This large difference in rise time between the two components is typical of vertebrates (Hoffman and Suckling, 1953 ; West, 1955 ; Vaughan Williams, 1959 ; Barets, 1961) but there seems to be no obvious reasons why the Lepidoptera should resemble the vertebrates in this respect. This rise time difference implies that two separate processes may be involved. In the case of the vertebrates the very fast spike responses is probably related to the need for a fast propagated spike to activate the whole fibre quickly for a contraction, and although propagated spikes have been claimed in the crustacea (Fatt and Katz, 1953) no such claims have been made for the active membrane responses in insects.

Falk (1961) suggested that the specific permeability changes which give rise to the action potential rising phase would have ceased by the time of the onset of the negative after potential. From this Falk argued that the after potential would represent a passive recharge of the membrane. Frankenhauser (1962) and Persson (1963) have rejected this hypothesis since measurements of the membrane resistance during the negative after potential indicate that permeability changes were still appreciable as late

as ten milliseconds after the action potential peak. Further, these authors point out that the first part of the negative after potential is not exponential, as would be expected during a passive recharge. These authors have suggested that the negative after potential is due to certain permeability changes in the membrane such as a small late increase in sodium permeability, or a non-specific general permeability increase. The negative after potential is a delay in the repolarisation process, and could be due to either or both of two main causes. If there was to be a temporary fall in the permeability of the membrane to potassium ions, this would result in a repolarisation delay, such a delay would also come about if the membrane developed a secondary permeability to sodium ions. The former would seem to be the more probable since sodium ions have two factors governing their movement. Firstly, a membrane which is relatively impermeable to them, but particuarly so during the repolarisation phase, and secondly, the active sodium pump which would be effectively ejecting sodium ions from the fibre after the peak of the action potential. Any secondary permeability to sodium would thus have to be very large to overcome these two damping factors. However, the permeability results of Falk (1961) and Frankenhauser (1962) show that after the action potential, permeability of the membrane to sodium is fairly low, certainly not of

the order needed. In section III of this thesis evidence will be presented to indicate that, in any case, sodium does not have the importance, in relation to the transport of charge in the Lepidoptera that it has in many animals which have been studied.

There is however, another totally different mechanism which could be responsible for the negative after potential, that of active transport. Macfarlane and Meares (1958a,b) found that the negative after potential was reversibly abolished by metabolic inhibitors such as 2:4 dinitrophenol and sodium azide. The authors suggested that the after potential could represent an active inward transport of cations or extrusion of anions, depending on energy from oxidative phosphorylation. They also found that the after potential altered in voltage and time course with change of temperature and they suggested that the thermolability of the after potential indicated that active transport was involved in its generation.

Evidence will be presented in section III of this work to indicate that active transport of ions is a phenomenon associated with generation of membrane potentials in some insects at least, and in the Lepidoptera it seems probable that active transport of sodium ions into the muscle fibre may well be the main factor contributing to the negative after potential.

The end plate potentials of the Lepidoptera are

remarkable in being of rather low amplitude, generally falling in the range 14 to 25 mV. The largest portion of the action potential is the active membrane response, which typically falls in the range 23 to 30 mV. Other Lepidoptera investigated by Belton (1958) are similar. The situation in which the active membrane response is larger than the end plate potential is the reverse of the trend in other insects, but is similar to the condition in the vertebrates (see Table 2).

Del Castillo et al (1953) showed that in the locust, the size of the end plate potential was directly proportional to that of the resting potential. This relation has been tacitly understood to be a trend in other insects as well, so that between insects there may be thought to exist a constant relationship between size of end plate potential and size of resting potential.. The published results which have been compiled in Tables 2 and 3, and the graphs obtained from them (Figure 33) show that the ratio of the end plate potential to resting potential in insects is actually far from constant, and that the ratio of end plate potential to active membrane response is also quite variable. It is possible that in an individual insect the end plate potential/resting potential relation may be constant, but there is certainly no overall trend suggesting that there is a fixed constant ratio for all insects. What does emerge from the collected results in

Table 2 is that the ratio of resting potential to action potential is fairly constant over a wide range of values. The graph from these results shows a linear trend in the relationship between action potential and resting potential, not only in insects, but in crustacea and vertebrates also. The extent of the depolarisation of the fibre may set the extent to which it can be depolarised or even reversed.

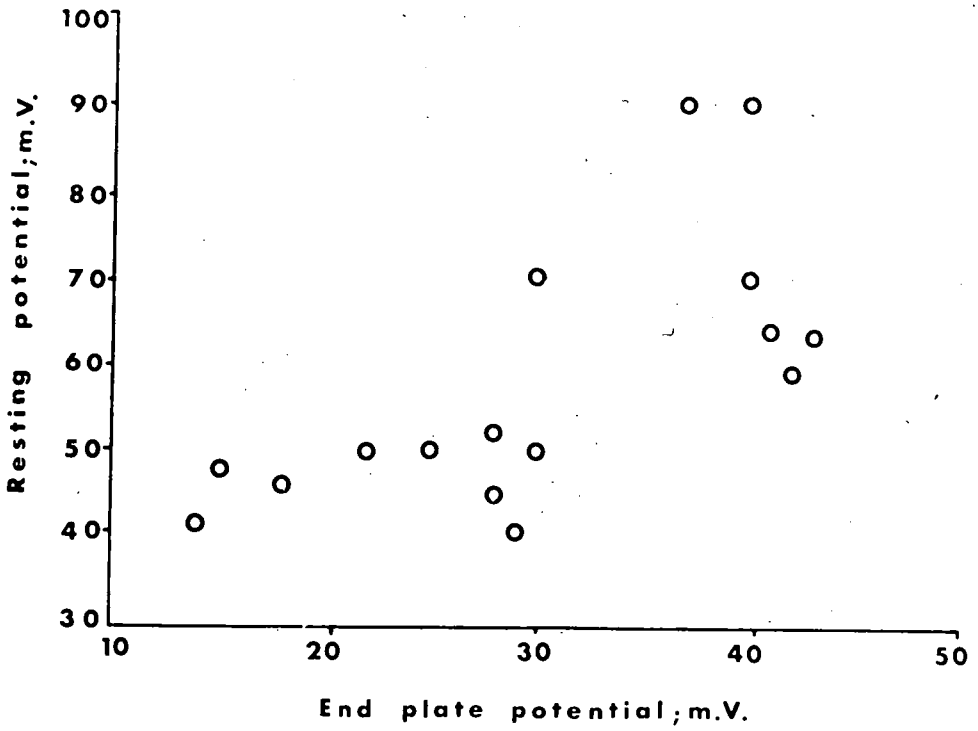
Hoyle (1957a) has reported that when action potentials fail to overshoot or even reach zero potential, this is nearly always associated with low resting potentials in the fibres concerned. The relation between resting and action potential discussed above would tend to agree with Hoyle's suggestion, in that one would expect to find low action potentials in muscle fibres with low resting potentials. However, it should be stressed that all data used in the above tables is based on mean values of resting and action potentials given by authors. Such mean values, especially in cases where action potential is equal to or slightly less than the resting potential, can hide some overshoots in individual fibres. Wood (1957b) has shown that in Carausius which has a low resting potential, many fibres had some (if small) overshoots of zero potential, even though the mean action potential was smaller in size than the mean resting potential. In Carausius there is thus a mean undershoot as there also

Table 2

Animal	End plate potential (mV)	Active membrane resp. (mV)	Ratio of epp/amr.	Reference
Carausius	29	18	1.6	Wood(1957a)
Locusta	41	28	1.5	Hoyle(1954)
Periplaneta	42	22	1.9	" "
Oxya	30	20	1.5	Hagiwara(1953)
Schistocerca	30	30	1.0	Usherwood(1963)
Arctia	28	26	1.1	Belton(1958)
Actias	28	30	0.9	" "
Philosamia	25	30	0.8	" "
Hyalophora	22	19	1.2	der Kloot(1963)
Telea	15	29	0.5	Original
Actias	18	23	0.8	"
Bombyx	14	26	0.5	"
Carcinus	40	40	1.0	Fatt & Katz(1953)
Portunus	30	40	0.75	" " "
Cambarus	30	30	1.0	Furshpan(IN Hoyle, 1957b)
Panulirus	40	38	1.1	" " "
Rana	40	65	0.6	Nastuk(1953)
Teleost	37	83	0.5	Barets(1961)

Table 3

Animal	Resting potential (mV)	Action potential (mV)	Ratio of A.P./R.P.	Reference
Carausius	41	39	0.95	Wood(1957a)
Locusta	64	69	1.08	Hoyle(1954)
Periplaneta	58	66	1.13	" "
Oxya	50	50	1.00	Hagiwara(1953)
Schisto-cerca	50	60	1.20	Usherwood(1963)
Arctia	45	54	1.20	Belton(1958)
Actias	52	56	1.08	" "
Philosamia	50	55	1.10	" "
Hyalophora	50	42	0.84	der Kloot(1963)
Telea	48	44	0.92	Original
Actias	46	41	0.90	"
Bombyx	42	40	0.95	"
Carcinus	70	80	1.14	Fatt and Katz(1953)
Portunus	70	70	1.00	" " "
Cambarus	70	60	0.86	Furshpan(in Hoyle 1957a)
Panulirus	70	78	1.11	" "
Rana	90	125	1.37	Nastuk(1953)
Teleost	90	120	1.33	Barets(1961)
Rabbit	75	100	1.33	Vaughan Williams(1959)
"	78	90	1.15	West(1955)
Dog	85	105	1.23	Hoffman & Suckling (1953)


Resting potential; m.V.
100
90
80
70
60
50
40
30
10
20
30
40
50
End plate potential; m.V.

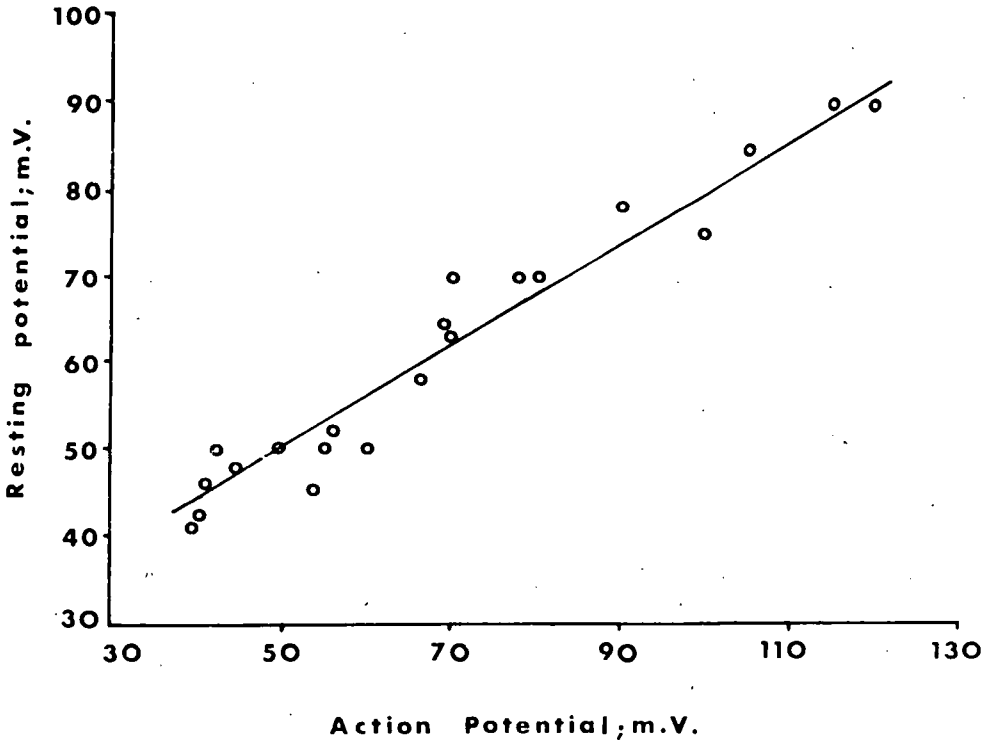

Resting potential; m.V.
100
90
80
70
60
50
40
30
30
50
70
90
110
130
Action Potential; m.V.

Figure 33

is in Lepidoptera.

The extent of contraction of a muscle fibre is said to be related to the extent of depolarisation of the fibre membrane by Hoyle, (1957a). The action potential in herbivorous insects is small, hence one would expect a relatively weak contraction to accompany each action potential in respect of the fast system only. The slow and fast responses can combine in certain cases (see Figure 32 and Wood, 1958). The result of this combination of responses is a superimposition of the fast depolarisation upon the slow depolarisation which results in a response which not only has a high degree of depolarisation, but overshoots zero potential to a considerable degree. In normal activity of the animal these two responses may well combine when extra depolarisation is needed for extra tension development. With the responses combined, the extent of depolarisation in Lepidoptera is as high as in most other insects with distinct overshoots and high depolarisation.

The very small magnitude of the twitch resulting from single nerve stimulation requires some comment. Nagai (1953) stimulated single crustacean muscle fibres and showed that the maximal contraction did not occur with only one stimulus. He suggested that in Arthropods, the contractile material of the fibre was not fully

activated by simple single depolarisations, and that at least more than one was necessary. Hoyle (1955b) has suggested that this may also be the case in insects. This can explain quite well the small nature of the single muscle twitches, and the great difference in tension developed between a single twitch and a tetanus. In Telea polyphemus the tetanus/twitch ratio was high, two estimates being 8 : 1 and 12 : 1. Wood (1958) and Hoyle (1955b) also reported high tetanus/twitch ratios in Carausius and Schistocerca respectively. This great difference in tension development is not easily explained in terms of simple mechanical summation. A concept of progressive development of full activation of the muscle contractile material such as Hoyle (1955b) and Nagai (1953) suggested seems to fit Arthropod results much more easily. This concept of faciliation of the contractile system seems a much better postulation than one of progressive increase in transmitter release or mechanical summation. Facilitation used in this context is defined as the potention of a second response either electrical or mechanical by a previous response. Summation is defined as a simple addition of either electrical or mechanical responses. The fast responses in the Lepidoptera show no facilitation, either electrical or mechanical, indeed a slight drop in height in the electrical record is usually noticed when several action potentials follow each other quickly (see

Figure 31). Wood (1957a) has reported summation of fast responses in Carausius if they are sufficiently close, but states that facilitation of the mechanical response or the active membrane response does not occur. He noticed that the end plate response, which is a graded phenomenon would facilitate [redacted] when pairs are elicited close together, and explained his results on this basis.

The small mechanical result of a single twitch contraction in Lepidoptera makes it evident that in normal functioning of the flexor tibialis muscle, any movement of the leg, no matter how small, would involve more than just single twitches. Movements of the leg probably involve long trains of impulses in both the fast and slow axons, bringing about a tetanus in the muscle. By means of the slow axon being used in conunction with the fast axon, very fine muscles movement is made more possible than would be the case if the fast axon only was present.

The slow responses in Lepidoptera are like those in Carausius (Wood, 1958), and certain Crustacea (Furshpan, 1955) and are rather small compared to the fast response, being only about one quarter of the amplitude of the latter. No inflexion is found in the rising phase, hence the slow response can be considered to be an end plate potential with a rather slow time course, about 40 milliseconds. Repetitive stimulation of the slow axon produces a fusion of the slow responses into a plateau-like tetanus. The

greater the frequency of stimulation, the higher the plateau, that is, the greater the depolarisation. Since the slow responses are graded, this behaviour is indicative of a process of facilitation.

The lack of effect of agents well known for their pharmacological effects upon vertebrate neuromuscular junctions is very striking. Since the insect neuromuscular junction, like other synapses, possesses a primary synaptic cleft (Edwards et al 1958a,b), neuromuscular transmission will almost certainly be effected by chemical agents. This is borne out by the diphasic nature of the action potential obtained from the muscles. The evidence above shows that this transmission agent is certainly not acetylcholine, even though this substance is abundant in Lepidoptera in the secondary sex glands (Morley and Schachter, 1963) and in the central nervous system of other insects (Lewis and Smallman, 1956). Roeder and Weiant,(1950) and Wood (1958) working upon the cockroach and stick insect respectively reported that acetylcholine was not the neuromuscular transmitter in those insects.

SECTION III

BLOOD AND MYOPLASM IONIC COMPOSITION AND THE EFFECT OF IONS ON MEMBRANE POTENTIALS

INTRODUCTION

It is now over a century since Matteucci and du Bois-Reymond showed that electrical currents could be obtained from living muscle fibres, and that the electrical effects observed were related in some way to the contraction of the muscle. The electrical potentials which are known to exist across the membranes of nerve and muscle cells have been the object of much investigation, particularly in relation to their origin and behaviour during activity. The most documented theory of the nature and origin of such membrane potentials centres around a concept of passive transport of some of the common cations of nerve and muscle, the ions moving along their electrochemical gradients with no expenditure of metabolic energy. At the beginning of this century, Bernstein(1902,1912) suggested that the resting fibre membrane was permable to potassium ions, but not to sodium ions, and that these ions were distributed according to a Donnan equilibrium, which resulted in the establishment of a potential difference across the membrane. Boyle and Conway(1941), after analysis of frog muscle and blood gave further evidence to support

the original idea of Bernstein. After various modifications to account for overshoots in action potentials, and active exclusion of sodium ions in fibres at rest, this original theory, generally called the Ionic Hypothesis has survived remarkably well to this day. The fullest statement of the ionic hypothesis is given in the review by Hodgkin (1951), supplemented by reconsiderations in Hodgkin (1958,1964).

There has always been a certain amount of criticism of the ionic hypothesis (Falk and Gerard,1954 ; Grundfest et al, 1954 ; Tobias, 1950) mainly from results of injection experiments, in which no resting potential elevation was noticed when potassium was injected inside fibres, or from the observation that the resting potential was not totally abolished when internal ions were removed from fibres. This work is rather old, and has not been followed up, but more recently new criticism of the ionic hypothesis has arisen over results which are not easily reconciled with the classical theory. Wood(1963) working on Carausius,Periplaneta & Locusta has shown that even though the relation between E_k and resting potential is fairly close in these insects, the action potential is more confused. The theoretical overshoot(E_{Na}) which this author calculated from analysis of internal and external sodium concentrations in muscle fibres bore no relation

to the actual values which were measured with internal electrodes. Belton and Grundfest(1962 a + b) showed that the internal and external potassium concentration in the muscles of Tenebrio molitor, using the Nernst equation, gave a theoretical E_k of only - 17 mV. The actual recorded values for resting potential ranged from - 50 mV to - 70 mV. Thus Wood found a large discrepancy involving sodium ions while Belton and Grundfest have found a large discrepancy involving potassium ions. In addition, Keynes(1962,1963) has recently shown that the position of the chloride ion in Sepia axons is far from ideal. His anlysis has shown that the chloride distribution in Sepia is different from what had been formerly supposed, giving a calculated E_{Cl} of only 39 mV. This value is much lower than the resting potential actually measured, and Keynes has proposed a system of active transport of chloride ions to explain these results, a simple Donnan equilibrium could not apply. Recently, Wood(1965) has found that chloride ions had little effect on resting potential in Locust and Cockroach muscle fibres, and that no correlation existed between the ratio K_i/K_o and Cl_o/Cl_i. Such a correlation should be found in theory.

The evidence outlined above in relation to the theoretical aspects of the ionic hypothesis very clearly

underlines the necessity of not only carefully measuring the effects of ions upon membrane potentials, but also of careful measurement of ion concentrations both in the blood of the animal, and in the myoplasm. This is important since many ions have very well known specific effects on neuromuscular transmission, hence measurement of both internal and external concentrations of these various ions will show to what extent the theoretical aspects of the ionic hypothesis are followed in insect muscle, and will also show how the muscle fibre membrane behaves under external ionic stress.

In all the experiments in this section involving sodium, potassium, and chloride ions, in addition to measurements of membrane potentials, internal ionic concentrations have been measured also, allowing theoretical considerations to be taken into account when discussing membrane behaviour.

As a result of a series of investigations on insect neuromuscular transmission, particularly involving magnesium, calcium, and potassium ions, Hoyle(1953,1954,1955a) concluded that neuromuscular transmission in insects was essentially similar to the process in vertebrates. As far as the locust and cockroach are concerned, this is very probably the case, since such insects have blood ionic concentrations similar to those found in the

vertebrates. However, when typical herbivorous insects are examined, a rather different ionic concentration pattern is encountered. The ionic concentrations in the haemolymph of insects are thought to be related to their diet (Bone, 1944 ; Clark & Craig, 1953 ; Duchateau et al, 1953). The result is that in typical herbivores, the blood sodium is low, and the blood potassium is very high. Both the sodium:potassium and calcium:magnesium ratios are well below unity. This is a complete reverse of the situation found in carnivorous and omnivorous insects and the vertebrates (Duchateau et al, 1953 ; Hoyle, 1955a). The only previous complete investigation into the neuromuscular mechanisms of a herbivorous insect was that of Wood (1957a, b ; 1958) upon Carausius. Even though the blood potassium in Carausius is high, with resultingly low resting potentials, the muscles were found to be efficient and possessed considerable contractile powers. This is strange in the light of the very high magnesium concentration of the haemolymph. Hoyle (1955a) found that only 20 Meqt. magnesium was enough to greatly reduce the end plate potential in locust, but this latter concentration is only about one fifth of the magnesium in Carausius haemolymph (Wood, 1957b). Although Carausius is a herbivore its ionic concentrations are modest compared with the very unusual composition of ions found in some herbivores. The Lepidoptera are extreme examples of herbivores with unusual

blood concentrations (Duchateau et al,1953). In some cases sodium is present in only vanishingly small concentrations, with a potassium/sodium ratio in the haemolymph as high as 50:1. The calcium/magnesium ratio is also often of this order. No data, however, is available concerning the intracellular muscle ion concentrations in most insects, and the little data there is has only very recently been published (Wood,1961,1965; Carrington and Tenney,1959 ; Belton and Grundfest,1962 a + b).

This section describes the results of ionic analysis of both the haemolymph and the muscle cells in the four species of Lepidoptera under investigation, as well as the effects of certain ions upon the membrane potentials of the muscle fibres. In the cases of sodium,potassium, and chloride ions, intracellular muscle ion analysis has also been performed to allow discussion of the theoretical aspects of the ionic hypothesis.

Are there endoplasmic reticulum spaces in this muscle — —

— releasing calcium as in vertebrates.

ANALYTICAL PROCEDURE

Analyses of haemolymph concentration of the main ions were carried out on all four species of moth. A small prick was made in the arthrodial membrane of one of the thoracic segments, and from the drop of haemolymph that oozed out a small measured quantity was taken up in a pipette, and then mixed with a small quantity of distilled water. The adults of all species contained enough haemolymph for several samples to be taken in a period of one week without harming the insects. Potassium, sodium, and calcium were estimated in the samples by means of an 'Eel' flame photometer. For magnesium and phosphate estimations, the proteins of the haemolymph were first precipitated by the addition of trichloroacetic acid, then after centrifuging, a protein free fluid was obtained. Magnesium was estimated by the method of Heagy(1948), using Titan Yellow, the red colour produced being estimated in an 'Eel' colorimeter.

The procedure for intracellular muscle ion analysis was much more lengthy. The optimum weight of muscle tissue needed for a three ion analysis was in the region of 4 to 8 milligrams. If much below this quantity, the chloride analysis was found to give fluctuating results. The muscle to be anlysed had to be excised carefully to cause as little damage as possible to the muscle fibres.

The cuticle below the flexor tibialis was removed in a strip and the scalpel inserted and drawn down either side of the muscle which was lifted out on its apodeme. The muscle was then quickly washed in distilled water to remove contaminating ions from the outside and from the extracellular muscle spaces. The latter proved very easy since the muscle fibres tended to fan outwards from the apodeme when their insertions were cut. After the washing the muscle was quickly scraped off the apodeme, blotted, and placed in a preweighed tube, then reweighed. The tubes of muscle tissue were placed in a rack inside an oven maintained at 105° C., and the muscles were dried to constant weight (usually taking about 16 hours) and then reweighed. The exact weight of muscle water could then be determined. The dried muscles were dissolved in a drop of concentrated nitric acid and when the reaction was complete the resultant solution was completely dried. Twenty microlitres of concentrated nitric acid were added to this and mixed at room temperature for one hour. Ten microlitres of this were removed for chloride ion anlysis, and the remaining ten microlitres were diluted to one or two c.c. (depending on the weight of the original sample) and this solution was used for potassium and sodium analysis. The two latter ions were estimated using an 'Eel' flame photometer as for haemolymph analysis. The chloride ion was estimated in

the first part of the sample using a modification of the Volhard back-titration (Wigglesworth,1938 ; Shaw,1955), in which a measured excess of silver nitrate was added to the sample on a 'fluon' tile. This was titrated against sodium thiocyanate issuing from a capillary burette, using ferric ammonium sulphate as indicator. During the titration the bubble was stirred by a fine jet of air from a capillary tube attached to an air pump. At the end point the bubble turned a maroon colour. The procedure for analysis of haemolymph chloride ion was similar, the haemolymph being transferred to concentrated nitric acid and silver nitrate on the tile.

All results of intracellular ion anlysis were expressed in terms of mM per kilogram tissue water. Although the extracellular muscle space was not a hindrance to the accuracy of ion analysis from a contamination point of view (see above) it was considered interesting to determine the muscle space for two main reasons. If the muscle extracellular space was large it would be necessary to take this into account when evaluating internal ions in terms of weight of muscle water, since the space would certainly be fluid filled, and also if the space was large it would be possible for internal ions to leak into it, hence possibly affecting electrical records from deep fibres in the muscle.

The extracellular muscle space was determined by a modification of the inulin clearance method (King and Wotton,1956). When warmed in the presence of HCl and resorcinol,inulin forms a cherry red colour which can be measured in a colorimeter. The whole muscle was allowed to soak overnight in a 1% inulin solution made up in saline (to prevent osmotic interference). The muscle was then washed and placed in an HCl/resorcinol mixture. Inulin from the extracellular space was extracted by squeezing the muscle and soaking. The cherry red colour produced was measured in an 'Eel' colorimeter against standards and the quantity of inulin in the sample was found. Since this came from the extracellular space, the latter was easily found. Two determinations were carried out in Sphinx ligustri giving 5% and 7% for the extracellular muscle space. Wood(1963) using a different inulin method obtained values around 4% for this space in Carausius, Locusta, and Periplaneta. The extracellular muscle space is thus very small compared to the whole bulk of the muscle and has been ignored in evaluating results.

RESULTS OF ANALYSIS

The results of haemolymph analysis of all four species of moth are given in Table 4. The results show fairly good agreement with the findings of earlier workers, with the exception that calcium is slightly lower than found earlier. From these results experimental salines were constructed for

all four species, using stock solutions already made up in one molar strengths. All salines were made isotonic with the lepidopteran haemolymph by the addition of 34.2 gms per litre (100 mM-) of sucrose (Belton,1958). These salines all differed from the 'moth saline' of Belton(1958) in containing more magnesium and less sodium. Belton claimed that his 'moth saline' corresponded to haemolymph concentrations in Actias selene (Duchateau et al,1953), but in fact he employed in his saline 30 mM sodium, instead of 4.8 mM, and 10 mM magnesium instead of 30 mM magnesium. His 'moth saline' in fact did not correspond to any of the lepidopteran haemolymph analyses in Duchateau et al(1953). The results of internal ion analyses are given in Table 5. The only other investigation in Lepidoptera (and one of the very few in any insect) is that of Carrington and Tenney (1959) on Telea polyphemus and this can be seen to be in reasonable agreement with the results in the table.

In considering the above ionic analysis results, an important question arises as to what extent the ions are free or bound. Insect blood has high concentrations of amines,amino acids, and proteins and it is possible that some of these may have a binding effect upon the ions present. Wood(1957a) after studying the effect of various cations upon the titration curve of blood amino-acids in Carausius concluded that the only ion likely to be bound in any significant manner was magnesium, and he made the

Table 4.

The results of ionic analysis of Haemolymph. All concentrations expressed as millimoles per litre.

HAEMOLYMPH IONS in mM/L						
SPHINX LIGUSTRI (L)						
K	Na	Ca	Mg	Phosphate	Cl	Author
49.8±1.3 [12]	3.6 ±.3 [9]	4.9 ±.3 [8]	36±2.5 [10]	14.5 ± 0.7 [13]	61.3±2.8 [13]	Original
48.4	4.3	7.5	-	-	-	Drilhon, 1934
52.8	2.6	8.2	24.6	-	-	Duchateau et al. 1953
TELEA POLYPHEMUS (CR)						
41 ± 1.7 [6]	3.3 ±.3 [8]	3.1 ± .14 [6]	29 ± 3.1 [7]	7.5 ± 1.0 [6]	67.5 ± 3 [8]	Original
34.6	9.8	8.1	-	-	-	Drilhon, 1934
54.1	2.5	5.8	36	-	20.8	Carrington and Tenney, 1959
BOMBYX MORI (L)						
41.3±1.2 [9]	9 ± .5 [11]	7.7 ± .4 [9]	42.3± 4 [8]	15 ± 2.1 [9]	68.2±3.2 [8]	Original
35.9	12.2	-	-	-	-	Tobias, 1948
41.5	11.3	12	34.7	-	-	Duchateau et al. 1953
ACTIAS SELENE (HUEBNER)						
47.2±1.5 [7]	9.1± .5 [7]	8.7± .9 [8]	26± 2.6 [6]	-	75.7 ±3.3 [6]	Original
51.3	4.8	15.7	30	-	-	Duchateau et al. 1953

necessary slight allowance for this in making up salines. An allowance of 5 mM in the magnesium concentration has thus been made in salines used in this work. As far as myoplasmic ions are concerned it is probable that the sodium,potassium, and chloride results represent the free state, but since both calcium and magnesium are involved in the contractile process it is very likely that the figure quoted for internal calcium does not represent the free state.

In constructing experimental salines for the work in this section the various ions had to be altered in concentration by altering their chlorides. In the experimental ptoassium salines, zero potassium was obtained by omitting KCl and using choline chloride to compensate for chlride loss. Above the normal blood concentration of KCl, potassium was raised by addition of potassium sulphate. Sodium salines were altered in a similar manner using choline chloride to maintain blood chloride concentration. The replacement of chloride itself presented the greatest problems. Although glutamate has been used in the past, Boistel and Fatt (1958) have shown that glutamate caused a large increase in membrane conductance. Acetylglycine was found by these authors to cause repetitive nerve discharge. The main disadvantage of sulphate is that it tends to remove some of the ionised calcium in the saline (Hodgkin and Horowicz,1959). As far as the resting potential is

Table 5.

Results of analysis of myoplasm. All concentrations expressed as millimoles per kilogram tissue water.

TISSUE IONS (mM Kg. Muscle water)								
SPECIES	K	Na	Ca	Cl	K_i / K_o	Na_o / Na_i	%Muscle Water	AUTHOR
SPHINX LIGUSTRI (L)	84.4 ± 7 [8]	20.7 ± 1.6 [8]	9.7 ± 1 [6]	14.5 ± 1.9 [4]	1.69	0.174	81.4 ± .82 [12]	Original
TELEA POLY-PHEMUS (CR)	78.9 ± 5.1 [5]	5 ± .7 [5]	7.2 ± .9 [5]	15 ± 1.4 [8]	1.92	0.660	77.2 ± 1.5 [6]	Original
.. ..	77.3	17.6	—	—	1.43	< 1	—	Carrington Tenney, 1959
BOMBYX MORI (L)	97.8 ± 8.7 [7]	12.4 ± 1.2 [9]	16.7 ± 2.8 [5]	13.3 ± 1.3 [8]	2.37	0.726	78.6 ± 1.3 [7]	Original
ACTIAS SELENE (H)	115.6 ± 5.2 [8]	16 ± 2.2 [10]	13.5 ± 1.3 [9]	14 ± 1.4 [8]	2.43	0.568	83 ± 1.2 [9]	Original

concerned this is no real problem since a fifteenfold alteration in external calcium only affects resting potential by about 11 mV. For most chloride replacement experiments, sulphate was used, and the calcium concentration was doubled (see Wood,1965).

Roeder and Weiant (1950) and Wilson (1954) both added some sucrose to the salines they used in the cockroach. Hoyle (1953;1955a) and Hagiwara (1953) added no sucrose to the salines they used in various Orthoptera and neither author noted any adverse effects upon the muscle fibres. Wood (1957b) however, noticed that when sucrose was not added to the saline for Carausius, the action potential declined in magnitude. Belton (1958) used salines containing sucrose for the Lepidoptera, and although there is no evidence that lepidopteran preparations are adversely affected by osmotic swelling, all salines used in this work contain 100 mM/litre. sucrose. For general purposes, a moth saline was constructed containing the following ions in mM per litre. K,50;Na,5;Ca,5;Mg,35; Phosphate 10. The pH of this saline was 6.4.

RESULTS OF EXPERIMENTS ON EFFECT OF IONS UPON MEMBRANE POTENTIALS.

Effect of Potassium Ions

As early as 1902, Bernstein postulated that the resting plasma membrane was selectively permeable to

potassium ions, and since that date a series of distinguished contributions to the literature (Hodgkin and Huxley,1939, 1945 ; Curtis and Cole,1940 ; Hodgkin and Katz,1949 ; Hodgkin,1964,1951) have clarified the position of the potassium ion as well as supplying a convincing weight of experimental data in support of a modified version of Bernstein's original idea. If the membrane was infinitely permeable to potassium ions then the membrane potential in the resting state would be related to their distribution according to the Nernst equation

$$E_k = \frac{R\,T}{F} \log_e \frac{(K)i}{(K)o}$$

Where Ki and Ko are the respective potassium concentrations inside and outside the fibre, R the gas constant, T the absolute temperature, and F the Faraday. The only assumptions involved are that the activities of potassium are the same on either side of the fibre (the work of Hodgkin and Keynes 1950, supports this) and that the membrane is infinitely permeable to potassium ions.

Preliminary investigations upon Telea polymphemus and Bombyx mori have shown that alteration of external potassium concentration affects the resting potential after a certain delay. A replacement time of 30 minutes was allowed before records were taken. Results of a typical experiment series are shown in Table 6 and Figure 34.

The resting potential was at its highest value in low potassium salines, and for a threefold increase in external potassium the membrane was depolarised by 80%.

Since the preliminary experiments involved no measurements of the intracellular potassium concentration, it was not possible to see whether the resting potential results followed the Nernst equation or not. More detailed investigations were hence carried out in Sphinx Ligustri and Actias selene, in which internal potassium content of the muscles was anlysed at each external potassium concnetration, in addition to the measurement of resting potentials over a wide range of external potassium concentrations. The dorso/ventral flight muscle prepration was used in the case of Sphinx, the flexor tibialis in Actias. A replacement time of 30 minutes was considered rather inadequate so the preparations were bathed in the experimental salines for six hours prior to recording. The results of two separate experiments are shown in Figures 35 and 36 and Tables 7 and 8. These graphs of observed resting potential plotted against the logarithm of the external potassium concentration have the same general hyperbolic shape as that for Bombyx mori and that found in many other excitable tissues (Hodgkin,1951). In Sphinx, for a threefold increase in potassium concentration, the muscle fibre membrane was

Table 6. The effect of external potassium concentration upon resting potential in *Bombyx mori*.

External Potassium in mM.	Number of Samples.	Mean resting potential(mV) ± S.E.
0	16	51.0 ± 1.0
10	9	43.6 ± 1.6
50	10	41.8 ± 1.4
70	14	22.6 ± 1.1
100	16	16.8 ± 0.6
150	15	8.6 ± 0.8
200	14	4.6 ± 0.4

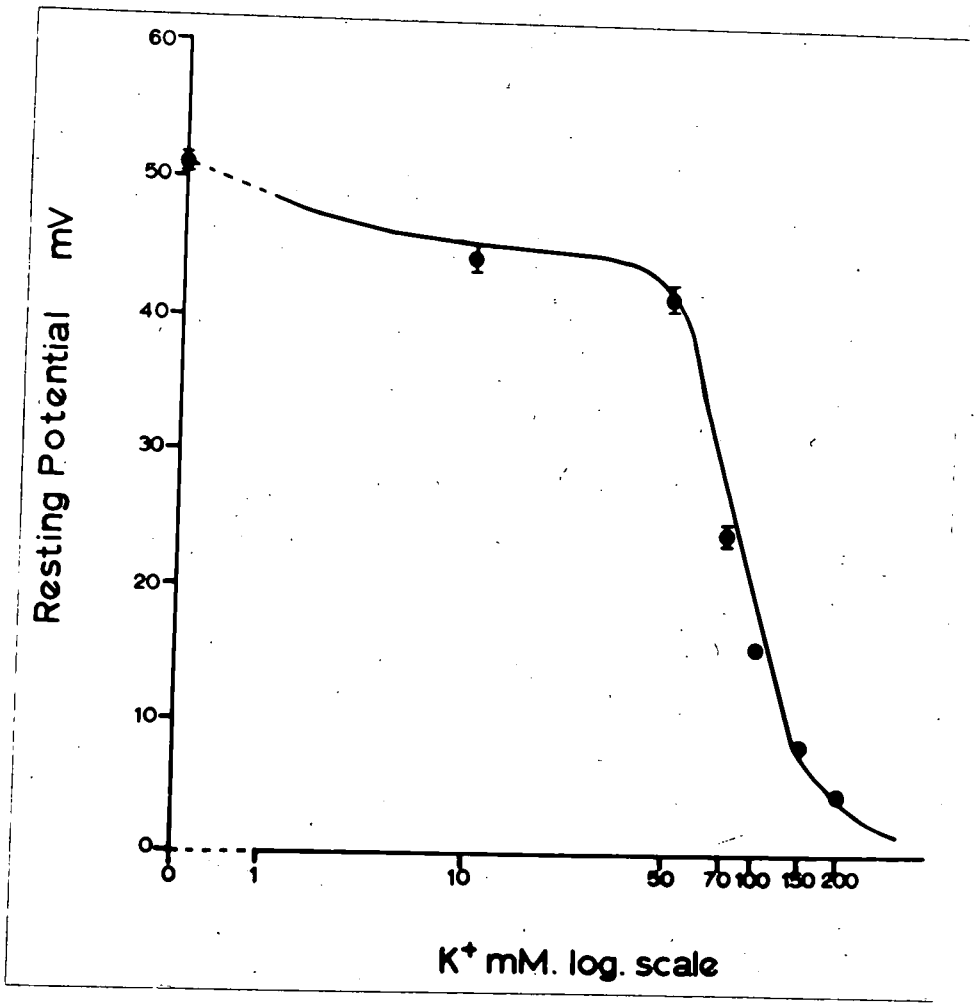


Figure 34. The effect of potassium ions on the resting potential of Bombyx mori muscle fibres.

depolarised by 60%,and the corresponding figure for Actias being 65%. The values for the concentrations of intracellular potassium are shown in Tables 7 and 8. As the external potassium was increased there was a corresponding increase in internal potassium, showing that the membrane was permeable to potassium ions. However, the rate of increase of internal potassium was smaller than that of the external potassium, so that in high potassium salines the condition arose where internal potassium first equalled then fell below external potassium concentration, so that the potassium gradient became reversed. From the values obtained for internal potassium it was possible to plot the theoretical membrane potentials which should have applied at the various external potassium values by using the Nernst equation. The theoretical E_K was found in both Actias and Sphinx to be almost a straight line. This would tend to support the idea that the insect muscle fibre membrane behaves in the strict theoretical manner with respect to potassium permeability. However, this is certainly not the case in either Actias or Sphinx. In these insects the potassium outgoing resting potential became up to +8 mV in Sphinx and +4 mV in Actias. No such positive resting potential has been recorded in either insect, neither approach more than - 10 mV to the zero

Table 7. The effect of external potassium concentration upon the resting potential in Sphinx ligustri.

External K^+ in mM	Internal K^+ in mM per Kgm. tissue water Mean ± S.E.	Theoretical E_k (mV)	Resting Potential Mean ± S.E. (mV)
1	45.4 ± 3.0	- 96.1	53.6 ± 1.2
5	61.4 ± 3.2	- 63.2	52.0 ± 1.0
20	71.0 ± 4.2	- 31.9	49.9 ± 1.5
50	84.4 ± 7.0	- 13.4	47.4 ± 1.0
70	96.0 ± 3.3	- 7.9	34.7 ± 0.8
100	111.2 ± 3.0	- 2.7	25.4 ± 1.2
150	130.1 ± 4.6	+ 3.6	18.2 ± 0.7
200	145.6 ± 8.3	+ 8.0	10.7 ± 0.6

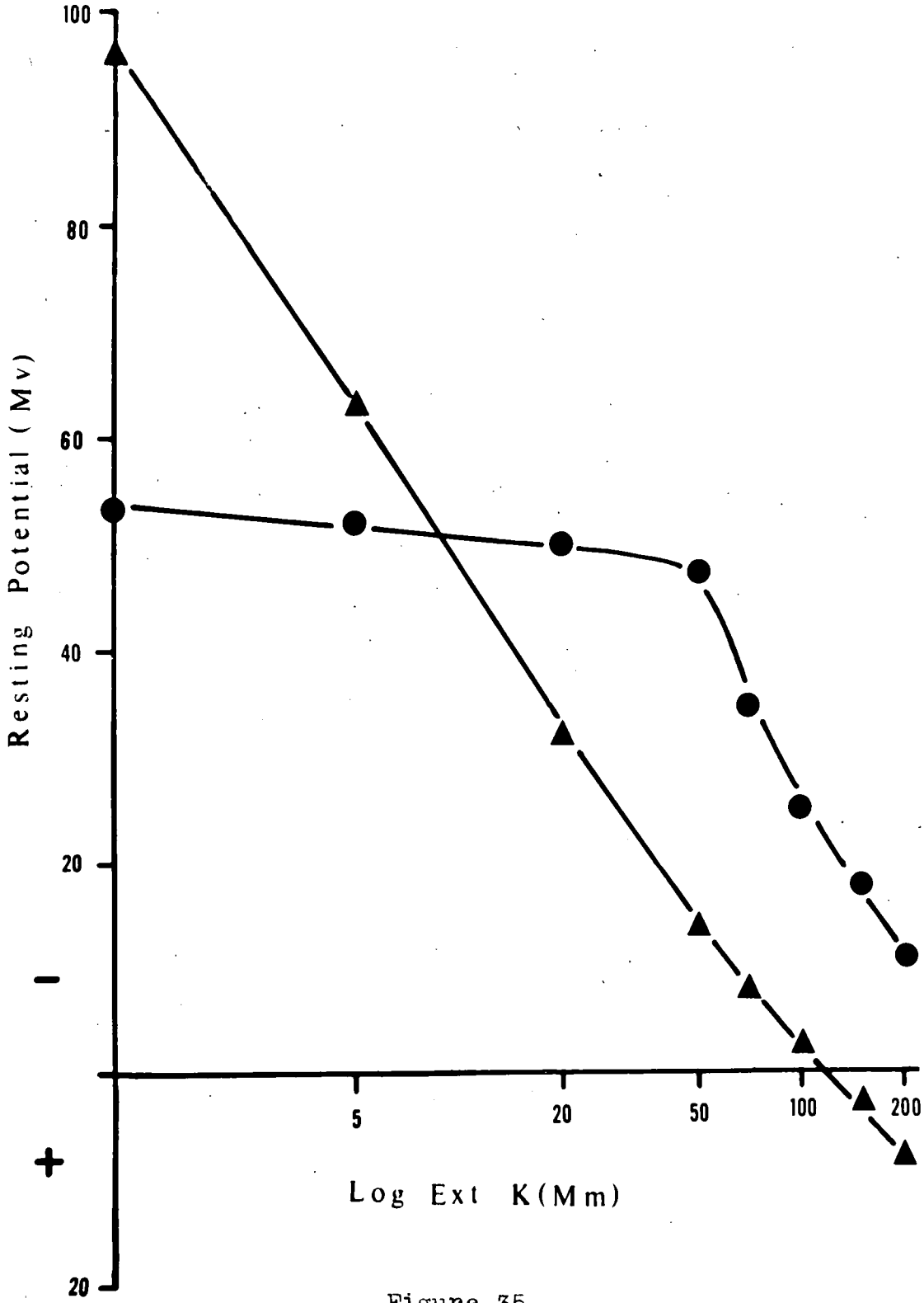

100
80
60
40
20
Resting Potential (Mv)
−
+
20
5
20
50
100
200
Log Ext K(Mm)

Figure 35

Table 8. The effect of external potassium concentration upon the resting potential in Actias selene.

External Potassium In mM.	Internal K^+ in mM per Kgm. Tissue water. Mean ± S.E.	Theoretical E_k (mV)	Resting Potential Mean ± S.E. (mV)
1	61.9 ± 5.5	- 103.9	54.4 ± 1.1
5	74.6 ± 4.6	- 68.0	54.1 ± 1.0
20	80.6 ± 6.4	- 34.9	51.5 ± 1.4
50	115.6 ± 5.2	- 21.0	46.4 ± 1.3
70	131.4 ± 4.2	- 15.9	32.9 ± 1.6
100	149.7 ± 7.1	- 10.2	21.9 ± 1.1
150	157.1 ± 2.2	- 1.2	15.2 ± 0.7
200	171.1 ± 5.0	+ 3.9	12.0 ± 0.9

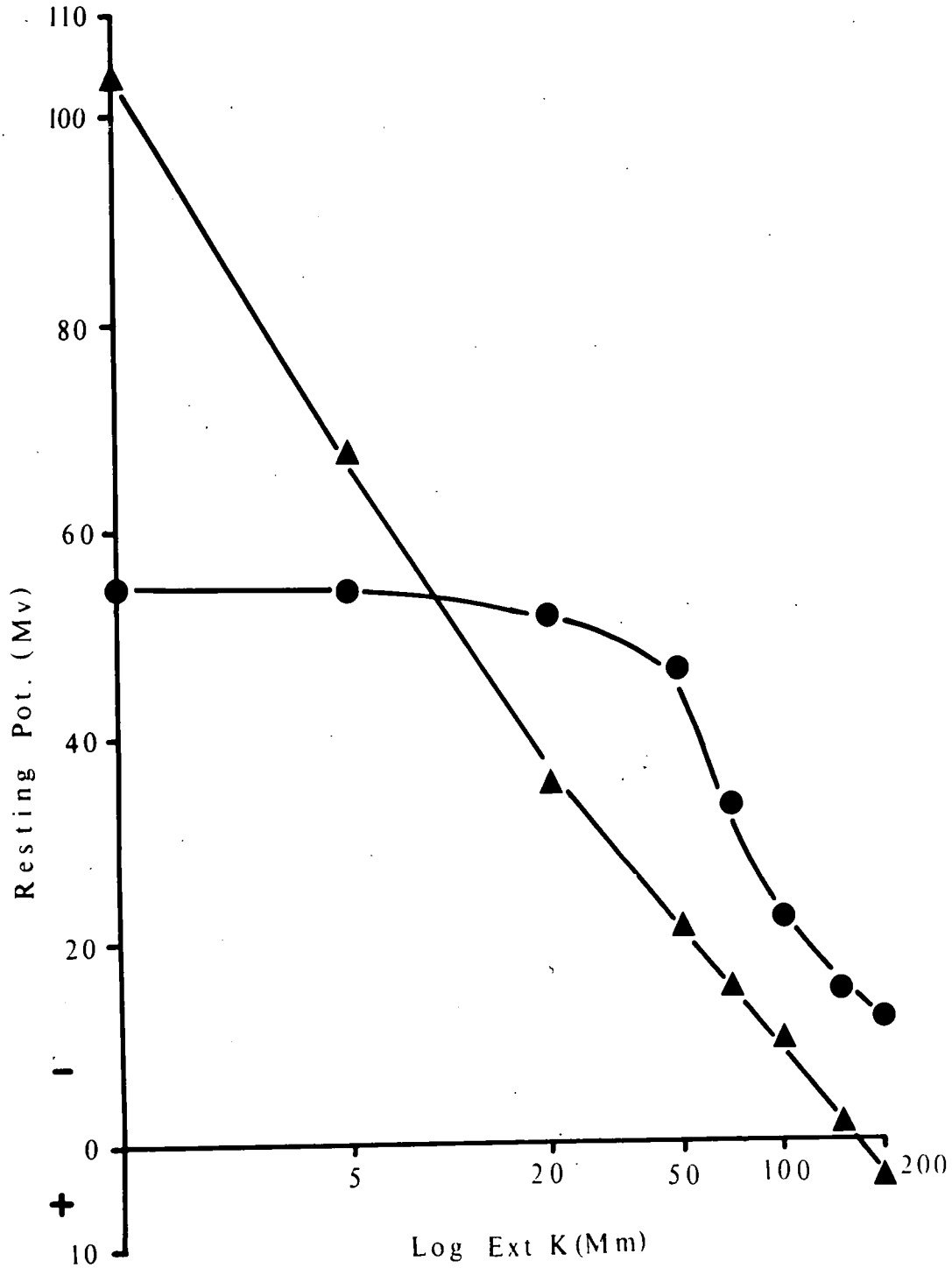


Figure 36

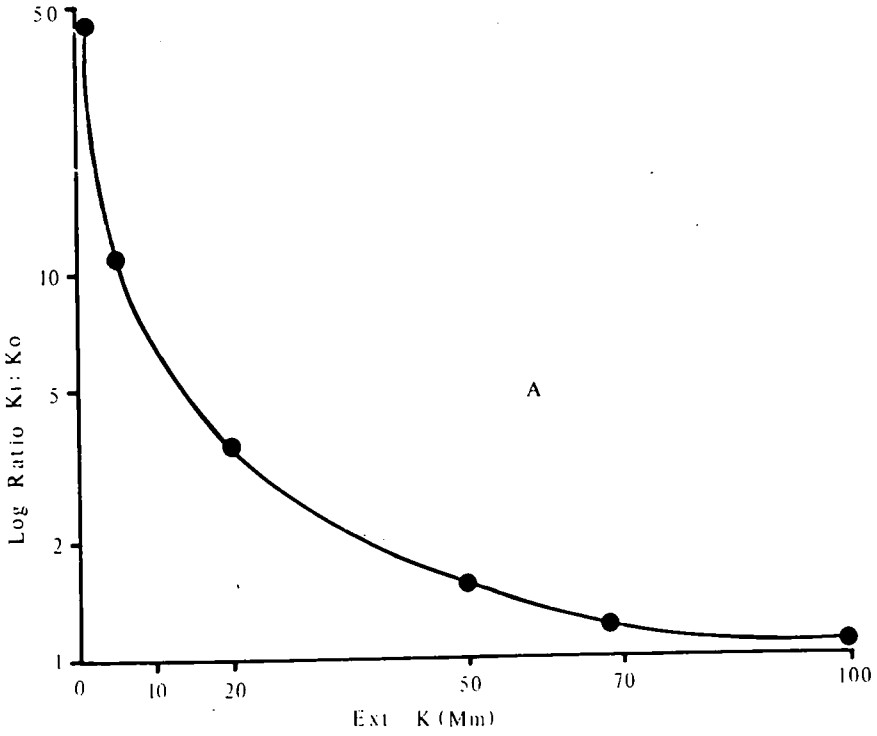
potential level. In both insects it is obvious that there is little relation between resting potential predicted from the potassium ion gradient, and the actual observed potential. The two sets of values diverge most widely at the very low and the high external potassium concentrations. The normal blood level of 40 to 50 mM potassium is nearer the theoretical than most other parts of the graph. In ImM external potassium the deficit between observed resting potential and predicted resting potential may be as high as 50%. In high potassium salines the reverse was the case, the observed resting potential being greater than the theoretical potential. This resting potential 'surplus' amounted to about 19 mV in Sphinx and 16 mV in Actias. In the middle range of external potassium, which is the range met with in the blood, the graphs of observed and calculated resting potential approach parallel conditions, with a linear deficit of 22 to 30 mV in Sphinx and 15 to 25 mV in Actias. At no point is there any obvious correlation between observed and calculated resting potentials. Belton and Grundfest(1962 a + b) found that in Tenebrio the muscle fibre membrane did not behave like a potassium electrode. In normal conditions they found a large resting potential deficit from 33 to 53 mV.

Since the ratios K_i/K_o were known for Sphinx and

Actias it was possible to plot these against external potassium concentration. The graph (Figure 37) shows the rate of change of the permeability of the membrane under the influence of externally applied potassium. The ionic hypothesis stipulates that the resting fibre membrane is readily permeable to potassium ions. Thus K_i/K_o for a wide range of external potassium values should be a straight line when plotted against external potassium, and the slope of the line, i.e. the rate of decrease of K_i/K_o should be proportional to the slope of the resting potential value divided by a constant (R.T./F), and should be constant itself. The graphs in Figure 37 are far from this ideal condition. As external potassium is raised, the membrane permeability to potassium increases rapidly in an expotential manner. The membrane appears to be unable to keep potassium out and the ratio K_i/K_o falls dramatically from 63 to 0.8 for a two hundredfold increase in external potassium. The permeability of the lepidopteran muscle fibre membrane thus deviates greatly from the conditions of the Nernst equation. The permeability of the membrane even in the physiological range is evidently much lower than had formerly been supposed for insect material. Recently, Maisky(1963) showed that in frog muscle fibres the resistance (i.e. permeability) varied with external potassium. Raising external potassium

Table 9. The effect of external potassium upon the ratio of external to internal potassium in Actias selene and Sphinx ligustri.

External Potassium in mM.	K_i/K_o in Actias selene. (A)	K_i/K_o in Sphinx ligustri (B)
1	61.9	45.4
5	14.9	12.3
20	4.0	3.6
50	2.3	1.7
70	1.9	1.4
100	1.5	1.1
150	1.1	0.87
200	0.85	0.73


50
10
5
2
1
Log Ratio Ki: Ko
A
0
10
20
50
70
100
Ext K (Mm)

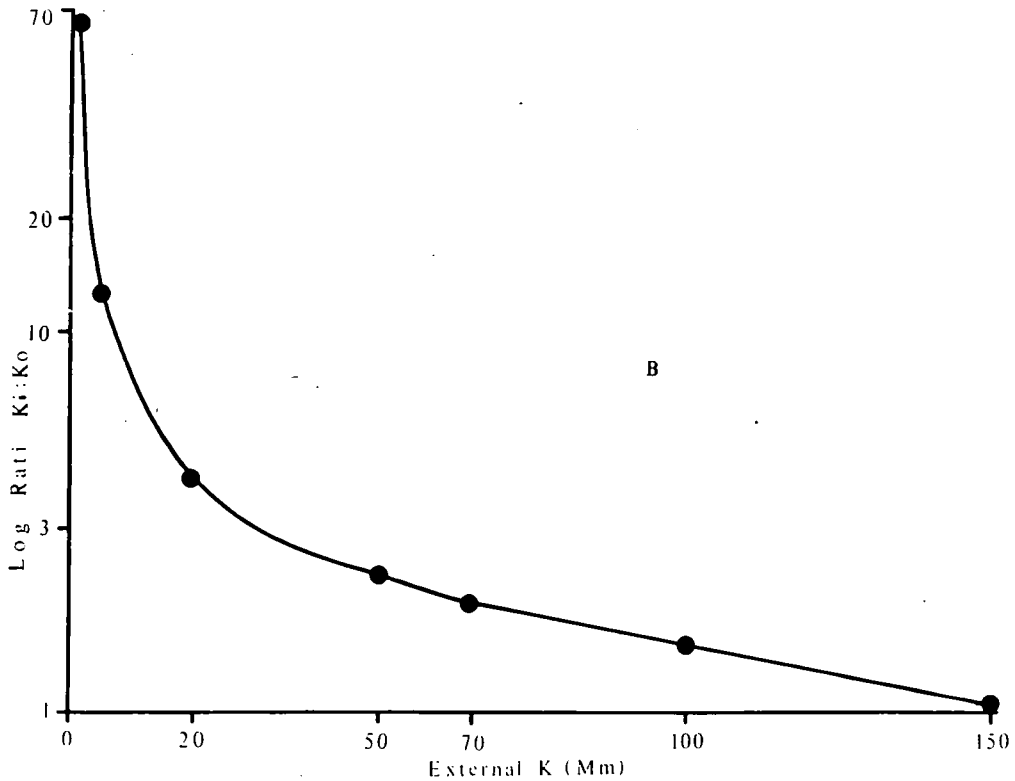

70
20
10
3
1
Log Rati Ki:Ko
B
0
20
50
70
100
150
External K (Mm)

Figure 37

to 100 mM caused a sevenfold fall in membrane resistance.

Alteration of external potassium concentration was found to affect the action potential, but only after a delay of 40 to 60 minutes. High external potassium salines had a depressant effect on the action potential which declined in amplitude and increased in time course as the potassium was raised(Figures 38 and 39). The largest action potentials were recorded in zero potassium salines in Telea polyphemus,and these had an amplitude of 63 mV with an overshoot of zero potential of about 8 mV, and a rate of rise of 12.6 volts/second. The normal action potential in 50 mM external potassium was 44 mV ± 1.2 with a rate of rise of 9.2 volts/second. As the external potassium was raised further, the action potential continued to decline in amplitude, and the production of the active membrane response became progressively delayed from the end-plate potential (Figure 38 C). At 100 mM external potassium the rate of rise of the action potential was only 4 volts/second, while at 150 mM external potassium the active membrane response had disappeared completely, leaving only a very attenuated end-plate potential. An interesting point about the lepidopteran action potential is that the active membrane response and the end-plate potential,

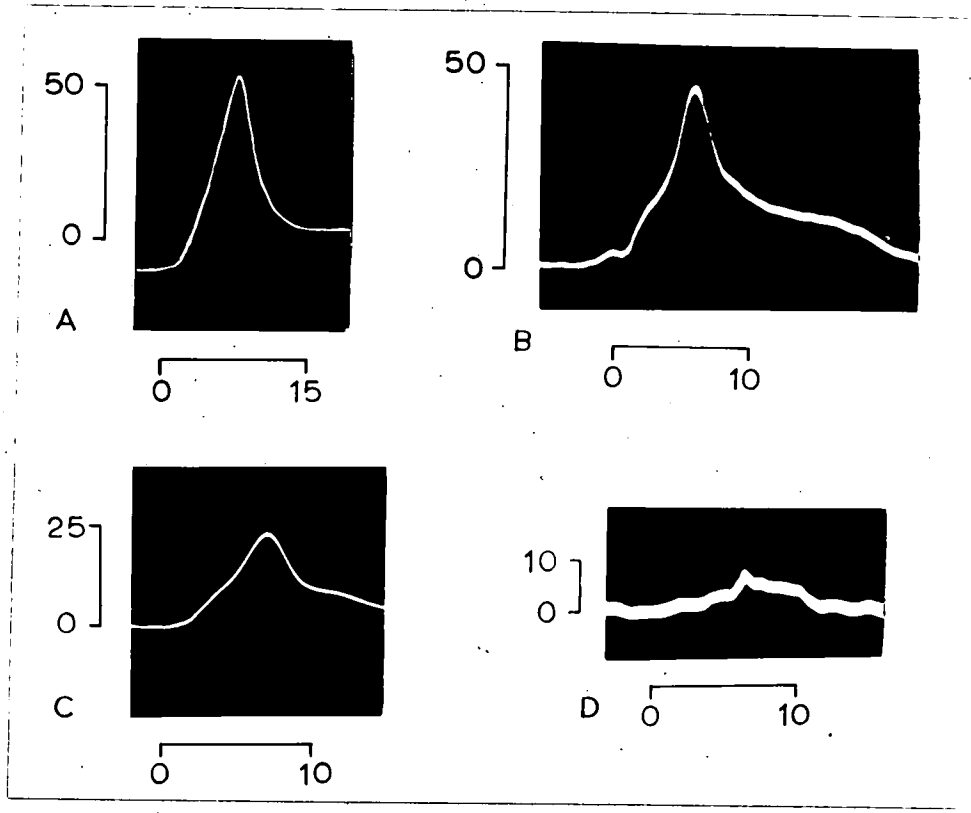


Figure 38. The effect of potassium ions upon the action potential of Telea polyphemus. A, zero potassium; B, 50 mM; C, 100 mM; D, 150 mM. Calibrations in millivolts and milliseconds. (A retouched).

over a wide range of potassium salines, have different rates of rise. In normal saline (potassium 50 mM) the rates were 12 and 6.4 volts per second respectively. Increase in external potassium did not have a uniform effect on rise time, the active membrane response being more readily reduced in rise time than the end-plate potential. Hoyle(1955a) and Wood(1957a) have reported that in high potassium salines the amplitude of the active membrane response declined in simple correlation with the amplitude of the end-plate potential. In Telea polymphemus (Figure 40) the active membrane response declined much more rapidly in high potassium salines than did the end-plate potential... In very high external potassium, the end-plate potential was always present, no matter how small, but the active membrane response required a critical value of the end-plate potential, about 10 mV, before it was produced.

Potassium thus affects the action potential of Lepidoptera in a manner similar to that already reported by Hoyle(1955a) and Wood(1957b) in other insects. The magnitude of the end-plate potential is roughly proportional to the concentration of external potassium, but this is most probably mediated by the effect of potassium ions upon the resting potential itself. As can be seen in Figure 40, both the end-plate potential and the active

Table 10. The effect of potassium ions upon the action potential of Telea polyphemus.

External K^+ Concentration (mM)	Mean Action potential ± S.E. (mV)	Number of Samples.
0	63.0 ± 1.0	7
50	44.0 ± 1.3	12
100	25.3 ± 1.4	5
150	9.0 ± 0.6	3

Table 11. The effect of potassium ions upon

o The junctional potential

0 The active membrane response

in Telea polyphemus.

External K^+ Concentration (mM)	Mean junctional potential ± S.E. (mV)	Mean active membrane resp. ± S.E. (mV)
0	22.0 ± 0.6	41.0 ± 1.2
50	15.0 ± 0.7	29.0 ± 1.0
100	12.0 ± 0.8	13.0 ± 1.2
150	9.0 ± 0.6	Zero

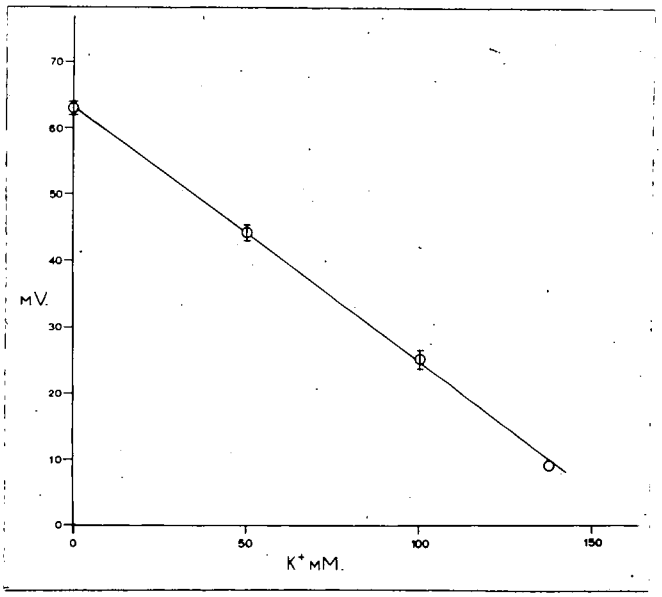


Figure 39. The effect of potassium ions upon the action potential of Telea polyphemus.

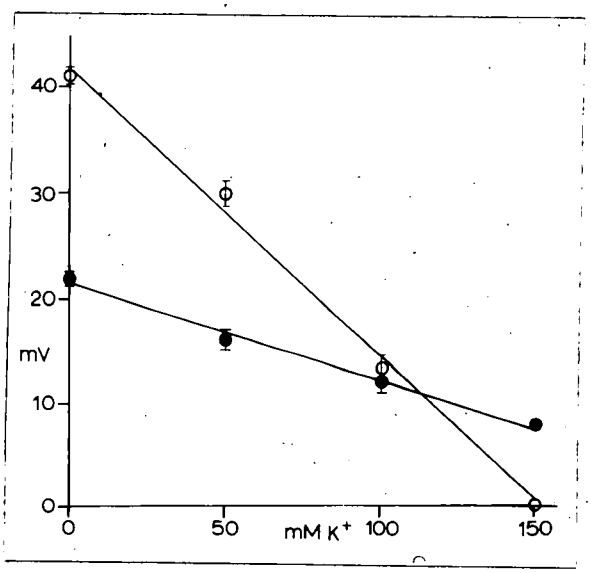


Figure 40. The effect of potassium ions upon the junctional potential(full circles) and active membrane response(hollow circles) in Telea polyphemus.

response have a straight line relationship with the concentration of external potassium ions, and when resting potential is plotted against both the active membrane response and the end-plate potential, although the curves are not completely linear, the relation of resting potential to both components is fairly similar. The alternative hypothesis, that potassium ions directly affect the actual transmitter mechanism producing the end-plate potential is discussed by Hoyle(1955a) and rejected. The results here would tend to support his argument. Hoyle has claimed that in the locust the active membrane response declined progressively with increase of external potassium, but did not disappear until the preparation was ageing. The results here however, differ from those of Hoyle in that at 150 mM external potassium no sign of the active membrane response was ever evident, even in fresh preparations. Wood(1957b) found that the active membrane response in the stick insect persisted up to 100 mM external potassium but above this it tended to disappear. However, the active membrane response sometimes persisted up to150 mM potassium but rather rarely (personal communication).

The effect of Sodium and Quaternary ammonium ions

Alterations of the sodium concentration in the bathing salines were found to affect the magnitude of both the resting and action potentials in Bombyx mori muscle fibres after a delay. The graph in Figure 41 shows the results of a typical experiment in this species. Up to about 100 mM external sodium the graphs of both the resting and action potential are very clear, and above 100 mM external sodium they only diverge to a small extent, the action potential graph rising more steeply. Even at a concentration of 200 mM external sodium however, the action potential is only a matter of 4 mV larger than the resting potential. The parallel nature of the two graphs suggests that the effect of sodium on the action potential may only be a secondary effect, being mediated by the effect of sodium on the resting potential. In section II of this thesis, arguments were advanced to suggest that in the normal ionic condition of the heamolymph the size of the action potential may be related directly to the size of the resting potential, and this may be the case here.

Figure 42 shows the effect of alteration of external sodium ion concentration upon the action potential in Bombyx mori. In high sodium concentrations the rate of rise of the action potential was slightly increased, and the repolarisation phase was slightly more rapid, but the

effect of sodium on the actual amplitude of the action potential was small.

It is interesting to note that sodium ions had an obvious effect on the resting potential. A similar effect was noticed by Wood(1957b) in Carausius, but Narahashi and Yamasaki (1960) found that sodium ions had no measurable effect on the resting potential of cockroach giant axons. Although there is one other reference in the literature to the effect of sodium ions on the resting potential (Huxley and Stampfli(1951) sodium only affecting the resting potential by 1 - 3 mV in frog muscle, observations of this nature are not general. Until more information is available the effect of sodium on the resting potential can be considered essentially as a specialised feature of the neuromuscular physiology of herbivorous insects.

In most excitable tissues so far investigated, the resting potential is explicable almost exclusively in terms of the distribution of potassium ions, and it is difficult to see how sodium could fit into the classical ionic hypothesis relating to the resting potential without invoking a concept of a multi-ion electrode in the muscle fibre membrane, an explanation which seems the most likely in herbivorous insects. Such an explanation has already been advanced for Tenebrio molitor muscle fibres by Belton and Grundfest(1962a,b), although the data presented

Table 12. Magnitudes of the resting and action potentials in different sodium concentrations

mM sodium per litre.	Number of samples.	Mean resting potential in millivolts ± S.E.	Mean action pot. in miilivolts.
1	9	38.4 ± 0.8	37.8 ± 1.4
9	10	41.8 ± 1.4	40.1 ± 2.1
50	9	42.0 ± 0.6	42.1 ± 1.1
100	10	44.5 ± 0.6	44.8 ± 1.4
150	7	46.5 ± 0.8	48.3 ± 1.1
200	10	48.6 ± 0.5	52.6 ± 1.8

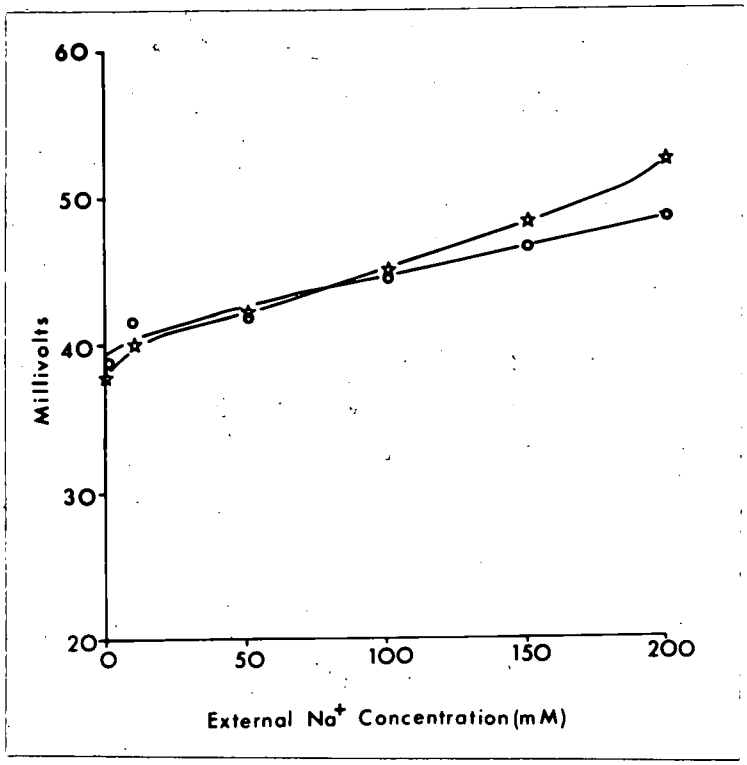


Figure 41. The effect of external sodium concentration on the resting potential(circles) and action potential(stars) in Bombyx mori.

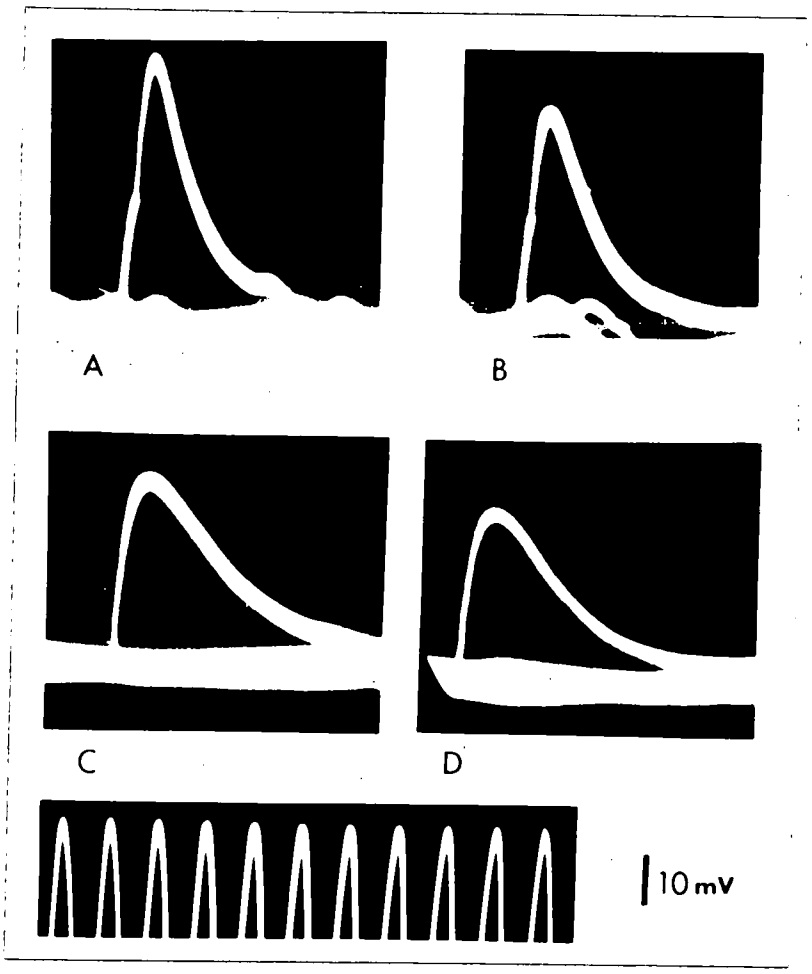


Figure 42. The effect of sodium ions on the action potential in Bombyx mori. A, 200 mM/l; B, 100 mM/l ; C, 50 mM/l ; D, 1 mM/l. Calibration 500 cycles/second.

by these authors is not altogether clear, and some of the results rather confusing. One possibility is that Na^+ ions exert a direct effect on the membrane, and modify responses to other ions.

Apart from its unusual effect on the resting potential, the sodium ion is also rather remarkable in Lepidoptera since its effect on the action potential is rather small. Wood(1957b) noted that in Carausius, a two hundredfold increase in external sodium ions caused only a ten millivolt change in the action potential. In Bombyx mori a two hundredfold increase in external sodium concentration caused only a 14.8 millivolt change in the action potential. It is postulated by the ionic hypothesis that the membrane of an excitable cell is relatively impermeable to sodium ions in the resting state. During activity, the sodium ions enter the fibre along their electrochemical gradient, and if the fibre is freely permeable to sodium ions, the membrane approaches the state of a sodium electrode, and its membrane potential will be predictable by the sodium version of the Nernst equation, in which:

$$E_{NA} = \frac{R.T}{F} \log_e \frac{(Na)o}{(Na)i}$$

where (Na)o and (Na)i are the sodium ion concentrations outside and inside the fibre respectively. The value obtained for E_{Na} will show the extent to which the membrane can be expected to deviate from zero potential in a positive

direction, and is the calculated E_{Na} or the calculated overshoot.

In many excitable tissues, the general trends of the ionic hypothesis outlined above have been confirmed, particularly the part relating the extent of the reversal of the membrane potential to the concentration of sodium ions present(Hodgkin and Katz,1949 ; Nastuk and Hodgkin, 1950 ; Huxley and Stampfli,1951 ; Cole,1949 ; Draper and Weiderman,1951). Compared to such findings as these, the effects of sodium ions on the action potential in Lepidoptera and in Carausius are very small indeed so further experiments were devised to see to what extent sodium ions followed the Nernst equation in the generation of the action potential. Bombyx mori muscle was again used, but intracellular sodium was now measured at each external sodium concentration, so that the theoretical sodium electrode potential could be calculated.

The results of a typical experiment are given in Table 13 and in the graph, Figure 43. Wood(1963) working on Carausius,Locusta, and Periplaneta found that the muscle fibres of these insects possessed the ability to maintain a fairly constant intracellular sodium ion concentration in spite of wide alterations in the concentration of the external sodium ions. Bombyx mori muscle fibres do not appear to possess quite this degree of regulation. A two hundredfold increase in external sodium ion concentration

produced a 3.5 times gain in the concentration of intracellular sodium ions. Although this gain in internal sodium is slightly higher than that found by Wood(1963) in Carausius it was still very small indeed, and the trend in these results is similar to the trend found by Wood. In evaluating his results, the latter author was able to rationalise the internal sodium concentration for the purposes of calculating overshoots, but in the results presented here, the theoretical overshoot has been calculated using the sodium concentration experimentally determined for each different external sodium value in view of the higher variation in internal sodium.

From the values of internal and external sodium concentration the theoretical overshoot was determined for each external sodium concentration, and these were plotted along with the values of the actual overshoot, determined experimentally with intracellular electrodes against the logarithm of the external sodium concentration. In many excitable tissues, particularly vertebrate nerve and muscle, and in the squid giant axon, the overshoot of the action potential has been found to approach the theoretical E_{Na} determined from internal and external sodium concentrations. However, in Bombyx mori not only is it obvious that the theoretical E_{Na} bears no relation to the observed overshoot, but the actual shapes of the two graphs are different.

Table 13. The effect of external sodium ions on intracellular sodium concentration and on the overshoot of the action potential in Bombyx mori muscle fibres.

External sodium concentration in mM per litre.	Resting Potential in millivolts	Action Potential in millivolts	Action Potential minus resting potential(millivolts)	Intracellular sodium in mM per Kgm. tissue water	Ratio of Na_o to Na_i	Calculated overshoot (E) in mV. Na
1	38.4 ± 0.8	37.8 ± 1.4	- 0.6	6.3 ± 0.7	0.16	- 46.4
9	41.8 ± 1.4	40.1 ± 2.1	- 1.7	12.4 ± 1.2	0.73	- 8.0
50	42.0 ± 0.6	42.1 ± 1.1	Zero	13.4 ± 1.1	3.73	+ 33.1
100	44.5 ± 0.6	44.8 ± 1.4	+ 0.3	15.2 ± 1.3	6.58	+ 47.4
150	46.5 ± 0.8	48.3 ± 1.2	+ 1.8	19.7 ± 1.5	7.61	+ 51.1
200	48.6 ± 0.5	52.6 ± 1.8	+ 4.0	22.3 ± 1.4	8.96	+ 55.12

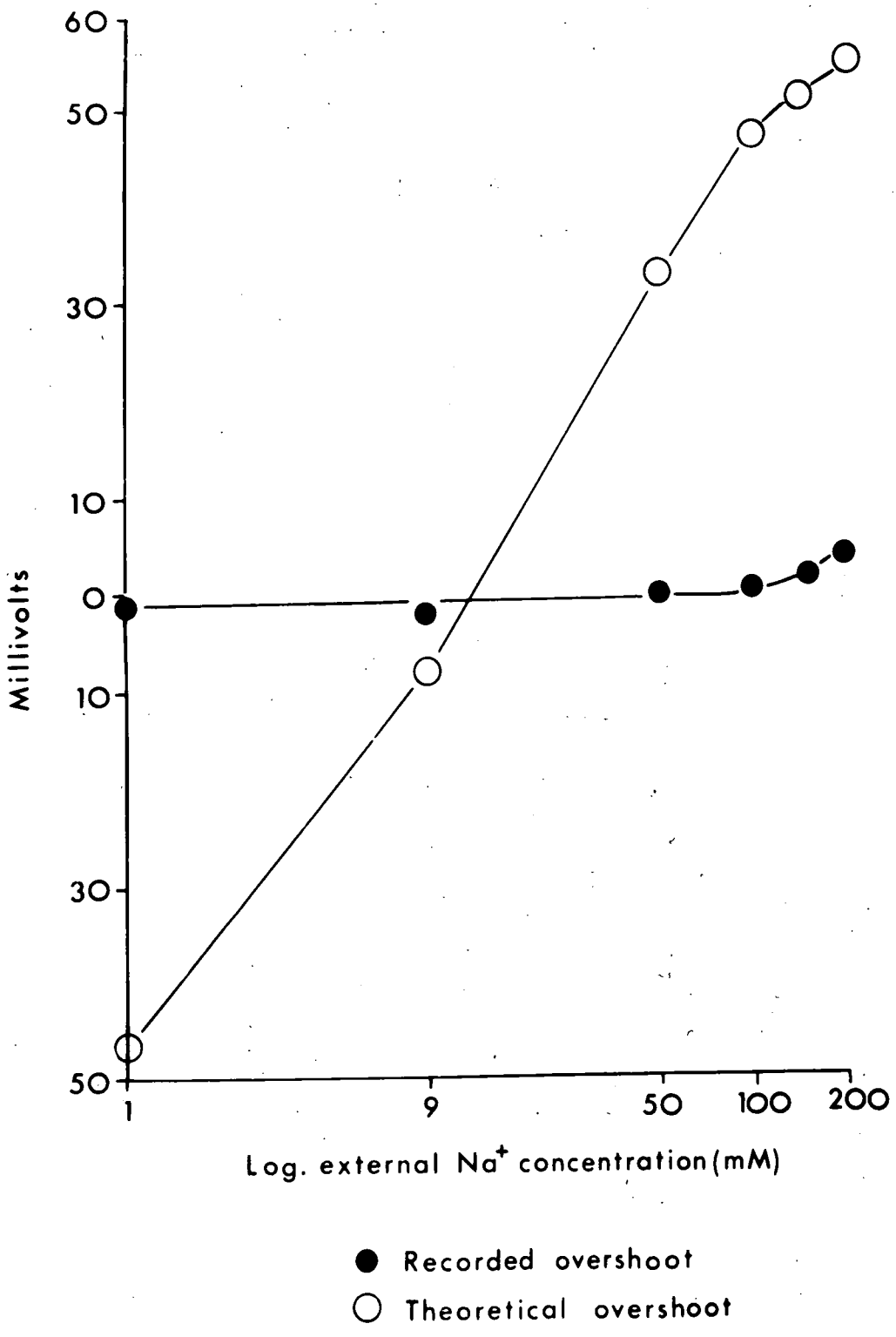

60
50
30
10
0
10
30
50
Millivolts
1
9
50
100
200
Log. external Na+ concentration (mM)
Recorded overshoot
Theoretical overshoot

Figure 43

Wood(1963) found similar results in Carausius, Locusta, and Periplaneta muscle fibres. It is interesting to note that the two graphs only cross each other (that is, the theoretical overshoot approaches the observed overshoot) at about the value of sodium normally found in the heamolymph of the insect. A parellel situation has already been described in relation to potassium ions in which the theoretical E_K approaches nearest to the observed resting potential value in the normal physiological range for potassium in the heamolymph.

An even more interesting point, brought out in Table 13 is that in the normal state, the internal sodium concentration of the muscle fibre is greater than that of the haemolymph sodium concentration. This is a reversal of the ideal state proposed in the ionic hypothesis and is true of all Lepidoptera studied in this investigation(see Tables 4 and 5), and was also noticed by Carrington and Tenney (1959) in Telea polymphemus although these authors made no further comment on their sodium results. In Bombyx mori it is only at about 15 mM external sodium concentration that the external sodium equalls the internal sodium. Below this value of external sodium all calculations with the Nernst equation give a negative value ("Undershoot") for the predicted overshoot of zero potential("Negative" action potential). The actual observed undershoot persists up to about 50 mM external sodium, at which concentration

the predicated E_{Na} is 30 mV positive.

The Lepidoptera are the only animals so far investigated to have a reversed sodium gradient(reversed that is, in terms of the ionic hypothesis). This finding must certainly cast doubt on the actual function of the sodium ion in relation to the action potential in the Lepidoptera. With a distribution of this nature, sodium ions could certainly make little or no contribution to the inward current generation of the action potential, since, during membrane activity there would be no sodium influx along the sodium gradient since this gradient is outgoing. If passive transport were solely involved, the muscle fibre would tend to lose sodium ions during activity. On the other hand, if active transport of sodium ions against their gradient was involved, then sodium ions could make some contribution to the generation of the action potential, although the contribution would be small since sodium is present in such small concentrations in the haemolymph of herbivorous insects.

Although sodium ions are regarded as being indispensable for the generation of action potentials in vertebrate material(see Hodgkin,1951,1964) this is not necessarily the case in the invertebrates. In the latter it is often possible to substitute other ions for sodium in attempts to elucidate the role of sodium without loosing the action potential in the muscles. In an attempt to solve the anomaly

of the sodium ion in Lepidoptera, experiments were performed in which the sodium content of the salines was entirely replaced by equivalent amounts of various quaternary ammonium compounds in Bombyx mori.

Fatt and Katz(1953) working on Carcinus and Portunus and Wood(1957) working on Carausius have shown that the excitability in the muscle fibres of these animals could be maintained in the absence of sodium ions when the latter were replaced by equivalent amounts of various quaternary ammonium compounds. Choline chloride maintains the normal excitability without causing a fall in the resting potential in many excitable tissues(Lorento de No,1949 ; Nastuk and Hodgkin,1950 ; Wood,1961). In the crustacea however, Fatt and Katz(1953) reported that choline not only maintained the resting potential of the muscle fibres but also increased both the amplitude and duration of the action potential enormously. This latter phenomenon has not been recorded elsewhere. In Bombyx mori and Telea polymphemus, choline maintained the normal level of polarisation of the muscle fibres, the choline resting potential being 38.4 ± 0.8 mV compared with the normal 41.8 ± 1.4 in Bombyx mori. The action potential was not affected by replacing the sodium with choline. In these circumstances choline would appear to be an inert cation which is perfectly capable of totally replacing sodium in the Lepidoptera.

Acetylcholine, the well known vertebrate neuromuscular

transmitter (Dale,Feldberg, and Vogt,1936 ; Brown,1937) was found to have no noticeable effect on either the resting or action potential of either Bombyx mori or Telea polymphemus when added to salines with or without sodium present.

Three other quaternary sammonium compounds have also been employed, tetrabutyl,tetraethyl, and tetramethyl-ammonium chloride. The tetrabutyl-ammonium salt could only be obtained commercially as the bromide. This was converted to the chloride by a modification of the method of Fatt and Katz(1953). The bromide was shaken with silver chloride in acidified methanol, the quaternary ammonium chloride then being recrystallised from distilled water. To determine the percentage of retained water, the salt was titrated against silver nitrate,using potassium chromate as an indicator. In the following experiments, the membrane potentials were recorded from flight muscle preparations of Bombyx mori.

Tetrabutyl ammonium ions(TBA)

After a short period of vigorous spontaneous activity (see Figure 44j), TBA ions in concentrations as low as 10 mM caused the muscle fibres to become inexcitable after only about 20 to 30 minutes. This inexcitability could not be reversed by returning the preparation to normal saline. The onset of inexcitability was accompanied by a

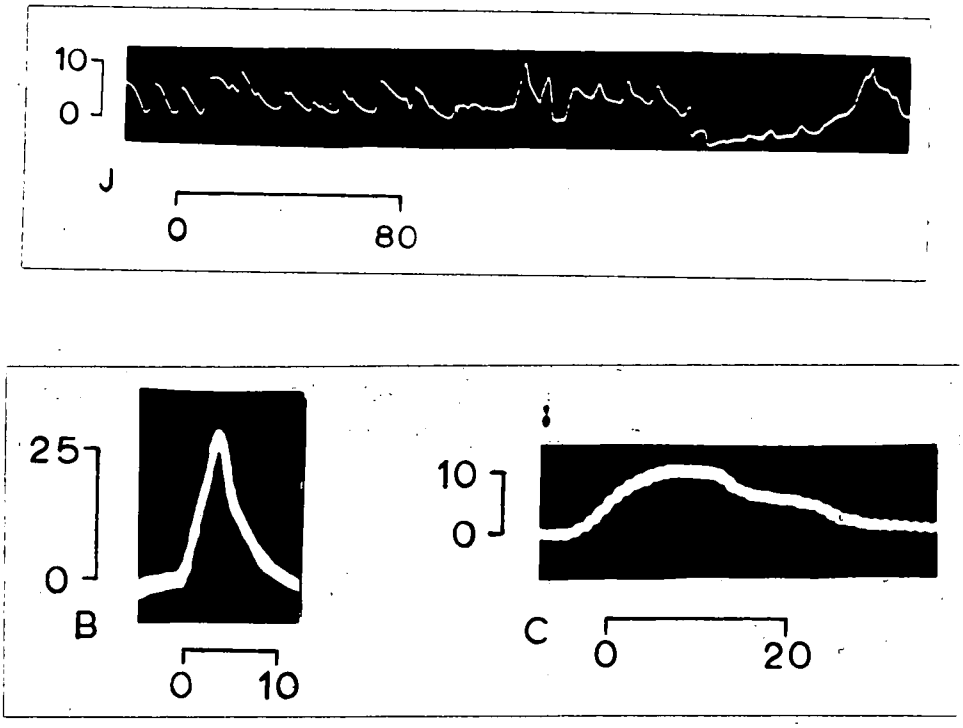


Figure 44, The effect of Tetrabutyl ammonium chloride ions on the muscle fibres of Bombyx mori.

J. Spontaneous miniature oscillations of membrane potential in 10 mM TBA.

B. Action potential recorded at the time of addition of TBA ions to preparation.

C. Action potential recorded from same fibre as(B)after 60 minutes in 50 mM TBA ions.

Calibrations in milliseconds and millivolts

progressive fall in the value of both the resting potential and the action potential(Figure 44b,c). The active membrane response soon disappeared leaving only a small end-plate potential. Similar effects were reported by Wood(1957b) in Carausius muscle, and Fatt and Katz(1953) in crustacea. Increase in the concentration of TBA ions resulted in an acceleration of the decline of the resting potential. This is shown for four concentrations of TBA in Figure 45. Figure 46 shows the effect in increase of TBA ions on the rate of decline of the resting potential measured as the time taken for the resting potential to fall to one half its original value. As TBA concentration was increased, the rate of decline of the resting potential increased. Increase in the concentration of TBA ions progressively reduced the time taken for inexcitability to set in the muscle fibre.

Tetreathyl ammonium ions(TEA)

TEA ions were found to affect both the resting and action potentials in Bombyx mori. Salines with TEA ions up to about 150 mM/litre caused an irreversible fall in the value of the resting potential, similar in nature to some earlier reports(Fatt and Katz,1953;Wood,1957b). The rate of decline of the resting potential for four different concentrations of TEA ions is shown in Figure 47. For a sixfold increase in TEA ions, the rate of decline of the resting potential

Table 14. The effect upon the resting potential in Bombyx mori of various concentrations of Tetrabutyl ammonium ions.

	Time after addition of TBA saline(mins.)	Mean Resting potential ± S.E. (millivolts)
TBA 10 mM per litre.	0	39.3 ± 2.4
	60	33.0 ± 1.7
	120	23.7 ± 2.0
	180	15.5 ± 1.8
TBA 20 mM per litre.	0	42.1 ± 1.6
	30	36.0 ± 1.4
	60	25.5 ± 1.5
	90	19.3 ± 1.0
TBA 30 mM per litre.	0	41.8 ± 1.5
	30	33.2 ± 1.6
	60	21.6 ± 0.8
	90	13.0 ± 0.8
TBA 50 mM per litre.	0	41.6 ± 2.9
	20	32.4 ± 1.4
	40	25.4 ± 1.0
	60	14.3 ± 0.7

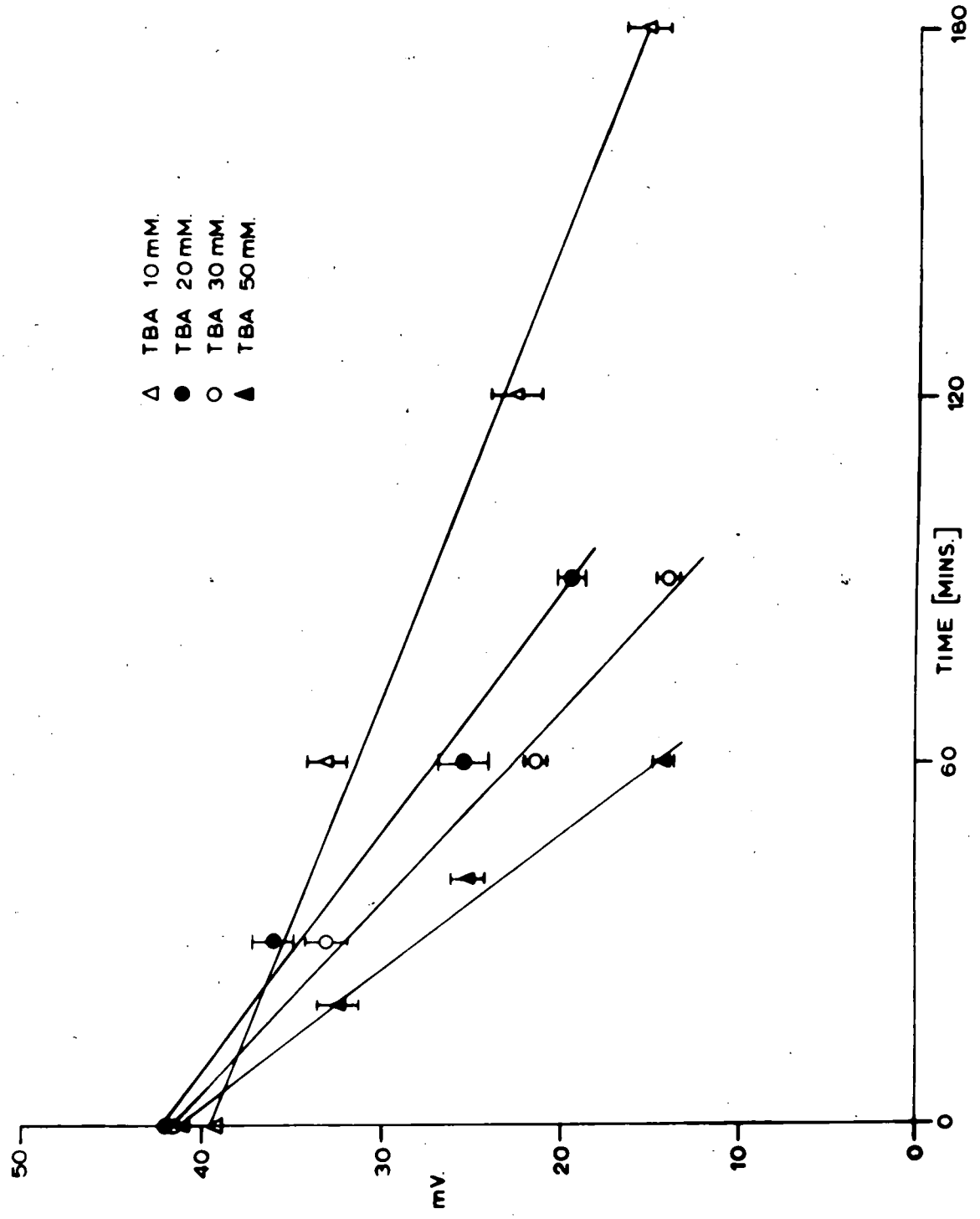

Δ TBA 10 mM.
● TBA 20 mM.
○ TBA 30 mM.
▲ TBA 50 mM.
mV
50
40
30
20
10
0
0
60
120
180
TIME [MINS.]

Figure 45

Table 15. The effect of increase in TBA ions on the rate of decline of the resting potential.

TBA concentration (mM per litre)	Time in mins for fall of resting potential to 50% initial value	Rate of decline of resting potential (mV.per hour)
10	150	8.0
20	82	15.3
30	64	19.7
50	48	26.2

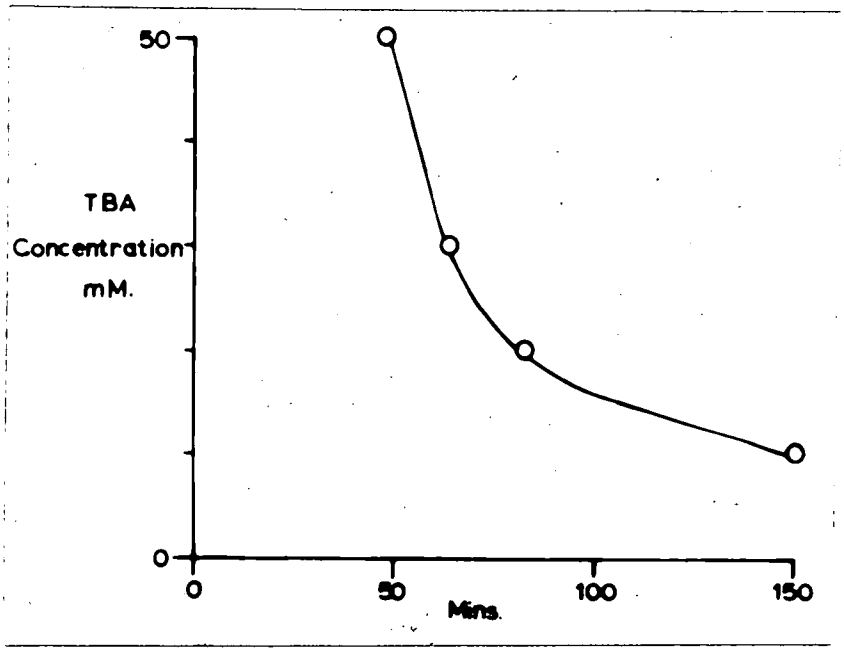


Figure 46. The effect of TBA ion concentration on the decline of the resting potential in Bombyx mori muscle fibres. Measured as the time taken for the resting potential to decline to 50% of its original value.

increased from 5.5 mV/hour to 13.8 mV/hour.

TEA ions caused the muscle fibres to become highly excitable, the fibres showing considerable spontaneous activity which consisted of miniature end-plate potentials (Figure 48d). At 25 mM TEA ions, the period of increased spontaneous activity lasted about 90 minutes, and was then followed by total inexcitability. As the TEA concentration was increased, the initial period of increased spontaneous activity was progressively reduced, until at about 150 mM TEA/litre there was no observable period of high activity at all, the fibres passing from the normal state into total inexcitability. This inexcitability was irreversible even when the preparation was returned to salines with increased sodium content.

TEA ions caused a progressive fall in the amplitude of the action potential, (see Figure 48 E to I), and in high TEA concentrations(100 to 150 mM/litre) the action potential was greatly lengthened in time course, being extended from 13.3 milliseconds in 25 mM to 30.3 milliseconds in 100 mM(see Figure 49 k to o). Conversely, the rate of rise of the action potential increased with increase in TEA concentration, and it would appear that TEA ions progressively delayed repolarisation of the membrane. In 100 mM TEA, some fibres showed a tetanic-like fusion of the action potential, after only one or two stimuli followed by tetanic mechanical responses in the muscle if sufficient fibres were affected.

Table 16. The effect of various concentrations of Tetraethyl ammonium ions upon the resting potential of Bombyx mori muscle fibres.

TEA 25 mM per litre	Time after addition of TEA saline(Mins)	Mean resting potential, mV ± S.E.
	0	40.4 ± 1.1
	60	38.5 ± 1.3
	90	33.8 ± 1.0
	120	28.8 ± 1.6
	150	25.6 ± 1.1
	180	23.0 ± 1.4
	120	20.8 ± 1.3
TEA 50 mM per litre	0	40.0 ± 1.4
	30	34.8 ± 1.2
	60	30.6 ± 1.1
	90	25.0 ± 1.0
	120	22.2 ± 0.8
	150	17.6 ± 0.8
	180	16.5 ± 1.4
TEA 100 mM per litre	0	40.9 ± 2.0
	30	36.0 ± 1.5
	45	32.0 ± 1.7
	75	26.7 ± 1.1
	105	22.7 ± 1.2
TEA 150 mM per litre	0	38.1 ± 1.4
	20	32.9 ± 0.5
	40	28.7 ± 0.7
	60	22.9 ± 0.9
	80	20.3 ± 0.9
	100	16.8 ± 0.8
	120	15.0 ± 0.7

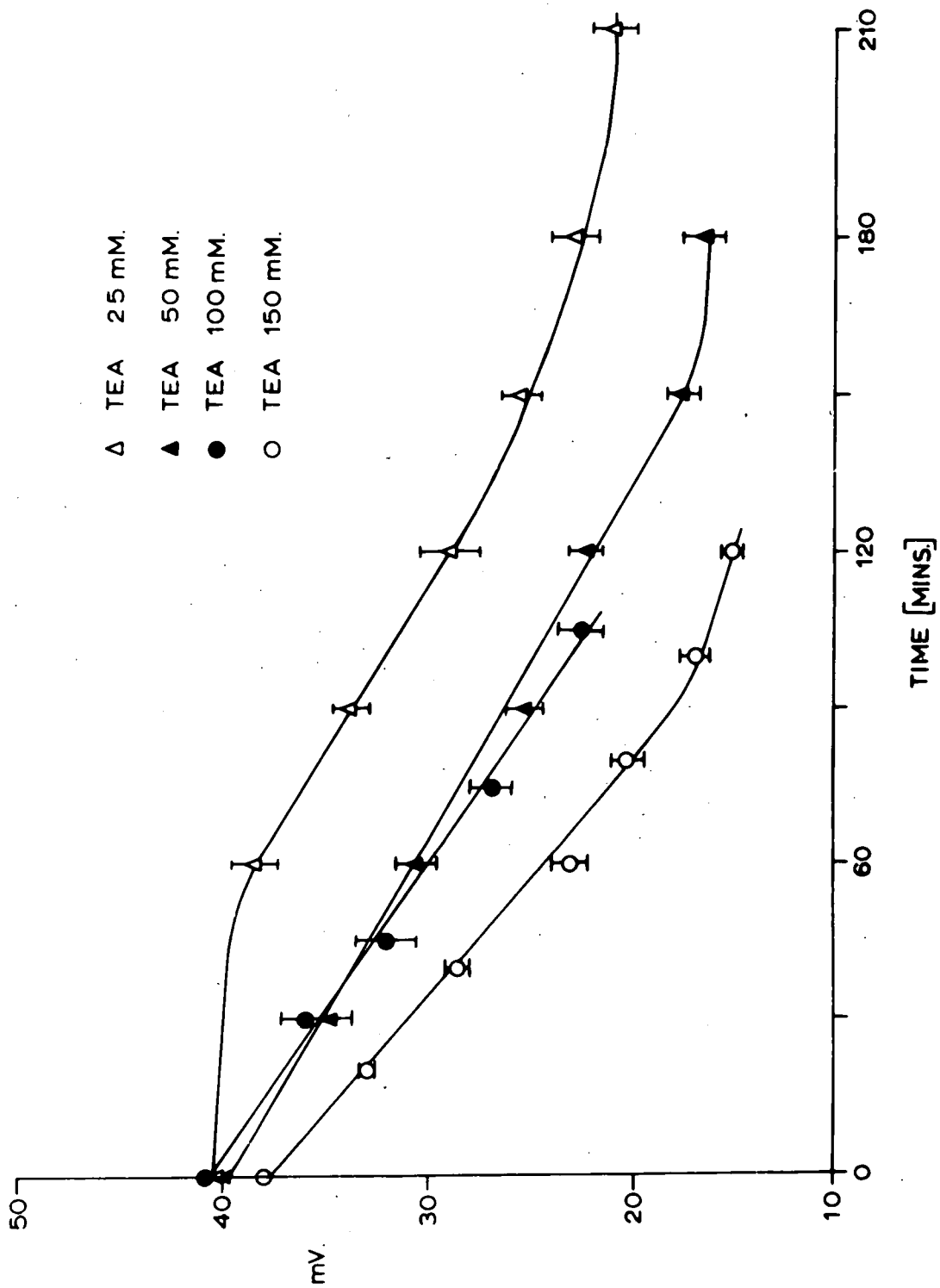

Δ TEA 25 mM.
▲ TEA 50 mM.
● TEA 100 mM.
○ TEA 150 mM.
50
40
30
20
10
mV
0
60
120
180
210
TIME [MINS.]

Figure 47

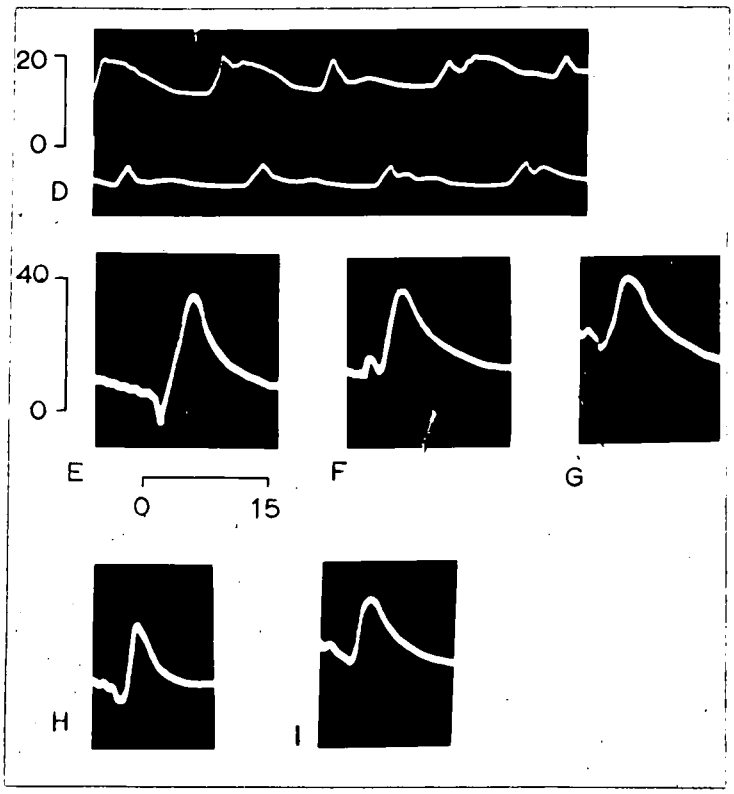


Figure 48. The effect of tetraethyl ammonium ions on Bombyx mori muscle fibres.

D. Spontaneous miniature end plate potentials in 25 mM TEA ions.

E to I Progressive fall in the amplitude of the action potential in 25 mM TEA ions.

Calibrations in milliseconds and millivolts.

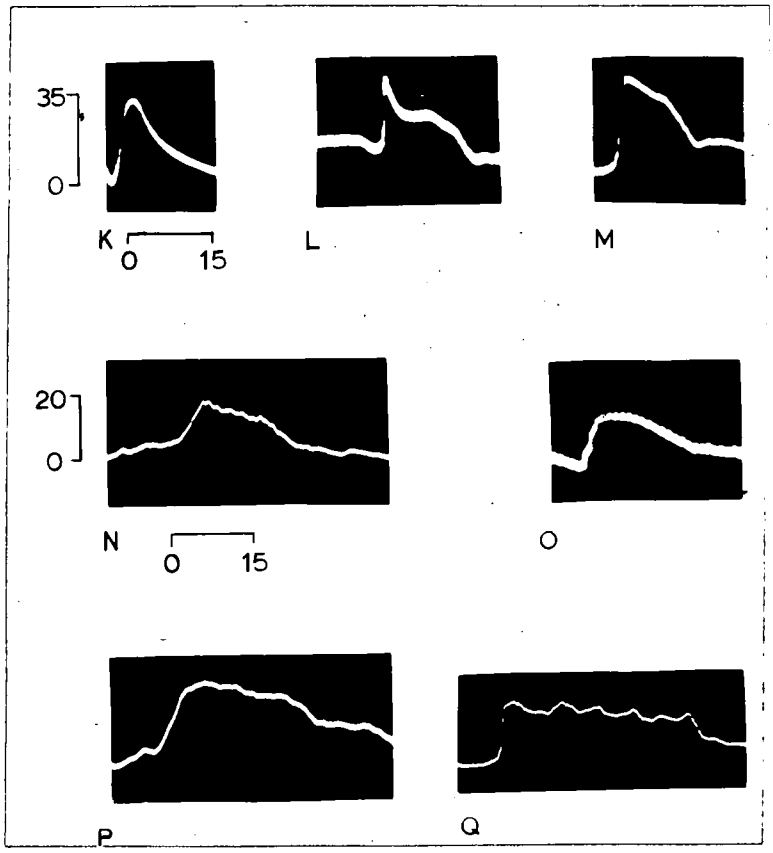


Figure 49. The effect of 100 mM TEA ions on the action potential in Bombyx mori muscle fibres.

K to O. Progressive lengthening of the time course in a single fibre.

P & Q. Tetanus like response from a muscle fibre after a single stimulus.

Calibrations in milliseconds and millivolts

To examine the fall in action potential amplitude, action potentials were recorded at a standard time of 20 minutes after the preparation was transferred to the TEA saline. For a fourfold increase in TEA ions, the action potential declined by one third of its amplitude(Figure 50).

Tetramethyl ammonium ions(TMA)

TMA ions possessed none of the properties of TBA and TEA ions for increasing excitability and reducing the resting potential value. Of the three quaternary ammonium ions examined in detail, TMA most resembled sodium in its effects on membrane potentials. In TMA ions the membrane remained polarised at about the normal level. However, increase in TMA ions had a greater elevating effect on the resting potential than sodium ions(see Figure 51). For a hundredfold increase in sodium, the resting potential rose by 5.1 mV, the corresponding figure for TMA ions was 12.6 mV. This effect on the resting potential was completely reversible by return to sodium containing salines.

The effect of chloride ions.

According to the ionic hypothesis, the resting muscle fibre membrane is readily permeable to potassium and chloride ions which exchange across it solely by diffusion under the conditions of a Donnan equilibrium. The resting membrane potential, E_r, is related to the relative concentrations of potassium and chloride ions by the equations.

Table 17. The effect of Tetraethyl ammonium ions upon the action potential of Bombyx mori muscle fibres. Action potentials recorded at a standard time of 20 minutes after the addition of the TEA saline.

TEA concentration (mM/litre)	Number of readings	Action potential mV ± S.E.
0	10	36.0 ± 1.0
25	11	27.7 ± 0.7
50	11	21.7 ± 1.3
100	13	18.4 ± 1.4

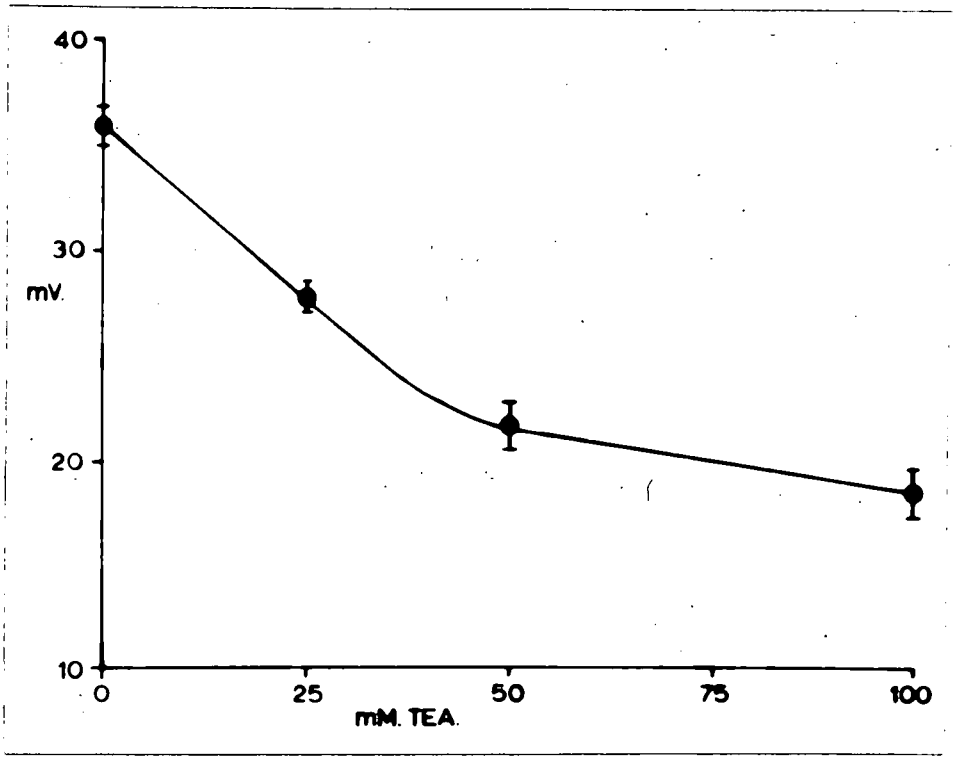


Figure 50. The effect of TEA ions on the action potential of Bombyx mori muscle fibres.

Table 18. The magnitude of the resting potential of Bombyx mori muscle fibres in different concentrations of sodium and tetramethyl ammonium ions.

Concentration (mM/l.)	Number of samples.	Mean resting potential ± S.E.
TMA ions.		
0	13	32.0 ± 1.0
25	10	37.4 ± 1.6
50	9	41.4 ± 0.9
100	9	45.3 ± 1.9
150	10	51.0 ± 2.0
Sodium ions		
1	9	38.4 ± 0.8
9	10	41.8 ± 1.4
50	9	42.0 ± 0.6
100	10	44.5 ± 0.6
150	7	46.5 ± 0.8
200	10	48.6 ± 0.5

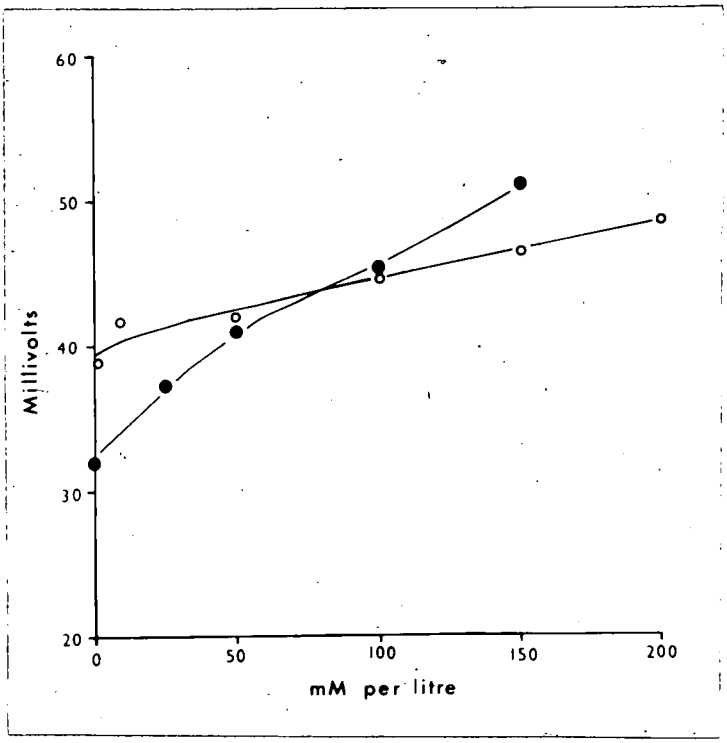


Figure 51. The effect of sodium ions(hollow circles) and TMA ions(full circles) on the resting potential of Bombyx mori muscle fibres.

$$E_r = \frac{R\,T}{F} \log_e \frac{(K)i}{(K)o} = \frac{R\,T}{F} \log_e \frac{(Cl)o}{(cl)i}$$

A reciprocal relation will thus exist between the ratios of potassium and chloride ions so that

$$\frac{(K)i}{(K)o} = \frac{(Cl)o}{(Cl)i}$$

There have been very few investigations into the role of chloride ions since, until recently, the chloride ion has always been regarded as the passive complement to potassium ions. In the Lepidoptera however, the potassium ion does not behave in a manner expected from the classical ionic hypothesis, and it is possible that chloride ions are equally unusual in their effects in this group.

Recently, several investigators have re-examined the whole question of the chloride ion, and the results they have obtained are far from what had formerly been supposed. Robertson(1961) working on Nephrops muscle found that there was a fairly large discrepancy between the ratios of internal to external potassium and external to internal chloride. Keynes(1963) found that in the squid giant axon the internal chloride was higher than formerly thought, and that the theoretical membrane potential E_{Cl} did not approach the actual recorded resting potential. This author proposed a system of active transport of chloride ions to explain his results. Wood(1965) found that in the cockroach and locust leg muscles, the theoretical E_{Cl} calculated from

Table 19. The effect of chloride ions upon the resting potential and upon intracellular chloride in Sphinx ligustri.

Haemolymph ions in mM/litre. Cl^-	K^+	Na^+	Tissue ions in mM/Kgm.tissue water Mean ± S.E. Cl^-	K^+	Na^+	(Cl)o/(Cl)i	(K)i/(K)o	Resting potential mean ± S.E.	E_{Cl} mV.	E_r mV. (Goldman)
200	50	3.6	17.3 ± 1.6	96.9±5.6	19.6±1.3	11.61	1.94	- 49.7 ± 1.9	-61.7	-19.9
140	50	3.6	14.5 ± 1.9	84.4±7.0	21.2±1.0	9.93	1.69	- 47.4 ± 1.0	-57.1	-16.0
100	50	3.6	14.4 ± 1.4	81.3±3.6	18.9±1.2	7.02	1.63	- 45.3 ± 1.6	-48.8	-14.4
50	50	3.6	12.6 ± 0.6	80.7±2.2	20.5±1.9	3.97	1.61	- 43.0 ± 2.4	-34.7	-13.4
20	50	3.6	11.4 ± 1.0	79.4±2.3	20.9±1.7	1.75	1.59	- 40.1 ± 2.4	-14.2	-12.4
5	50	3.6	11.0 ± 1.3	77.9±2.0	19.2±1.8	0.45	1.56	- 37.1 ± 1.7	+19.9	-11.6
1	50	3.6	10.5 ± 1.0	77.5±2.2	20.0±2.1	0.095	1.55	- 36.8 ± 1.3	+60.2	-11.4

the Nernst equation did not equal the observed resting potential, and that there was no reciprocity between the respective potassium and chloride ion distribution across the muscle fibre membranes.

The above results are all in conflict with the conditions proposed in the ionic hypothesis, and it was therefore decided to examine the relation between the resting potential and chloride ions in the muscle fibres of Sphinx ligustri. In these experiments the external chloride concentration was altered from 1 mM to 200 mM per litre, and at each concentration, the resting potential, along with intracellular chloride, sodium, and potassium were measured. With such information it was possible to calculate the theoretical E_{Cl} for each value of external chloride and to compare its value with the observed resting potential. It was also possible to calculate both(Cl)o/(Cl)i and (K)i/(K)o at each concentration of external chloride to check their reciprocity.

Results from a typical group of experiments are shown in Table 19. The graphs in Figures 52 and 53 are derived from these results. As can be seen from the table, alteration of the external chloride concentration over a wide range produced little alteration in the intracellular muscle chloride concentration. This suggests that either the muscle fibre membrane is only slightly permeable to chloride ions or that it is actively maintaining a more

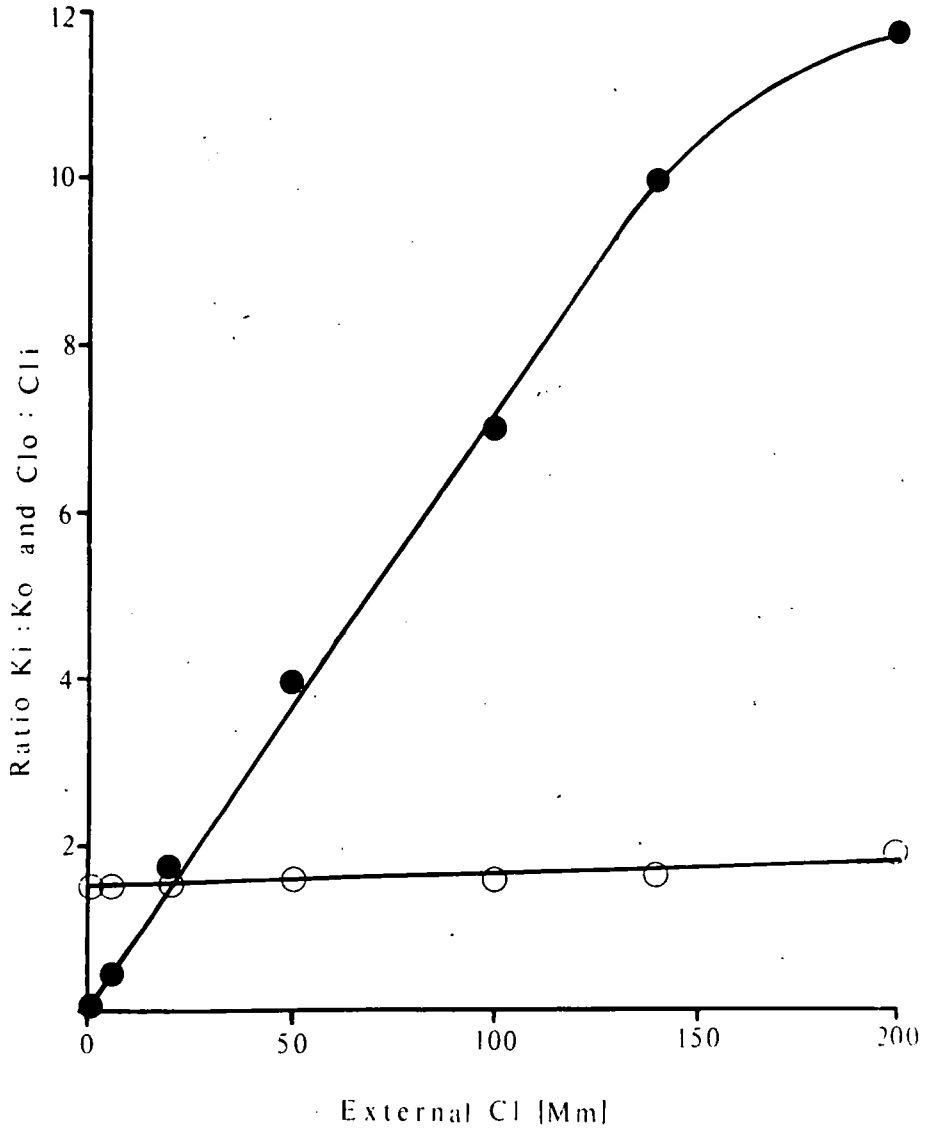

12
10
8
6
4
2
0
50
100
150
200
Ratio Ki : Ko and Clo : Cli
External Cl [Mm]

Figure 52

or less constant internal chloride. If the membrane were readily permeable to chloride ions, as is postulated in the ionic hypothesis, the ratio of external to internal chloride should be constant within a wide range of external chloride concentrations. This, however, is far from what is encountered here, the ratio of chloride distribution being seen to vary enormously. The intracellular potassium was little affected by the external chloride ions, and the ratio (K)i/(K)o was constant for the whole range of external chloride concentrations. In Figure 52, the ratios (K)i/(K)o and (Cl)o/(Cl)i have been plotted against external chloride. Far from being reciprocal, the two ratios are seen to be widely divergent, especially in the high chloride concentrations which form the normal physiological range in the Lepidoptera. There does not appear to be any passive reciprocity between the distribution of potassium and chloride ions, on the contrary, the chloride ions seem to be quite independent of the potassium distribution.

Alteration of the external chloride concentration affected the resting potential as is shown in Figure 53. For a two hundredfold increase in chloride concentration, the resting potential was raised by 14 millivolts, approximately a 30% change. The theoretical E_{Cl}, calculated from the chloride distribution by means of the Nernst equation was also plotted in Figure 53. Little correlation

can be seen between the resting potential actually recorded, and the predicted E_{Cl}. Correlation was greatest in high chloride concentrations(near the normal saline chloride of 140 mM per litre), but at low chloride concentrations the theoretical diverged greatly from the observed resting potential. In very low chloride concentrations, the ratio (Cl)o/(Cl)i became less than unity, thus the Nernst equation using such values produced a membrane potential with a reversed sign, becoming theoretically positive. No such positive potential was ever observed. The magnitude of the discrepancy between predicted and observed resting potential in these experiments is similar to the discrepancy encountered involving potassium ions.

The evidence of the relation of potassium ions to the resting potential, the lack of reciprocity of chloride and potassium, and the anomalous position of the sodium ion in the Lepidoptera seem to suggest that the membrane potentials in the Lepidoptera may not be governed by single ion fluxes as is thought to be the case in vertebrates, but may be more general in ion usage, possibly being related to all the major ions present, in the form of a muscle fibre membrane being a multi-ion electrode. The concept of a multi-electrode muscle fibre has already been postulated in Tenebrio by Belton and Grundfest(1962a,b) to explain their unusual potassium results. If this was also

the case in the Lepidoptera, the membrane potentials would not approach the values proposed by the Donnan equilibrium/Nernst equation system which works on the concept of single ion fluxes, but would be more readily explicable (at least for the resting membrane) in terms of the equation derived from the Constant Field theory of Goldman(Goldman,1943;Eccles,1953) in which

$$E_r = \frac{P_k(K)i + P_{Na}(Na)i + P_{Cl}(Cl)o}{P_k(K)o + P_{Na}(Na)o + P_{Cl}(Cl)i}$$

where P_k,P_{Na}, and P_{Cl} are the relative permeabilities of the muscle fibre membrane to the respective ions. This expression involves all the major ions thought to have some charge carrier action in excitable tissues, and should give an accurate description of the resting potential if passive diffusion alone were responsible for the generation of membrane potentials.

When the graph of E_r, derived from the constant field theory of Goldman(see Appendix for evaluation of relative permeabilities and calculation) is plotted against observed resting potential(see Figure 53), it is found to be very similar in shape to the latter, but smaller in value. The similarity of shape implies that the discrepancy in value is virtually constant, and the Goldman equation gives a much closer fit to the observed resting potential than does the predicted potential taking into consideration only the chloride ion. It is thus

possible that several ions may be involved in the generation of the resting potential, but in addition another constant factor seems to be involved. It is possible that active transport of ions against their electrochemical gradients may be involved, and this possibility is examined later in this section of the thesis.

Any possibility that chloride ions take a major part in the generation of the resting potential must be ruled out since, in the resting state, the muscle fibre membrane is much less permeable to chloride ions than to either sodium or potassium ions(see Appendix). Nor do chloride ions appear to have much effect on the Lepidopteran action potential. Incidental observations of action potentials from Sphinx ligustri muscle in various external chloride concentrations showed that these action potentials possessed no unusual features except in very low chloride concentrations, when the action potential appeared to be rather slow in repolarisation with the result that the time course of the action potential was slightly prolonged.

The effect of Calcium and Magnesium Ions on the Resting Potential

Increase in the external calcium concentration resulted in a slight increase in the size of the resting potential, in Bombyx mori muscle fibres. An increase in external

calcium from 0 to 15 mM(2 x normal for the haemolymph) resulted in an 11 mV increase in the resting potential (see Table 20).

Magnesium ions exerted little effect on the resting potential(Table 20). It is hoped in the future to complete studies on the effects of calcium and magnesium on the action potential in Lepidoptera. In Carausius morosus recent investigations seem to indicate that the magnesium ion may be involved in the charge carrying system of the action potential in muscle fibres(Wood,1957b) and in nerve (Treherne,1965a,b). Wood found that the action potential was impaired below 50 mM Magnesium in Carausius, a concentration far above that required to paralyse most neuromuscular systems. Depression of the action potential only started to occur at about 150 mM magnesium. This effect of magnesium on the action potential seems to suggest an active role for the ion, and since magnesium is often the most abundant ion present in herbivorous insects (Duchateau et al,1953) it is possible that magnesium ions may be used in action potential generation.

Studies on the Metabolic Inhibition of muscle fibres.

In the investigation of the effect of potassium ions on the resting potential, it was seen that a large discrepancy existed between the resting potential actually measured, and the resting potential calculated from the ratios of the potassium ion distribution. A similar, but rather smaller

Table 20. The effects of calcium and magnesium ions on the resting potential in Bombyx mori muscle fibres.

Concentration in mM per litre	Resting potential (Mean ± S.E.)	No of Records
Calcium		
15	47.7 ± 1.2	8
10	45.7 ± 1.1	7
7.5	42.5 ± 1.3	6
2.5	39.5 ± 0.6	7
0	36.2 ± 1.0	6
Magnesium		
200	39.8 ± 1.4	11
150	42.8 ± 0.7	15
100	41.7 ± 1.6	12
50	41.9 ± 1.2	14
0	42.4 ± 1.0	12

discrepancy was also seen when the effect of the chloride ion upon the resting potential was investigated. There was a possibility that in these cases, passive transport of ions was not the only mechanism involved in the generation of the resting potential. The observed resting potential might not have been completely linked to the ionic distribution, but also linked with the metabolism of the cell itself. To investigate the possibility of active transport of ions being also involved in the generation of the resting potential, the effect of certain metabolic inhibitors was investigated.

According to the ionic hypothesis, if the metabolism of a lepidopteran muscle cell were to be inhibited, we would expect to find only a very small and constant fall in the resting potential as sodium ions began to leak into the fibre from the saline. Ling and Gerard(1949) however, found that when frog muscles was soaked in a 5 mM/litre solution of the metabolic inhibitor b-indolyl acetic acid, the resting potential fell in two stages, a rapid 'A' fraction, followed by a plateau, then a less rapid 'B' fraction. The authors attributed the 'A' fraction of the resting potential directly to the metabolism of the muscle fibre, and the 'B' fraction to a non-metabolic source, being only related to metabolism for the maintainance of the cell membrane. Their overall conclusion was that cell metabolism was responsible for the maintainance of a large portion of

the resting potential. This work is now rather old, and has not been repeated or confirmed elsewhere. In a more recent investigation,Persson(1963) found that the metabolic inhibitor 2:4 dinitro-phenol progressively reduced the amplitude of the action potential by about one sixth after only 30 minutes. It was decided to repeat these experiments to investigate the role played by metabolism in membrane potentials in the Lepidoptera.

Preliminary experiments were carried out upon Bombyx Mori. Figure 54 shows the results of an experiment in which the fibres of the flexor tibialis muscle were soaked in normal saline containing 2.5 mM/litre 2:4 dinitro-phenol, a chemical which uncouples oxidative phosphorylation. The resting potential fell in about two hours to half the normal value, which was maintained for about one hour, then a slow fall set in, the resting potential being 6 mV. after seven hours. There was no secondary rise in the value of the resting potential after the plateau period as reported in frog muscle by Ling and Gerard(1949), but these results followed the same general course as those reported by these authors. When the preparation was returned to normal saline after the experiment there was no recovery of the resting potential to the normal value.

Since the preliminary experiments seemed to confirm the findings of Ling and Gerard(1949), it was decided to carry out a more detailed investigation of metabolic inhibition in

Table 21. The effect of 2.5 mM/lite 2:4 dinitro-phenol on the resting potential of Bombyx mori muscle fibres.

TIME(MINUTES)	RESTING POTENTIAL (Mean ± S.E.)	NUMBER OF RECORDS
0	43.1 ± 1.7	10
15	39.0 ± 1.3	4
30	38.1 ± 2.3	8
45	36.5 ± 1.3	7
60	37.4 ± 1.5	5
75	32.2 ± 1.9	8
90	30.0 ± 2.2	6
105	27.8 ± 2.0	7
120	24.7 ± 1.1	10
135	24.1 ± 1.8	6
165	23.4 ± 1.9	7
180	22.5 ± 2.7	8
195	20.1 ± 1.9	8
210	18.6 ± 1.0	7
225	17.8 ± 1.5	6
240	14.1 ± 0.8	7
255	14.5 ± 0.6	6
270	13.7 ± 1.3	7
285	12.3 ± 1.2	6
300	9.2 ± 0.3	7
315	8.3 ± 0.8	7
330	8.5 ± 0.8	6
345	8.1 ± 0.9	7
360	6.1 ± 0.8	6
390	6.0 ± 0.5	6
420	5.0 ± 0.7	6

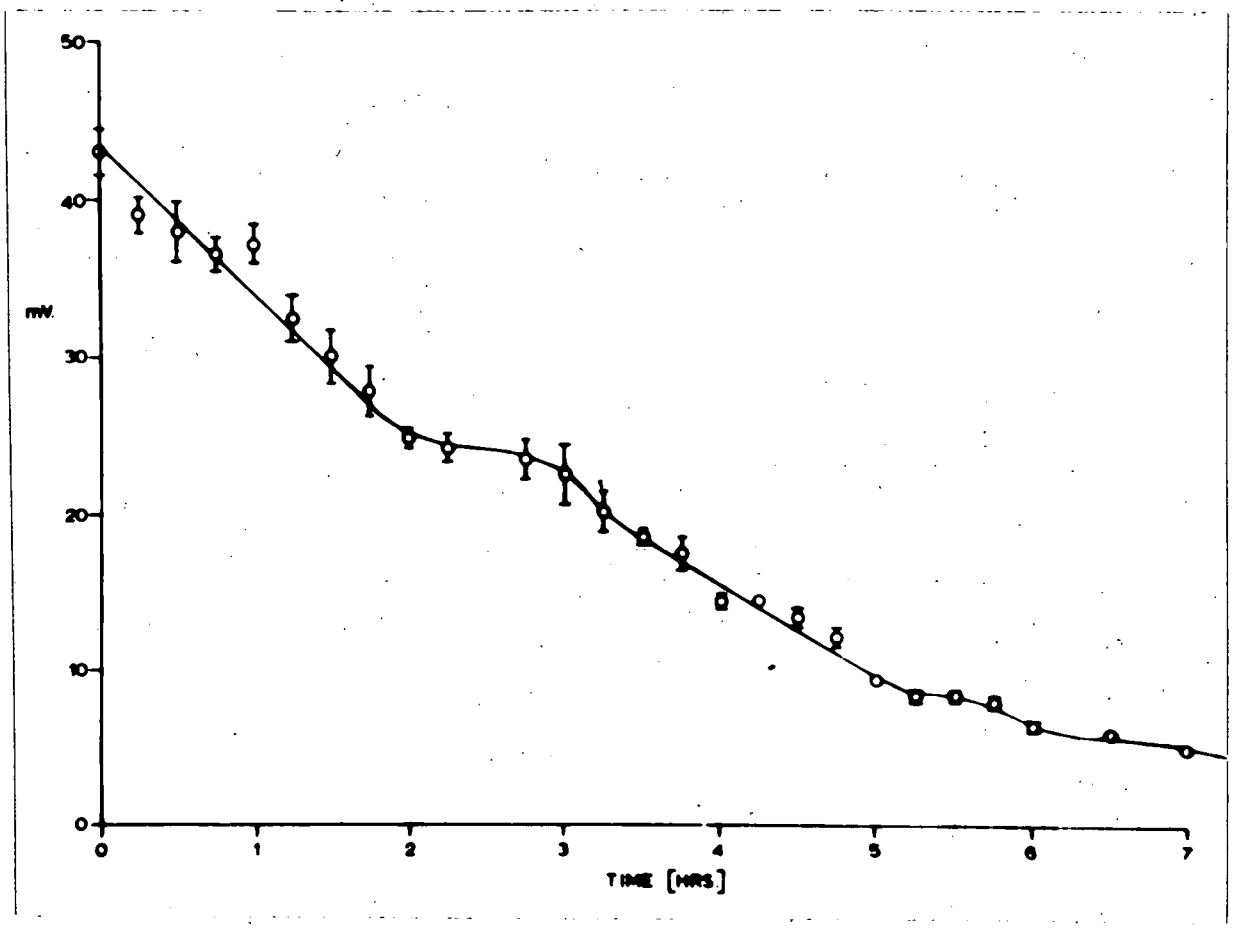


Figure 54. The effect of 2:4 dinitro-phenol on the resting potential of Bombyx mori muscle fibres.

Sphinx ligustri. Since it was important to know what effect 2:4 dinitro-phenol had on the permeability of the muscle fibre membrane, sodium and potassium analyses were performed at various stages in the experiments to follow any ion movements that may take place.

In the preliminary experiments it was noticed that the effects of inhibition were irrevsible, at least at the end of the experiments. A further group of experiments were performed to see at what point in the progressive onset of inhibition the effects became irreversible since reversibility in the early stages of the experiment would indicate whether an active transport process was involved.

In all investigations on Sphinx ligustri, the dorso/ventral flight muscle preparation was used. Figure 55 shows the results from two separate experiments. In the first (I), the muscle fibres were bathed in normal saline containing 0.5 mM/litre 2:4 dinitro-phenol, and the resting potential was recorded every 30 minutes. The results were essentially similar to those in Bombyx mori described above, the resting potential fell to about half its value in two hours, followed by a plateau, then a further fall to 7 mV after six hours. In the second experiment(2), recordings of resting potential were taken every 30 minutes, but after two hours, when the resting potential had fallen appreciably, the preparation was washed twice then returned to normal saline without the inhibitor. After a short delay the muscle

fibres made almost a complete recovery to 39 mV, about 90% of the original resting potential. This almost complete recovery of the resting potential suggests that in the early stages of the experiment some active transport process has been cut off, and that this is re-established by return to normal saline. In the latter stages of the experiment the reversibility is not observed, and it seems possible that this may be due to membrane damage. To check this, the effect of inhibitors on the membrane permeability was investigated. Sphinx ligustri muscle fibres were bathed in 0.5 mM/litre 2:4 dinitro-phenol saline, and every hour muscle samples were removed for sodium and potassium analysis by flame photometry.

Under the influence of metabolic inhibition, the muscle fibres tend to loose both sodium and potassium ions(see Table 23,Figure 56). Both ions tended to leave the muscle fibres rapidly at first, and then more slowly. After about four hours the internal sodium content of the fibres was between 4 to 8 mM per Kgm. tissue water, and the potassium content was 40 to 50 mM. The sodium and potassium content of the external saline was 5 and 50 mM/litre respectively. It thus appears that the internal ions declined and eventually equilibrated with the external saline, indicating that the muscle fibre membrane had become freely permeable under the influence of metabolic inhibition, The curves for the decline of these two ions in Figure 56 showed no plateau in the centre, which indicates that the exit of ions from

Table 22. The effect of 0.5 mM/litre 2:4 dinitro phenol on the resting potential of Sphinx ligustri muscle fibres, and the effect of return to normal saline.

Time (Minutes)	Resting potential (I) mV. Mean ± S.E.	Number of records	Resting potential (2) mV. Mean ± S.E.	Number of records
0	46.5 ± 1.7	8	43.9 ± 1.1	17
30	35.4 ± 1.1	9	33.1 ± 1.5	10
60	29.7 ± 1.3	9	27.7 ± 1.2	10
90	26.5 ± 1.2	12	23.2 ± 1.2	9
120	24.0 ± 1.0	11	20.6 ± 0.9	11
150	22.2 ± 0.5	9	21.3 ± 1.0	11
180	22.0 ± 0.8	11	20.1 ± 1.0	13
210	21.4 ± 0.7	17	24.2 ± 1.7	11
240	17.3 ± 1.5	12	29.4 ± 1.1	12
270	13.3 ± 0.9	7	32.2 ± 1.1	11
300	11.0 ± 0.9	9	35.7 ± 1.3	11
330	7.3 ± 0.5	7	39.0 ± 1.5	11
360	6.8 ± 0.4	12	- - -	-

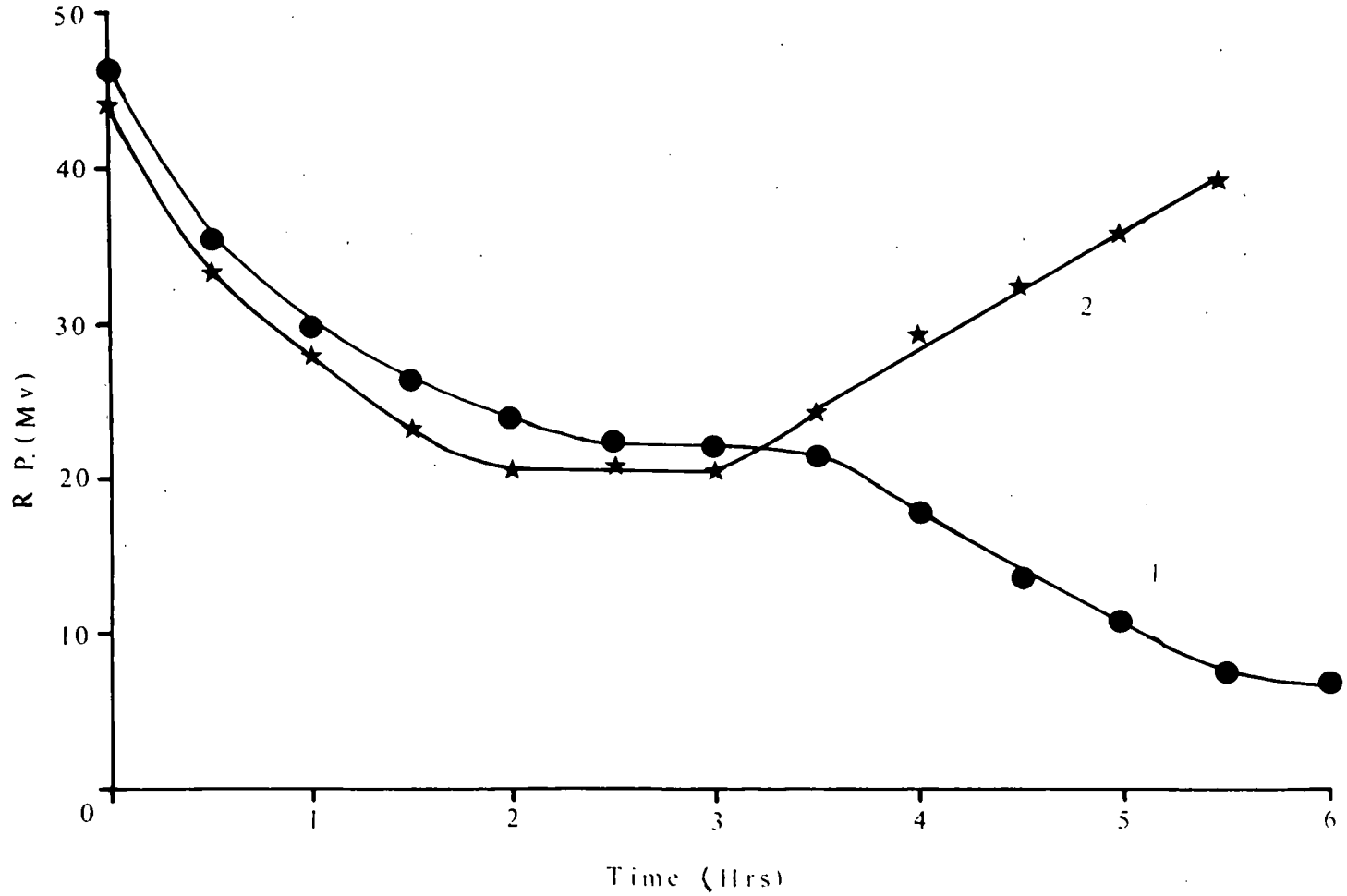


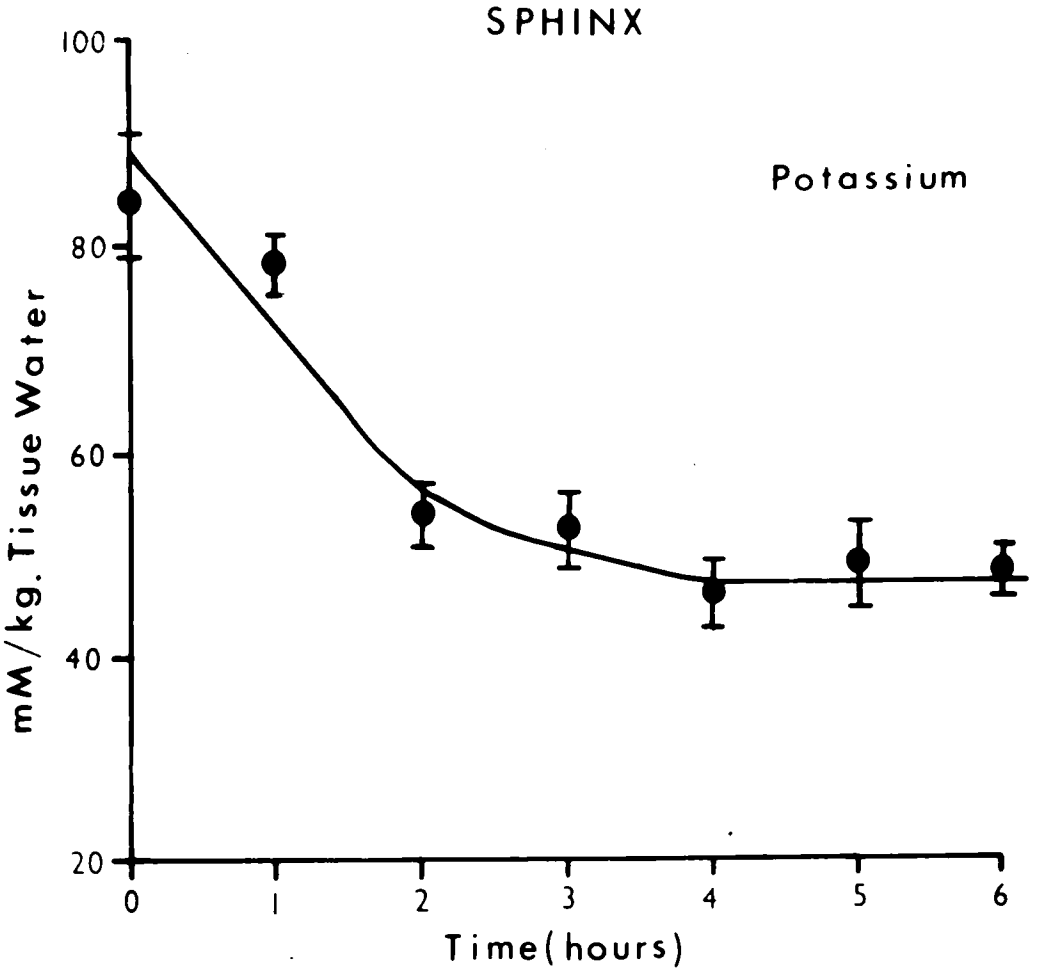
Figure 55

the muscle fibre is not fully related to the double decline of the resting potential seen in both Bombyx mori and Sphinx ligustri. It would seem that the resting potential fall is not all due to ionic loss.

Since both internal and external potassium were known in these experiments, it was possible to calculate the theoretical potassium electrode potential, E_k for each point on the graph, showing to what extent metabolic inhibition affected the E_k. These predicted potentials are shown plotted with observed resting potentials in Figure 27. In the latter stages of the experiment the calculated potassium electrode potentials approached either side of the zero potential line, so all the results have been plotted to indicate the scatter in both positive and negative directions. As the metabolism of the muscle fibres was progressively inhibited, the theoretical E_k began to approach more closely the observed resting potential. After 5 hours the scatter of results for the resting potential and the E_k became contiguous. This closeness between the observed and calculated resting potentials is remarkable since they differed by about 35 mV at the beginning of the experiment. This evidence would seem to indicate that the resting potential resulted from two sources, one metabolic, and one purely from distribution of potassium ions. As the metabolic fraction is cut out by progressive inhibition, the remaining resting potential approached the potential predicted from ionic distribution.

Table 23. The effect of 0.5 mM/litre 2:4 dinitro phenol on the intracellular potassium and sodium content of Sphinx ligustri muscle fibres.

Time (Hours)	Intracellular K^+ mM/Kgm.tissue water Mean ± S.E.	Number of records	Intracellular Na^+ mM/Kgm. tissue water Mean ±S.E.	Number of records.
0	84.4 ± 7.0	8	20.7 ± 1.6	8
1	78.7 ± 2.5	6	15.9 ± 1.3	6
2	54.2 ± 2.6	6	9.9 ± 0.6	6
3	52.8 ± 3.8	6	9.2 ± 0.7	6
4	45.5 ± 3.0	6	7.3 ± 0.5	6
5	49.5 ± 4.4	6	7.8 ± 0.5	6
6	48.2 ± 2.8	6	7.0 ± 0.5	6


SPHINX
Potassium
mM/kg. Tissue Water
100
80
60
40
20
0
1
2
3
4
5
6
Time(hours)

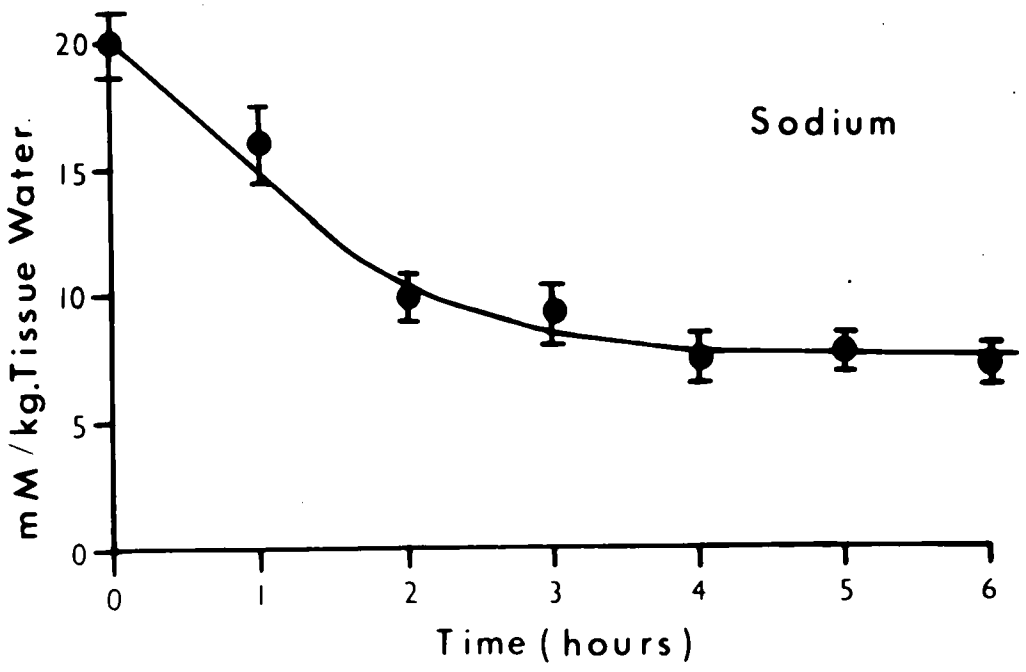

Sodium
mM/kg.Tissue Water
20
15
10
5
0
0
1
2
3
4
5
6
Time (hours)

Figure 56

This is also the conclusion Ling and Gerard(1949) drew from their results on frog muscle fibres, using different techniques.

To check that the results in these experiments resulted from the metabolic inhibition effects of 2:4 dinitro-phenol and not from other possible side effects of this substance upon the muscle fibre membrane alone, a further experiment was carried out using 1 mM sodium cyanide as the metabolic inhibitor. This latter substance has a more direct effect by irreversibly inactivating the metallo-protein enzymes of the cell involved in oxidation. The results, using the flexor tibialis preparation of Bombyx mori are shown in Figure 58. The resting potential fall was rather more rapid than that seen with 2:4 dinitro-phenol or b-indolyl acetic acid(Ling and Gerard,1949), but the shape of the graph was similar to that obtained with the other inhibitors. After three hours, 70% of the resting potential was abolished. This is taken as evidence that the inhibitors all act simply by cutting off cell metabolism, and eventually making the cell membrane more permeable, but not initially damaging the membrane(this will happen when osmotic swelling of the cell becomes great).

Ionic Depletion of Muscle.

An early investigation which bears upon the problem of ionic origin of the resting potential is that of Tobias(1950). In a series of experiments on frog muscle fibres, he was able

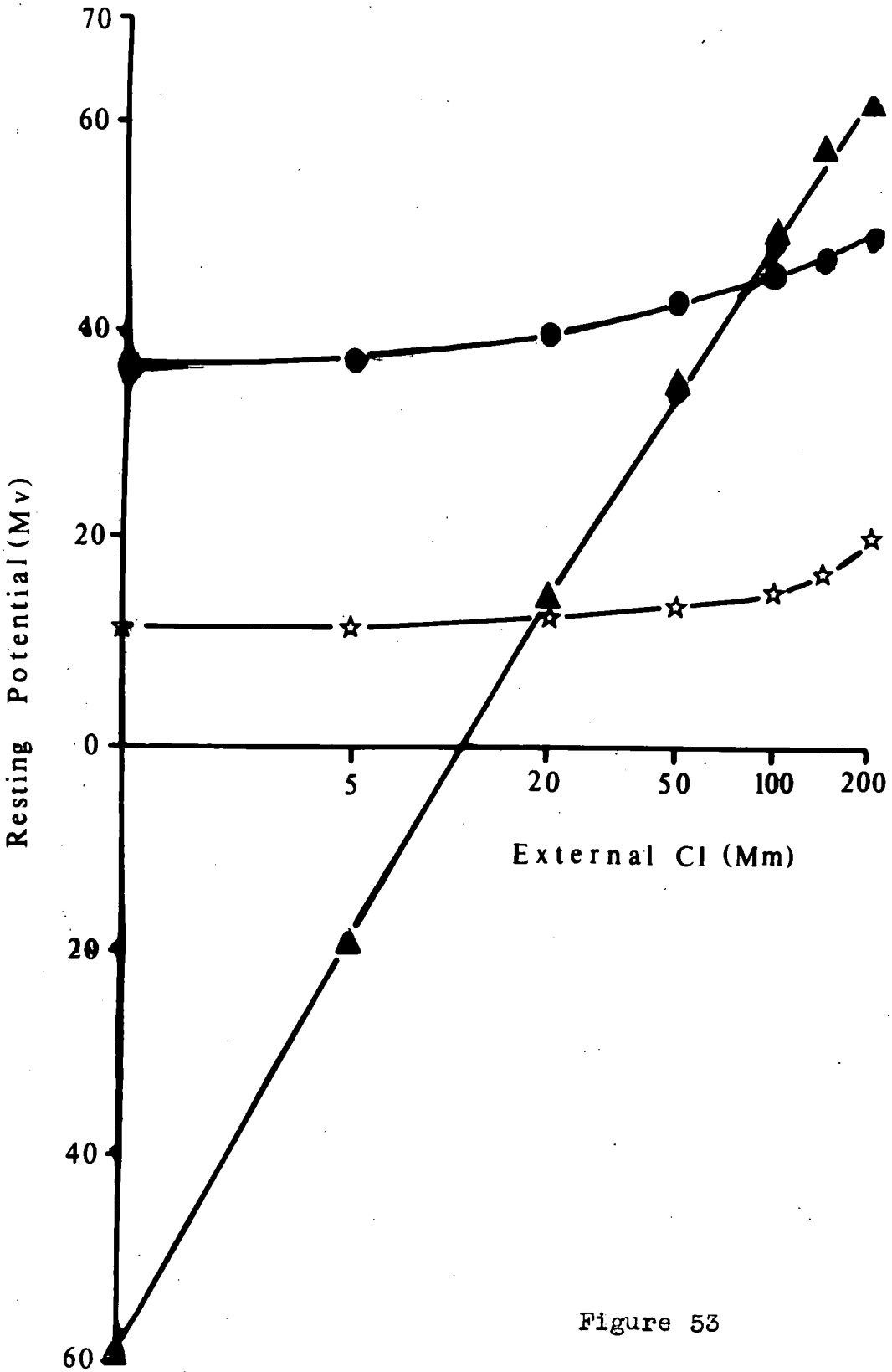

70
60
40
20
0
20
40
60
Resting Potential (Mv)
5
20
50
100
200
External Cl (Mm)

Figure 53

Table 24. The effect of 0.5 mM/litre 2:4 dinitro phenol on the theoretical potassium electrode potential, plotted with the observed resting potential in Sphinx ligustri.

TIME (Hours)	Observed resting potential(mV) ± S.E.	Number of records	Theoretical E_k from(K)i/(K)o mV.	Number of records
0	- 46.5 ± 1.7	8	- 12.4 ± 2.3	6
1	- 29.7 ± 1.3	9	- 11.3 ± 0.8	6
2	- 24.0 ± 1.0	11	- 1.8 ± 1.2	6
3	- 22.0 ± 0.8	11	- 1.9 ± 1.9	6
4	- 17.3 ± 1.5	12	+ 2.4 ± 1.8	6
5	- 11.0 ± 0.9	9	- 0.1 ± 2.4	6
6	- 6.8 ± 0.4	12	+ 1.1 ± 1.5	6

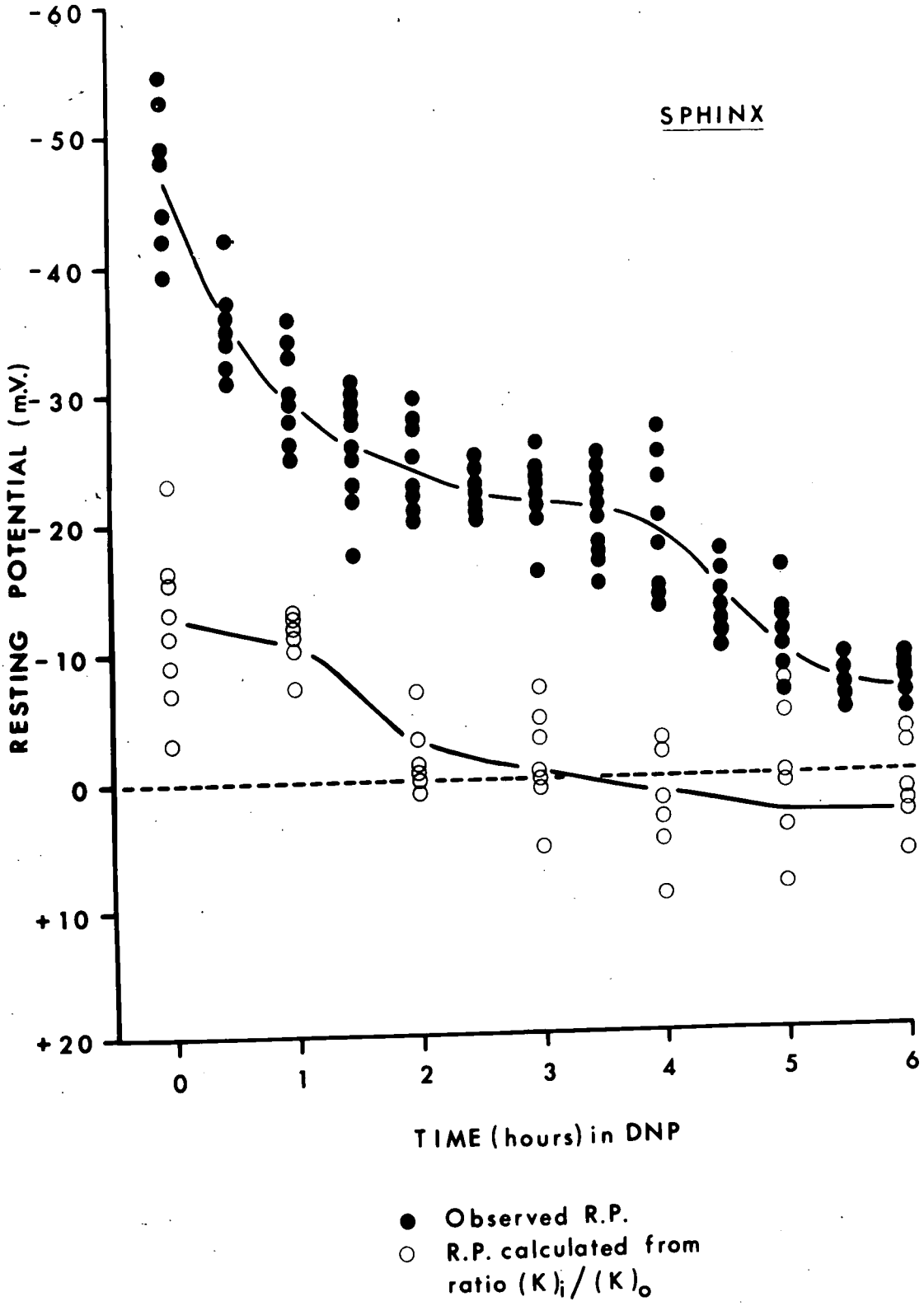

SPHINX
RESTING POTENTIAL (m.V.)
-60
-50
-40
-30
-20
-10
0
+10
+20
0
1
2
3
4
5
6
TIME (hours) in DNP
Observed R.P.
R.P. calculated from ratio $(K)_i / (K)_o$

Figure 57

to remove 99% of the intracellular potassium and 96% of the intracellular sodium by soaking the muscles for prolonged periods in cold distilled water. The fibres became oedematous and inexcitable as well as freely permeable, but still showed a resting potential about 40% of the original value. An e.m.f. generative process was still in operation since current could be drawn from the fibres for several hours. These results have been confirmed recently(Wood, personal communication). Tobias argued that a Donnan equilibrium could not be responsible for the potential since the membrane was freely permeable. The author concluded that the resting potential left was due to the remaining metabolism of the muscle fibres themselves.

This experiment has been repeated on the flexor tibialis muscle of Bombyx mori and Sphinx ligustri. The preparations were soaked for six hours in three changes of distilled water, the water being regularly circulated round the muscles. The results in both species are shown in Figure 59. After five hours, the muscle fibres of Bombyx mori still showed 12.5% of their original resting potential, after six hours the corresponding figure for Sphinx ligustri was 28% even though the muscles in both species were waterlogged and inexcitable. Analysis of muscle fibres of Sphinx after six hours showed that sodium had dropped to 8 $\pm$ 0.7 mM/Kgm. tissue,water, and potassium to 5.8 $\pm$ 1.0 mM/Kgm. tissue water. Since the remaining resting potential could not be attributed to ionic sources, a metabolic source seems the only plausible alternative.

Table 25. The effect of Sodium cyanide(1 mM/litre) on the resting potential of Bombyx mori muscle fibres.

Time (Minutes)	Resting potential Mean ± S.E. (mV).	Number of records.
0	39.3 ± 1.1	10
30	27.6 ± 1.1	7
60	24.9 ± 2.1	9
75	22.6 ± 2.3	8
95	19.7 ± 1.7	8
110	19.2 ± 0.9	8
125	18.0 ± 1.2	8
140	16.4 ± 0.8	8
155	14.1 ± 1.1	8
170	12.8 ± 0.6	6
185	11.7 ± 0.8	8

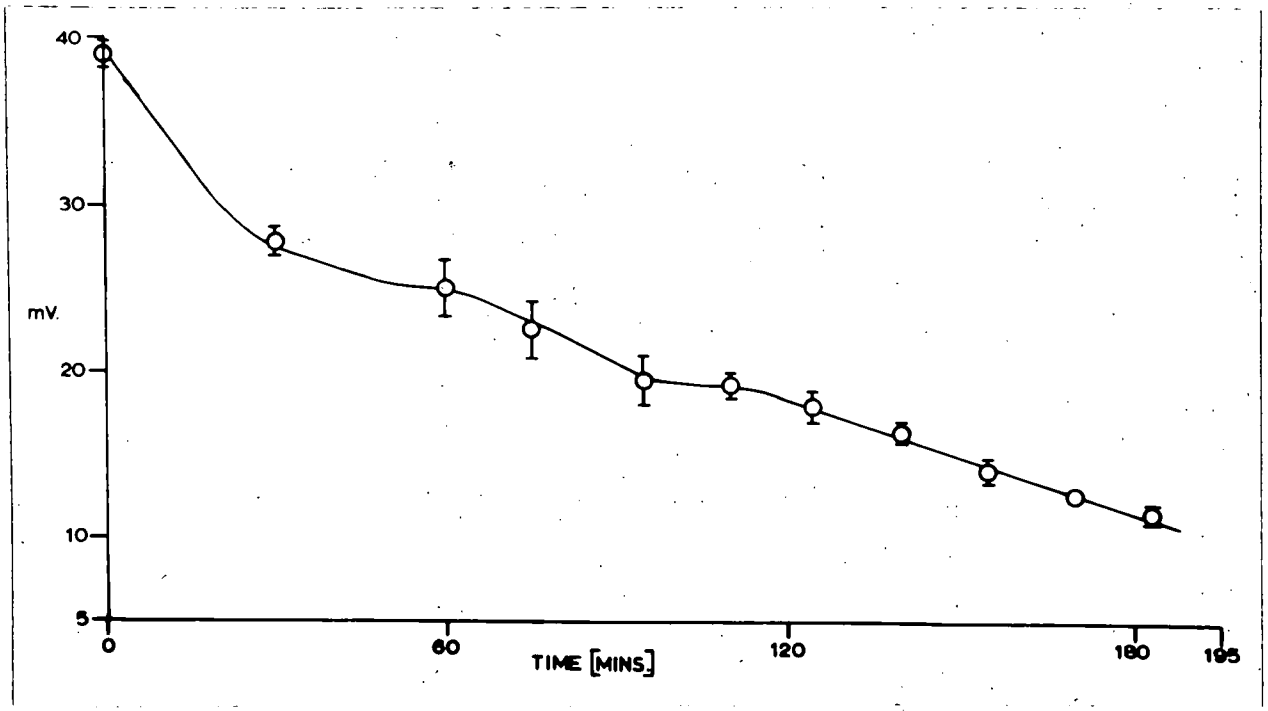


Figure 58. The effect of Sodium cyanide on the resting potential of Bombyx mori muscle fibres.

The ionic hypothesis is centred around a concept of passive transport of ions, the ions involved moving along their electrochemical gradients with no expenditure of metabolic energy(Boyle and Conway,1941;Conway,1946,1947; Hodgkin and Katz,1949;Hodgkin,1951,1964). The sodium pump (Dean,1941) is the only concept involved which has any reference to the metabolism of the muscle cell. Metabolic inhibition should thus have very little effect on membrane potential. The evidence of earlier workers, and the evidence presented above is not easily explicable in terms of a simple passive diffusion of the type postulated in the ionic hypothesis. It is interesting to note that in the above experiments, the fall in the resting potential followed a two phase course, which seems to suggest that the resting potential results from two sources, and not one.

One interpreation of these results is that the initial fall in the resting potential is directly due to the effects of the metabolic inhibitor upon metabolic processes which are in turn directly supporting the part of the resting potential which is subsequently abolished. If this was so, then removal of the inhibitor should lead to a restoration of the abolished potential. Evidence is presented above to show that this is occurs in these experiments. Such a restoration of the normal resting potential supports the argument that it is not the membrane which is being directly damaged by the inhibitor, but that the effect is on the cell metabolism only. If this supposition is correct, then the plateau present in

Table 26. The effect of prolonged soaking on the resting potential of muscle fibres in Bombyx mori and Sphinx ligustri.

Time (Minutes)	Resting potential mV, Mean ± S.E.	Number of results.
BOMBYX		
0	46.5 ± 1.2	9
10	37.0 ± 2.3	5
30	29.6 ± 1.3	8
60	21.4 ± 1.0	8
75	14.8 ± 1.0	6
105	12.1 ± 0.8	7
135	12.8 ± 0.9	11
165	10.3 ± 1.1	10
215	8.5 ± 0.7	8
285	5.3 ± 0.6	8
315	5.4 ± 0.5	5
SPHINX		
0	43.1 ± 1.5	15
30	37.6 ± 2.0	10
60	31.2 ± 1.4	19
90	27.0 ± 1.0	17
120	24.0 ± 1.1	19
150	22.1 ± 1.2	14
180	19.0 ± 1.1	20
210	18.0 ± 0.9	15
240	15.0 ± 1.2	17
270	13.4 ± 1.2	12
300	11.6 ± 1.3	14
330	12.0 ± 1.2	12

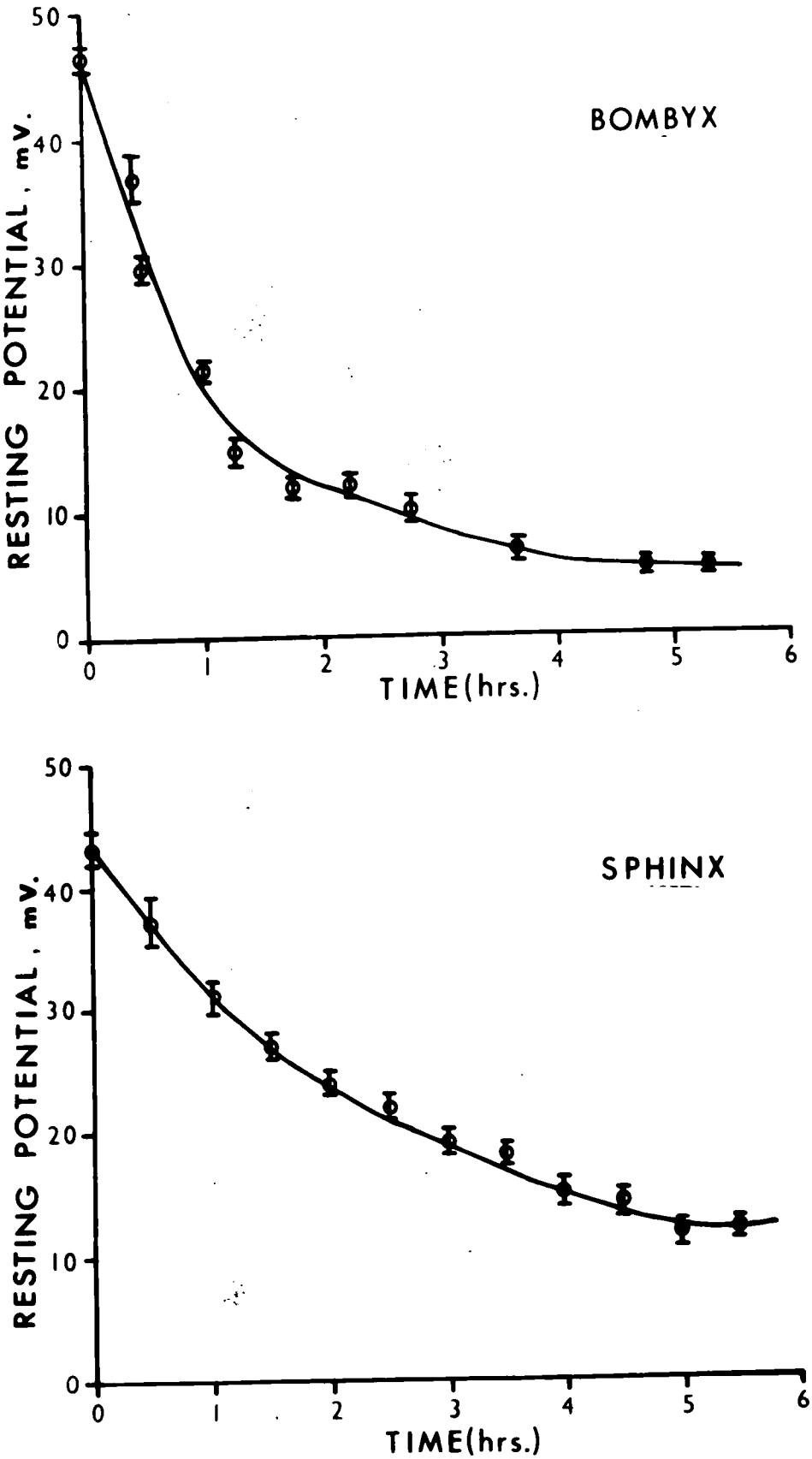

BOMBYX
RESTING POTENTIAL, mV.
50
40
30
20
10
0
0
1
2
3
4
5
6
TIME(hrs.)
SPHINX
RESTING POTENTIAL, mV.
50
40
30
20
10
0
0
1
2
3
4
5
6
TIME(hrs.)

Figure 59

the graphs would represent the resting potential resulting from non-metabolic(i.e. ionic) sources. This is confirmed by the fact that the theoretical E_k for the species investigated is roughly at the value of the plateau found in these experiments(see Table 27). The subsequent slow fall in resting potential from the plateau under prolonged exposure to metabolic inhibition may be due to progressive damage to the cell membrane caused by cessation of cellular metabolism, and the effect this probably has on membrane permeability. The observation that the second fall in the resting potential is irreversible adds weight to the argument that some physical change has set in within the membrane itself. This secondary fall is thus not related to the metabolism of the cell as such, but is more likely to be related to progressive osmotic stress in the cell. As the cell loses its sodium and potassium ions the second fall in membrane potential eventually sets in.

If the resting potential was entirely metabolically supported, one would expect that metabolic inhibition would cause the resting potential to decay away in ah exponential manner, whereas this is not seen. The results above fit much more closely into the interpreation of Ling and Gerard (1949) involving a dual source for the resting potential.

The experiments in which the muscles were soaked in distilled water can be considered as a reversal of the inhibition experiments. In these cases, the initial resting potential fall is probably due to the increased membrane

permeability and subsequent loss of intracellular ions. The plateau would then mark the level of the metabolically supported resting potential. This is maintained until progressive waterlogging and ionic stress inside the cell causes a cessation of cell metabolism, and then the metabolically supported part of the resting potential will begin to decline.

GENERAL CONCLUSIONS

The ions present in the haemolymph of an insect are thought to be related to the insect's diet(Bone,1944; Duchateau et al,1953). In omnivorous and carnivorous insects, the sodium to potassium and calcium to magnesium ratios in the haemolymph are usually greater than unity, and in this respect they resemble the vertebrates. Herbivorous insects, which ingest material rich in potassium and magnesium have haemolymph ion concentrations in which the sodium to potassium and calcium to magnesium ratios are less than unity, magnesium often being in concentrations greater than all the other ions together(Duchateau et al,1953; Tobias,1948;Wood,1957b;Carrington and Tenney,1959). The Lepidoptera are extreme examples of this 'herbivorous type' haemolymph (see Tables 4 and 5). In Table 27, the various electrode potentials for the normal haemolymph ionic concentrations in the four species of moth have been calculated and are shown next to the recorded resting and action potentials in these species.

There seems to be little relation between the observed resting potential and the theoretical potential calculated from potassium ion distribution. If the lepidopteran resting potential was developed in accordance with the Nernst equation, it should be proportional to the logarithm of the external potassium concentration, giving a straight line relationship. The relation found is rather hyperbolic, only approaching

ideal conditions in high potassium concentrations. Below the normal blood level of potassium, the graph falls increasingly away from the theoretical. Even though potassium ions have a smaller effect on resting potential in Lepidoptera than they have in other animals and even in other insects(Hoyle,1953,Wood,1957b,1963), part at least of the resting potential must be related to potassium ions, since alteration of external potassium does have an effect on resting potential, and so does depletion of internal potassium ions.

The nearest approach based on the Nernst equation to the observed resting potential is given by the chloride electrode potential in the haemolymph ion range. The results given in Table 27 are about 90% of the observed potential which is a reasonable fit. This fit between the two potentials is only fortuitous, since the chloride potential and the resting potential diverge widely outside the chloride range of the haemolymph.

It is interesting to note that the two supposedly related electrode potentials, E_k and E_{Cl} are not related even in the normal haemolymph range for these ions. This underlines the lack of reciprocity between these ions which is found at almost all experimental concentrations investigated.

The values quoted for E_{Na} in Table 27 are purely hypothetical since the basis on which they were calculated can not apply in the Lepidoptera owing to the reversed soidum gradient.

Table 27. The theoretical electrode potentials and observed membrane potentials in Lepidoptera.

Species	E_k (mV)	E_{Na} (mV)	E_{Cl} (mV)	Resting Potential (mV ± S.E.)	Action Potential (mV ± S.E.)
Bombyx mori	- 21.7	- 8.0	-41.2	41.8 ± 1.4	40.1 ± 2.1
Sphinx ligustri	- 13.3	-44.0	-36.3	44.5 ± 2.2	42.3 ± 1.8
Actias selene	- 22.6	-14.2	-42.5	46.4 ± 1.3	45.0 ± 2.3
Telea polympehmus	- 16.5	-10.5	-37.9	48.2 ± 1.4	44.0 ± 1.2

In no case, however, do the theoretical sodium electrode potentials approach the action potentials observed. In all species the action potentials were observed to overshoot zero potential on occasions, but the mean action potentials in all species showed slight undershoots of a millivolt or so. Such undershoots were never as large as the theoretical "undershoots" worked out from sodium distribution.

One mechanism which could go a long way to explain the unusual results above in terms of the conventional vertebrate ionic hypothesis would be a mechanism involving a diffusion barrier of some sort around the muscle fibres. Such a diffusion barrier could create a two compartment system around the muscle fibres. If such a barrier existed then the strange ionic configuration of the haemolymph would present no problems since the diffusion barrier could selectively concentrate ions such as sodium and calcium around the muscle fibre membrane, and could actively eliminate potassium and magnesium ions from this space. There is widespread evidence of a diffusion barrier around the nerves of insects(Hoyle,1952;Hughes,1953), Recently Treherne(1965a,b) has put forward a concept of a two-compartment system with active concentration and elimination of ions around the central nervous system of Carausius.

In the Lepidoptera, the central nervous system possesses a stout neural lamella which extends along the peripheral nerves and may act as a diffusion barrier. There is some physiological evidence in favour of a diffusion barrier

around the muscles in Lepidoptera, the most important evidence being the very long replacement times needed for the effects of external ions to be obvious on the membrane potentials. The small effects of sodium on the action potential and the observations of Wood(1957b) that the concentration of magnesium required for neuromuscular block in Carausius was very high indeed might also be considered to support such an interpretation although magnesium in low concentrations caused the resting potential to decline. In the Lepidoptera there is no histological evidence of a diffusion barrier around the muscles. The long replacement times for external ions may be due to the dense tracheolar network in the muscle, making penetration into muscle spaces rather difficult. However, there is no evidence that any barrier to diffusion caused by tracheal membrane is selective for certain ions, the delay in penetration of ions seems quite general.

The inulin clearance technique suggests that the intramuscular space in Lepidoptera is small, and Wood reported similar results in Carausius(1963). This space is considerably smaller than total haemolymph volume and it seems unlikely that such a small intramuscular space could regulate ions to the degree required in such an unfavourable haemolymph of that size.

Hoyle(1957b) considered the processes of neuromuscular transmission in insects to be essentially similar to the

vertebrate process. He considered the unusually large concentrations of magnesium and potassium to be specialisations due to diet, and thought that the insects were at the extreme point of tolerance to these ions. On this view, insects are just an extreme extension of the normal ionic hypothesis. Tolerance of magnesium is possible if the neuromuscular transmitter is not magnesium-sensitive. Magnesium reduces the quantity of vertebrate transmitter released from the nerve terminals(del Castillo and Engback,1954), but there is no evidence that magnesium has this effect in herbivorous insects. Tolerance of potassium is possible owing to the smaller effect which this ion has on the membrane potentials of herbivorous insects. Since the insect muscle fibre will contract in the absence of an active membrane response concentrations of potassium which abolish this but do not abolish the end-plate potential can be tolerated. In the Lepidoptera this concentration of potassium may be as high as 150 mM per litre. An explanation of insect neuromuscular transmission involving simple tolerance of ions may apply to non-herbivorous insects which are relatively near to the vertebrates as far as haemolymph ions are concerned, but it does not seem possible to extend the accepted ionic hypothesis to explain neuromuscular transmission in herbivorous where sodium and calcium are present in such very small concentrations while magnesium and potassium are present in such large concentrations. The suggestion that

magnesium may play an active part in membrane potential generation(Wood,1957b;Treherne,1965a,b) is a specialisation very different from accepted ionic theory, and in any case, the reversed sodium gradient is inexplicable in terms of the ionic hypothesis.

Herbivorous insects do not appear to be extensions and specialisations of the normal accepted ionic hypothesis, instead a quite different mechanism seems to be responsibl e for muscle membrane potentials in these animals. This leaves two alternatives. Either the membrane potentials are ionic in origin, but result from very different ions from those normally invoked in the ionic hypothesis, or the membrane potentials are either partly or wholly supported by active transport of ions involving expenditure of energy from cell metabolism.

The former alternative does not seem very likely. The major ions in the haemolymph of herbivorous insects are of the same type as in other animals, and none of the electrode potentials from these ions seem to fit closely to the observed resting potentials(with the fortuitous exception of the chloride potential). Even when the main ions are all taken into account in Goldman derived equations the fit is not close, although the fit is closer than the Nernst equation involving single ions. This does suggest that the muscle fibres are multi-ion electrodes.

The second alternative seems the most likely. The

membrane potentials are probably not totally supported by active transport of ions since metabolic inhibitors only partly abolish the membrane potentials in the muscle fibres, and the Goldman equation does show that the main ions make some contribution to the resting potential. The plateau noticed in the metabolic inhibition experiments probably represents the junction between two separate potentials which form the resting potential. The tendency for the potassium electrode potential to approach the observed resting potential under the influence of metabolic inhibition also suggests a twofold origin for the resting potential: part active transport which is easily abolished by metabolic inhibitors, and part passive which is only abolished as the cell organisation breaks down.

Such a mechanism is very different from accepted ionic theory and represents a specialisation in herbivorous insects to ensure that the muscle fibres operate efficiently in an unfavourable ionic medium imposed on the animals due to food source.

SUMMARY

1. All four species examined had a similar type of neuromuscular anatomy, the metathoracic tibialis (flexor) muscle being supplied by one nerve from the metathoracic ganglion.

2. The muscle fibres were multiterminally innervated. End-plates were distributed along the fibres at intervals of approximately 60μ, and were of the Doyère-cone type.

3. Examination with intracellular electrodes showed two types of electrical response in the muscle fibres. Fast, non-facilitating responses of about 40 mV were concerned with twitch contractions and rapid movements, while slow readily facilitating responses of about 10 mV were concerned with slow graded movements and the maintainance of tonus and posture.

4. These two responses resulted from separate axons, often visible as separate entities up to the end plate. The muscle fibres were thus polyneuronally innervated.

5. In all species the resting potential ranged from 40 to 50 mV. Although all species had some muscle fibres which showed a slight overshoot of zero potential, the mean action potential was always slightly smaller than the mean resting potential.

6. The rising phase of the action potential was composed of an

end-plate potential and an active membrane response, and the decay phase lasted up to 30 milliseconds due mainly to a prolonged negative after-potential.

7. Haemolymph analysis has shown that all four species are typical herbivores containing large concentrations of potassium and magnesium and having sodium to potassium and calcium to magnesium ratios less than unity.

8. Analysis of the myoplasm reveals high internal potassium and low internal sodium and chloride. Moreover, the ratio of internal to external sodium was greater than unity, and the ratio of internal to external potassium was low.

9. Potassium ions exerted a depolarising effect upon the muscle fibre membrane, and in high external potassium the active membrane response was abolished. The resting potential was only proportional to the logarithm of the external potassium concentration in the middle range. Above and below this large discrepancies appeared. The theoretical E_k did not approach the resting potential values in either normal or experimental salines.

10. Alteration of external sodium ions had a slight effect on both the resting and action potential. The theoretical E_{Na} was unrelated to recorded action potential and was negative up to 50 mM/litre external sodium.

11. Excitability could be maintained in the absence of

sodium. Tetramethyl ammonium ions were an adequate substitute for sodium, although the ethyl and butyl homologues caused increased membrane excitability followed by inexcitability. It is postulated that sodium ions are not essential for the development of the action potential.

12. Increase in external chloride ions slightly elevated the resting potential but had little effect on the action potential. It is suggested that the lepidopteran muscle fibre membrane is at least in part, a multi-ion electrode. There was no reciprocity between the ratios of internal to external potassium and external to internal chloride. The E_{Cl} bore little relation to the observed resting potential, except in normal saline. It is argued that this agreement is quite fortuitous.
13. The equation derived from the Goldman constant field theory gave a closer fit to observed resting potential than the Nernst equation. This seems to provide further evidence of a multi-ion electrode muscle fibre. However, a constant discrepancy was encountered.
14. Magnesium and calcium had very small effects on the resting potential. The effects of these ions on the action potential (if any) were not investigated.
15. Application of metabolic inhibitors resulted in a fall in the resting potential of the muscle fibres. In early stages this effect was reversible but irreversible at

later stages. The resting potential decline was bi-phasic, with a central plateau. It is suggested that the initial fall is due to direct inhibition of an active transport process. The plateau is taken as the level of resting potential due to ionic sources. In Bombyx mori and Sphinx ligustri the E_k is roughly equal to this value. As metabolic inhibition progesses, the resting potential approaches the value of the Nernst equation derived from potassium distribution.

16. Prolonged soaking in distilled water casued muscle fibres to lose internal potassium and sodium, and the resting potential also declined. A di-phasic decline of resting potential was found. It is suggested that the plateau in this case marks the level of the resting potential due to metabolic support. The secondary fall in resting potential may be due to damage of the muscle cell by progressive osmotic stress.

17. It is concluded that the membrane potential in the Lepidoptera has its origin in both passive distribution of ions across the muscle fibre membrane, and in active transport of ions maintained by membrane metabolism.

Appendix. The evaluation of relative permeabilities and the Goldman constant field equation.

If more than one ion were to be involved in the generation of the resting potential, then more than one ion must be taken into account when calculating potentials using the Nernst equation. In isolated preparations of the Loligo giant axon, which were gaining sodium and losing potassium, Hodgkin & Katz (1949) used the constant field theory of Goldman(1943) to calculate the resting potential. The equation they derived was :-

$$E_r = \frac{R\,T}{F} \log_e \frac{P_k(K)i + P_{Na}(Na)i + P_{Cl}(Cl)o}{P_k(K)o + P_{Na}(Na)o + P_{Cl}(Cl)i}$$

where P_K, P_{Na}, and P_{Cl} were the relative permeabilities of the membrane to the respective ions. To evaluate the equation, the relative permeabilities are needed.

In this investigation, experiments have been performed in which the external concentrations of potassium, sodium, and chloride have been altered, and the changes in internal ions measured in various Lepidoptera. Such ion changes allow us to calculate the relative permeability of the lepidopteran muscle fibre membrane to these ions. It has been assumed that the two different lepidopteran species used here differ little in their permeability characteristics, hence the sodium results from Bombyx mori have

been used to calculate sodium permeability.

Alteration of the external chloride concentration in Sphinx ligustri from 1 to 200 mM/litre produced an internal change of 7mM chloride ion(see Table 19).

Alteration of the external sodium concentration in Bombyx mori from 1 to 200 mM/litre produced an internal change of 16 mM in sodium ions(see Table 13).

Alteration of the external potassium concentration in Sphinx ligustri from 1 to 200 mM/litre produced an internal change of 100 mM potassium ions(see Table 7).

Taking the chloride permeability as unity, the relative permeabilities are

Chloride	Sodium	Potassium
1	16/7 = 2.3	100/7 = 14.3

When these permeabilities are used in conjunction with the results in Table 19(section III), we obtain the following results(at 20 ^{o}C) for the various external Cl concentrations.

At 1mM external chloride,

$$E_r = \frac{58 \log \ 14.3(77.5) + 2.3(20.0) + 1(1.0)}{14.3(50.0) + 2.3(3.6) + 1(10.5)}$$

$$\simeq 58 \log 1.573$$

$$= \underline{11.4 \text{ mV}}.$$

Using this calculation, the results at the other external chloride concentrations are :-

At. 5 mM external chloride

E_r = 11.6 mV.

At 20 mM external chloride

E_r = 12.4 mV.

At 50 mM external chloride

E_r = 13.4 mV.

At 100 mM external chloride

E_r = 14.4 mV.

At 140 mM external chloride

E_r = 16.0 mV.

At 200 mM external chloride

E_r = 19.9 mV.

These results for the constant field theory potential have been plotted along with the chloride electrode potential, E_{Cl} against the observed resting potential in Figure 53 (section III).

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