STUDIES ON MYO-NEURAL MECHANISMS IN ARTHROPODA

Thesis by

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RESUMÉ

PART I. Innervation Patterns of Crustacean Limbs.

In contrast to the vertebrates where the skeletal muscle is built up of large numbers of motor units, the limb muscles of decapod Crustacea are innervated by a small number of motor and inhibitory axons. It is possible to determine the details of the innervation and of the muscular response elicited by each of the several axons by isolating them in the nerve trunk. By such techniques, the previously determined innervation patterns of the walking legs in the four tribes of the Decapoda Reptantia have been confirmed and extended. Incomplete innervation patterns for the Decapoda Natantia and Stomatopoda have also been determined. Many characteristic features of innervation and associated motor and inhibitory effects were found to be common to all the Decapoda and the Stomatopoda. These physiologically derived anatomical data support the classification of the Decapoda into Reptantia and Natantia with division of the former into four tribes. A tentative evolutionary hypothesis is presented to explain the divergence of the different innervation patterns.

PART II. The Effect of Spaced Stimulation of Excitatory and Inhibitory Axons of the Crayfish.

If a single motor axon to one of the limb muscles of a crustacean is stimulated with pairs of shocks repeated at regular intervals, the resulting contraction is greater than that elicited by the same number of regularly repeated single shocks. This effect was previously studied in the fast and slow contractions of muscles in various decapod Crustacea. The authors concluded that the effect was much more pronounced in the fast than in the slow system. In order to extend these observations and to determine whether or not it is a true myo-neural junctional phenomenon,

the effect of unequally spaced stimulation of both the excitatory and inhibitory axons in the abductor of the dactylopodite was studied. The contractions resulting from such stimulation of the excitatory axon were more difficult to inhibit than were the normal contractions. Stimulation of the inhibitory axon with unequally spaced shocks resulted in a much greater reduction of the contraction than when it was stimulated with the same number of regularly recurring single shocks. It is concluded therefore, that the spacing effect occurs at the myo-neural junction and that it is not a mechanical effect at the level of the contraction.

PART III. Neuromuscular Mechanisms in the Grasshopper, Romalea microptera (Beauv.).

Insect myo-neural physiology has not been extensively studied but recently the existence of single and double innervation and of fast and slow contractions has been reported. The occurrence of peripheral inhibition has been claimed but the observations cannot be considered conclusive. In view of the phylogenetic relationship between the Crustacea and Insecta it might be expected that the two sub-phyla would have many characteristics of the myo-neural system in common. Both histological and physiological observations of the limb muscles of Romalea have confirmed this expectation. With one exception, these muscles are innervated by about six axons, each of which gives rise to a characteristic contraction. The majority of these contractions are of the fast type. True slow contractions have not been observed. There is no marked facilitation of either the contractions or the action potentials. The large 'jump' muscles of the third limb are specialized and exhibit only a single fast contraction type. No evidence for peripheral inhibition has been obtained.

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INNERVATION PATTERNS OF CRUSTACEAN LIMBS [1])

by

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INTRODUCTION.

In contrast to the innervation of vertebrate muscles, there are only a limited number of efferent axons innervating the muscles of the Arthropoda (PRINGLE, 1939; 1949; WIERSMA, 1941; 1946). By a technique of isolation of single axons in the legs of Crustaceans, it is possible to determine this innervation for a muscle and to compare it with that of other muscles in the same animal and with homologous muscles of other species. This type of approach is particularly significant since it has become clear that each of the axons to a muscle mediates a discreet mechanical response which can be determined by stimulation of the axon and recording of the myogram. The axons innervating the muscles of the lower leg are most suitable for this purpose as sufficient length of nerve is available for the isolation of single axons. For anatomical reasons, axon isolation in other muscles of the body, when possible at all, is much more difficult. In most cases however, the relatively small number of efferent axons can be determined by *in vivo* staining with methylene blue solution. The regular, parallel branching of the axons on the surface of the muscle makes observation relatively simple in the majority of the Crustacea. By this method it is often possible to confirm innervation patterns determined by stimulation of the single axons of the leg muscles.

Using these methods, innervation schemes for a number of the Decapod Crustacea have been determined. Characteristic differences exist between the different tribes whereas in general, members of a tribe have identical schemes. Previously the number of species investigated within both the Palinura and Anomura was too small to allow generalization. The present investigation was undertaken to remedy this deficit and to extend our knowledge to species of the Decapod Natantia and Stomatopoda. Since for the latter two groups no data concerning the mode of innervation were available, it became of considerable importance to determine, in the first place, whether these forms have indeed an innervation similar to that of the higher Decapoda. To date the highly characteristic peripheral inhibition is known to occur among the Arthropods with certainty only in the Reptantia Decapoda Crustacea. Fast and slow contractions occur in both Reptantia and Insecta. From the point of view of comparative physiology this makes the study of lower crustacean forms particularly interesting.

All the reported experiments were carried out at the Marine Station of the University of Hawaii on Coconut Island. We are indebted to the Director of the Honolulu Aquarium for supplying much of the material, and to the staff of the Department of Zoology and Entomology for their cooperation.

METHODS.

For a full description of the methods used in preparing single motor and inhibitory axons reference may be made to a previous paper (WIERSMA, 1941). The number of preparations necessary to establish an innervation scheme depends very much upon the animal investigated. One factor which greatly influences the ease of preparation is the diameter of

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3) Contribution no. 20 Hawaii Marine Laboratory.

the axons. Rock-lobsters were found to have the largest axons and about ten preparations were sufficient to establish a complete scheme. On the other hand, the small size of the axons of the shrimps was largely responsible for the failure to obtain a complete scheme, notwithstanding the much larger number of preparations. Besides the actual size of the axons, other factors which are of importance are the strength of the connective tissue surrounding them and the viability of the nerve-muscle preparations. The latter was sometimes found to be exceedingly short, especially in the Stomatopoda, and was the main reason why relatively few data were obtained from a large number of preparations. Attempts were made to extend this survival time by using, instead of the customary sea water for physiological solution, artificial solutions to bathe the preparations. They proved unsuccessful.

In most cases contractions and inhibitions were observed by visual inspection although in some cases kymograph recordings of the muscle action were made with an isotonic lever.

Methylene blue dissolved in sea water was regularly used to stain the axons on exposed muscle surfaces. The results were extremely variable and depended upon the species used. Thus, whereas in certain cases staining was of assistance in establishing the innervation schemes with certainty, in others the staining was at best poor and few data could be gleaned from these preparations.

RESULTS.

The results will be presented in an order in which the higher forms are dealt with first.

Brachyura.

The innervation scheme (Fig. 1) previously presented for the Brachyura (WIERSMA, 1941) is based on a number of species distributed throughout the tribe. Of the Hawaiian species suitable for investigation only those which presented features of special interest were used. The data collected were incomplete in several respects but in so far as determined, all were in agreement with the established scheme.

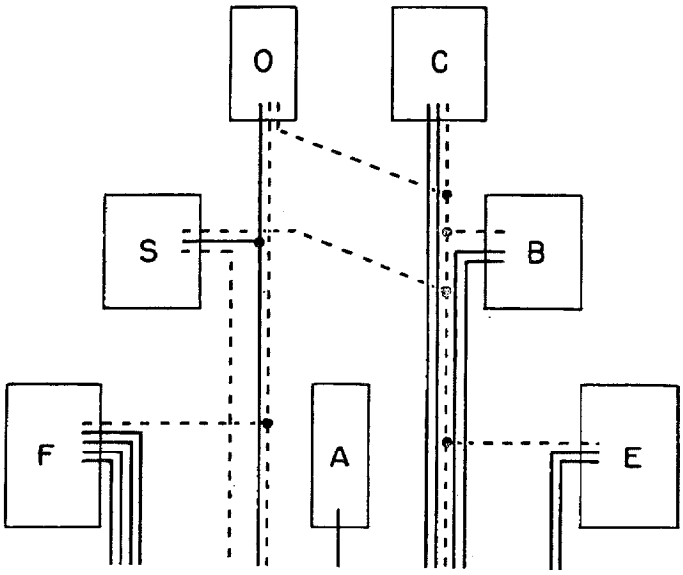

Fig. 1. *Brachyura.* Innervation of the seven distal muscles of the thoracic legs. O-opener; C-closer; S-stretcher; B-bender; F-main flexor; A-accessory flexor; E-extensor. Solid lines -motor axons; broken lines- inhibitory axons. The square bracket at bottom of diagram indicates that all the axons are included in one nerve bundle.

Portunus sanguinolentus (Herbst). A few preparations of the distal motor nerve supply of the swimming legs of this animal were made. These legs, with their changed function and reflex behaviour (HERTER, 1932), have the anatomical peculiarity that the opener muscle (abductor of the dactylopodite) is equal in size, or even somewhat larger than the closer (adductor of the dactylopodite). It was therefore of interest to determine whether or not the opener received only the customary single motor axon and the closer both fast and slow axons. The leg was opened in the meropodite. As in other crabs only a single nerve is present, and is easily split into six to ten preformed axon bundles. From one of these a single motor axon was prepared

which caused simultaneous contractions of both the stretcher (extensor of propodite) and opener and formed the sole motor innervation for both muscles. From another bundle, two motor axons for the closer were obtained. It may thus be concluded that the swimming leg has indeed the same innervation pattern as the other thoracic legs, and that the differences in function are not accompanied by a special distribution of the axons.

Dromidiopsis dormia (L). This large sponge crab was of interest as it belongs to the subtribe Dromiacea of which no representative has so far been investigated. The number of animals available was, however, limited. Furthermore the innervation of the bender (flexor of the propodite) showed a special functional arrangement which was of considerable interest and formed the basis for a separate series of experiments. For these reasons the determination of the distribution pattern of the axons has remained incomplete.

The motor innervation of the four distal muscles has been determined and is identical with that of all other decapods; double for closer and bender, one single common axon for stretcher and opener. Since the ischiopodite is sufficiently long to permit preparation of axons, it has been possible to find, in addition, that the extensor (of the carpopodite) is doubly motor innervated and the accessory flexor (of the carpopodite) receives a single motor axon. For the main flexor (of the carpopodite) the motor innervation is more difficult to study as it usually consists of four axons of which some may be small. In the present case the quadruple innervation has not been shown.

The investigation of inhibitory innervation has remained incomplete. The "true" stretcher inhibitor was found, and as usual accompanied the opener-stretcher motor axon. In the ischiopodite an axon was prepared which inhibited closer, bender and extensor. This axon is presumably the "common" inhibitor but its other functions were not shown. An axon was also found which inhibited contractions of the main flexor. This axon ought to be the "true" opener inhibitor, but inhibition of the opener was not obtained in any preparation.

It can be concluded that all positive findings are in agreement with those to be expected from the Brachyuran scheme but that some important data are lacking so that possible differences cannot be excluded.

Calappa hepatica (L). This species, which belongs to the subtribe Oxystomata, was the second of this group available for investigation. Previously *Randallia ornata* (Randall) was shown to possess two opener inhibitors (WIERSMA and ELLIS, 1942). In *Calappa* the innervation of the four distal muscles was determined in a few claws. Though the axons were rather thin (20—40μ) they could readily be prepared as the connective tissue was easily ruptured. The motor innervation is as presented for *Dromidiopsis*. Three inhibitors were found; one for the stretcher, one for the opener and a third which inhibited closer, bender and opener for certain, but its effect on the stretcher was not definitely established. The latter may be due to an early branching to the stretcher in some preparations. That the opener has a double inhibitory innervation indicates the similarity of innervation scheme of the four most peripheral muscles to that of the other Brachyura.

It may be well to point out now that our investigations of the Brachyura have been of a more exploratory nature than those used in the following species. We have presented the results for two reasons; to give the reader some insight into the techniques and difficulties encountered and thus form an introduction to the rest of the paper and because the results were just definite enough to warrant presentation as corroborative evidence for the Brachyuran scheme.

Anomura.

For the Anomura as a tribe, no scheme has so far been offered, although an almost complete scheme (Fig. 2) has been worked out for the coconut crab, *Birgus latro* L. (WIERSMA, 1949). In investigating the innervation of *Blepharipoda occidentalis* Randall, by histological staining methods, VAN HARREVELD (1939) found a number of axons which differed from those of *Birgus*. For this reason the innervation scheme of *Birgus* was not considered

as representative of the Anomura. In a recent investigation concerning the innervation of the four distal muscles of the limbs of *Eupagurus bernhardus* (L.), it was found that these

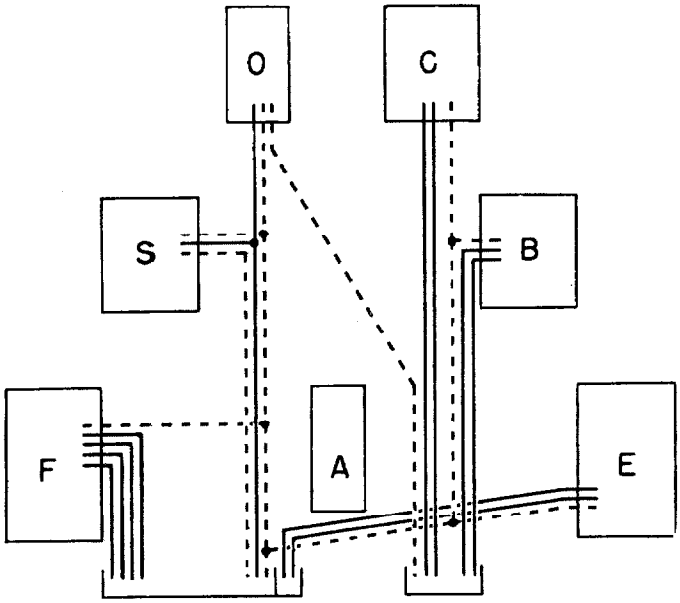

Fig. 2. *Birgus latro*. Innervation of the seven distal muscles of the thoracic legs. Notation the same as for Fig. 1. Square brackets indicate the nerve bundles. In this and all following figures the bundle on the right is the thicker.

differed from those of *Birgus* in that the common inhibitor did not innervate the opener and stretcher (WIERSMA, 1951a). It was thus of importance to determine the innervation of some other pagurid which might be expected to show further differences between the innervation schemes of the pagurids and *Birgus*.

Dardanus asper (De Haan), a large hermit crab, proved to be very suitable for investigation of the nerve supply to the muscles of the walking legs. In large specimens both second and third legs, and in small ones only the latter, could be used for exposing and preparing the nerve in the ischiopodite.

The motor axon supply was completely worked out for all seven muscles. In all but one preparation it was in complete agreement with that of all higher decapods. In this one limb the bender was innervated by three motor axons instead of the usual two. Although this was almost certainly an abnormality, believed to be due to a branch of the accessory flexor axon supplying the bender, it is reported because the bender differs in other respects from that of most species. In both *Dardanus* and *Dromidiopsis*, stimulation of either of the two bender axons resulted not only in contractions of different speeds but also of different directions. One rotates the propodite in one direction and the other the opposite way. A special study was made of this phenomenon, and of a number of preparations made for this purpose, none showed more than two bender axons.

The inhibitory axon supply was determined without undue difficulty. From the very first it became clear that *Dardanus* showed the features to be expected from the scheme of *Birgus* and not those found in *Eupagurus*. It could be shown in practically all preparations that the opener receives a double inhibitory supply. The "common" inhibitor prepared in the ischiopodite was found to have an inhibitory influence on the contraction of all seven muscles. It was also possible to show that both the opener and stretcher inhibitors innervated respectively only opener and stretcher (Fig. 6b).

Data from methylene blue staining have confirmed all these findings. It is especially noteworthy that the opener has a triple innervation and not a double one as was found in *Eupagurus*.

The distribution of the axons in the nerve bundles is remarkable and deserves special mention. Two main bundles are present; a thicker and a thinner one. In the ischiopodite the thinner bundle contains the four motor axons for the main flexor, the opener-stretcher axon, the stretcher inhibitor, the common inhibitor and in most preparations the two motor axons of the extensor. The latter three axons, in the Palinura and Astacura, are located in the thick bundle.

In a number of cases the diameter of the efferent axons was determined; motor axons measured 40—80μ, and inhibitors 30—50μ in diameter.

Palinura.

The scheme previously presented for this tribe (Fig. 3) was founded solely on that determined for *Panulirus interruptus* (Randall) (WIERSMA, 1941). It was therefore of con-

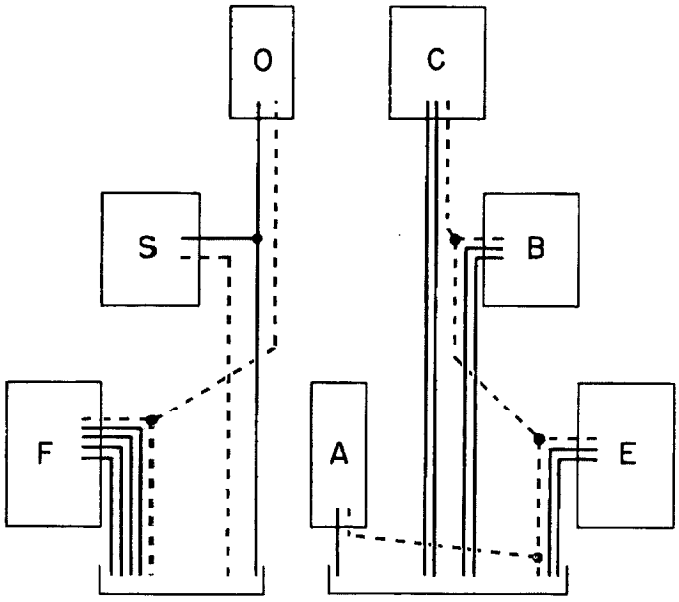

Fig. 3. *Panulirus interruptus*. Innervation of the seven distal muscles of the thoracic legs. Notation the same as for preceding diagrams.

siderable importance to investigate other species of this tribe. Available Hawaiian forms closely related to *P. interruptus* are *P. penicillatus* (Olivier) and *P. japonicus* (De Siebold).[1]) The slipper lobsters *Paribacus antarcticus* (Lund) and *Scyllarides squammosus* (Milne Edwards), belonging to the Scyllaridae, were also available. This family is classified by some authors (e.g. EDMONSON, 1946) together with the Palinuridae and Homaridae as families of the tribe Macrura. The difference between the Palinura and Astacura innervation schemes is at least as great as that between the other tribes. It was therefore of considerable interest to determine the scheme of the Scyllaridae, in order to obtain evidence regarding their systematic position.

[1]) LEONE (1950) found in serological investigations that *Panulirus interruptus* and *P. penicillatus* are very closely related, whereas *P. japonicus* shows greater serum differences.

Panulirus penicillatus and *P. japonicus*. Since there was no significant difference in any respect between these two species, they will be treated together. For both it was possible to establish a complete innervation scheme for the seven most distal muscles of the limbs. The large diameter of the fibres and the weak connective tissue made preparation of single fibres easier in these animals than in any other species investigated in this series.

In the ischiopodite the nerve consists of two bundles. In the smaller one are found the four motor axons of the main flexor and the single opener-stretcher axon. In the thicker bundle are found one motor axon for the accessory flexor, two for the closer and two for the bender. The two axons for the extensor are also often in this bundle but sometimes form a very thin separate bundle.

With regard to the inhibitory innervation, an unexpected result was obtained. The stretcher inhibitor was always located in the small bundle in close proximity to the opener-stretcher motor axon but the preparations differed with regard to the remaining inhibitory innervation. In many cases two additional inhibitors were found which showed the same distribution as in *P. interruptus*. One inhibitor, located in the thicker bundle, inhibited closer, bender, extensor and accessory flexor. The other, located in the smaller bundle, inhibited opener and main flexor. However, in a number of the preparations only one additional inhibitor was present. This axon combined the functions of the two above. It was located in the thicker bundle and inhibited every muscle but the stretcher. The two patterns occurred in different limbs of the same animal; the more anterior legs often showing the presence of only one inhibitor whereas in the more posterior legs two were found. In the latter case it is apparent that an early branching of the inhibitory axon took place and that there are in reality not three, but only two inhibitors in the limbs (Fig. 6*c*).

As mentioned before, the axon diameter can be very large. It is, however, difficult to measure the true diameter since there is a very thick axon sheath. Axon diameters exceed 200μ for the largest main flexor axons, and all motor axons exceed 100μ. The inhibitors are usually somewhat thinner but the stretcher inhibitor in particular is often well above 100μ. Methylene blue staining gave fair results which were in agreement with the physiologically determined scheme.

Scyllarides squammosus. In contrast to the rock-lobsters, it proved difficult to conduct the investigations on this animal. The connective tissue between the axons as well as that surrounding the bundles, is extremely tough. In addition, the axons themselves, although surrounded by a thick sheath, have a very fluid axoplasm and are thus easily compressed by the stimulating electrodes. Furthermore the legs are much shorter than those of the rock-lobsters and in order to expose sufficient length of nerve in the ischiopodite, it was necessary to remove part of the exoskeleton of the meropodite. This interferes with the functioning of the muscles in the meropodite, especially by damage to the proximal belly of the accessory flexor. It was therefore not surprising that its innervation was found in only a few cases. Again the short length of nerve available makes it difficult to prepare all four motor axons of the main flexor. As the main object was to locate the inhibitors, the fact that methylene blue staining of the main flexor showed a quintuple innervation was taken as proof that there are four motor axons present. The only motor axon regularly prepared in the small bundle was that of the opener-stretcher, although in several preparations one or more of the main flexor motor axons was prepared. In the large bundle, two bender, two closer and two extensor axons were found. In several instances an additional thick axon was isolated. It caused flexion of the carpopodite but in all cases this contraction stopped within a very short interval presumably because of the damage to the accessory flexor.

An inhibitory axon for the stretcher was regularly prepared from the thin bundle. This axon did not inhibit any other muscle, unlike the case in the Astacura in which it is common to both stretcher and closer (Fig. 6*d*). In a few cases another inhibitor, which inhibits the opener, was found in the thin bundle. At least once this axon was shown to inhibit contractions of the main flexor. In many preparations an axon present in the thick bundle inhibited both closing and bending and in some, extension in addition. In two

preparations it inhibited also the opener contraction resulting from stimulation of the unprepared thin bundle. In these two cases subsequent division of the small bundle resulted in preparation of a stretcher inhibitor but no opener inhibitor.

Axon diameter was less than in the rock-lobsters and the sheath was relatively thinner. The outside diameters of motor axons varied from 50 to 90μ; inhibitors from 30 to 60μ. Staining with methylene blue often gave good results. These were in all respects in agreement with those obtained in the rock-lobsters. Special mention of the double opener and quintuple main flexor innervation should be made.

Paribaccus antarcticus is somewhat smaller than the previous slipper lobster but otherwise resembles it closely in appearance. It proved, however, to be more suitable for axon preparation. The results were somewhat more complete than for *Scyllarides*. The motor axon for the accessory flexor could be prepared in the thicker bundle, as well as the pairs of axons for closer, bender and extensor. In addition an axon which inhibited only these four muscles was regularly found. In the thin bundle the opener-stretcher axon and several main flexor axons occur. The inhibitor of the stretcher was regularly obtained and was specific for this muscle. An opener inhibitor was found which, in a few cases, was shown to inhibit also the main flexor. In all respects this mirrors the findings in *Panulirus interruptus*.

Axon diameter was only slightly less than in *Scyllarides*. Methylene blue staining gave exactly the same results as in the three previously discussed palinurans.

Decapoda Natantia.

No data have been published on the innervation of the muscles of the legs of the shrimps. In this investigation it was found that of the available forms, two were suitable to a certain extent for the type of approach used. These forms were *Stenopus hispidus* (Olivier), belonging to the tribe Stenopidea, and *Spirontocaris marmoratus* (Olivier) belonging to the Caridea. Both diameter and survival time of the preparations were factors unfavourable in obtaining single axons. In investigation of the inhibitory supply, despite large numbers of preparations made, insufficient data were obtained to complete the distribution picture. However, our results did show that the general principles of the innervation scheme are the same as in the Decapoda Reptantia.

Stenopus hispidus. The bandana shrimps were collected at night on the coral reefs off Honolulu and Coconut Island. Difficulty was encountered in that they are very prone to autotomise the large chelipeds formed by the third pair of thoracic legs. The animals are very aggressive and concentrating them within narrow confines leads to severe fights with great loss of claws. The second pair of walking legs which has small chelae, was occassionally used but proved too small for purposes other than indicating that the pattern of innervation is similar to that of the third legs.

An advantage of the shrimps over the higher forms is that the ischiopodite is relatively long so that the nerve can be prepared in this joint and the innervation of the muscles of the meropodite can thus be studied. The number of muscles in the limb differs from that of the higher decapods. The dactylopodite is moved by opener and closer but the propodite is moved by three muscles which may be named stretcher, bender and rotator. The apodemes of the stretcher and bender are opposed to each other while that of the rotator attaches about half way between the two on the lower surface of the propodite. The contraction of this muscle causes the propodite to rotate about the longitudional axis. This is made possible by the fact that there is much more freedom of movement in this articulation than in most species where only one plane of movement is possible. (However, see above for walking legs of *Dardanus* and *Dromidiopsis*). The articulation between meropodite and carpopodite is similar to that of the Reptantia, with limited freedom of movement. As in the Reptantia, extensor, main flexor and accessory flexor occur in the meropodite. The latter muscle again is comprised of two bellies and causes a flexion which is not distinguishable from that of the main flexor.

On opening either the meropodite or ischiopodite, two main nerve bundles are found

which are almost equal in size. Stimulation of the slightly thinner one causes opening and the thicker closing. Isolating the axons is difficult because of their small diameter and delicate nature. Some fast axons had a diameter of 50μ, but inhibitors as thin as 15μ had to be prepared. A difficulty which occurs only rarely in the higher Crustacea but which is common in this animal, is cross-excitation of the axons. This occurred most likely only in cases where the axons were damaged distal to the stimulating electrodes. Such axons have a lowered threshold and occasionally stimulation of an isolated axon would lead to their discharge. Thus, for example, on stimulation of an axon which was presumably the fast closer, the extensor, bender and closer contracted.

Preparation of single axons was considerably easier in the thicker than in the thinner bundle. It was repeatedly shown that the closer possesses a double motor innervation; fast and slow. Twitch contractions, sufficient to cause complete closure of the claw, could be obtained with single shocks to the thick bundle. Usually these disappeared before the single axon was prepared. Slow contractions were elicited only after a number of impulses had reached the muscle. For the bender and extensor it was possible to find two motor axons in several instances. A single motor axon for the accessory flexor was found in the same bundle. These are most likely all the motor axons for these muscles.

The situation in the thinner nerve was more difficult to analyse. Stimulation of the whole nerve resulted often in movement of only the rotator. A single axon causing this movement could be prepared. After splitting the bundle, contractions of the main flexor could often be obtained. In some preparations it was possible to find two single axons which caused flexion. There was never any indication that more motor axons were present but despite this we cannot claim the absence of a quadruple flexor innervation. Particular attention was paid to the question of whether the stretcher and opener have a common motor axon, as in the Reptantia, or whether two separate axons are present, as in the Stomatopoda (see below). In many preparations we found that contraction of either stretcher or opener resulted from stimulation of thin bundles or even isolated axons. In such cases it was never possible to obtain another bundle or axon which caused contraction of the other

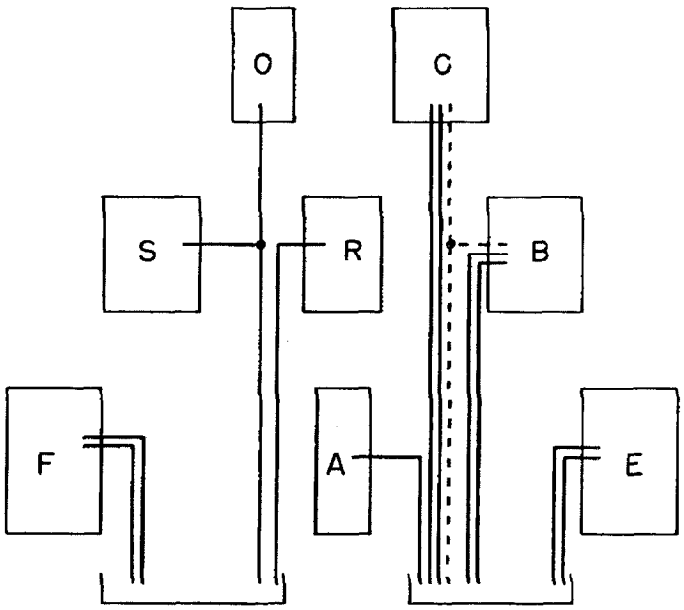

Fig. 4. *Stenopus hispidus*. Incomplete innervation of the eight distal musles of the third thoracic limb. R-rotator. Other notation the same as for preceding figures.

muscle. In a few cases a single axon was prepared which caused contraction of both opener and stretcher so it appears probable that the situation is as in the Reptantia. Reservation is necessary in the light of the above-mentioned possibility of cross excitation but we nevertheless consider it as established with enough certainty to be included in the tentative scheme (Fig. 4).

The presence of inhibitory axons has been demonstrated with certainty in some of these preparations. However, tracing of the inhibitory pattern has remained incomplete. It is definitely established that closer and bender share an inhibitory axon which runs in the thicker bundle. Whether this inhibitor can suppress the twitch contractions of the closer was not revealed. By the time the inhibitor was prepared the fast contractions were invariably fatigued so the inhibition had to be demonstrated against stimulation of the slow axon. In the small bundle no inhibitor has been prepared as a single axon. That at least one was present follows from the above-mentioned observation that stimulation of the whole bundle did not result in any visible contraction of the main flexor, stretcher or opener, but that after splitting, such contractions were often obtained. It was remarkable that the rotator always contracted on stimulation of the whole bundle and that attempts to suppress this contraction by simultaneously stimulating the thick bundle failed. It should be kept in mind however that because of bender contractions, it is difficult to determine rotation. Parts of the thicker bundle which did not cause bender contractions did not inhibit the rotator.

Methylene blue was not very successful for determining the number of axons in these preparations. The best results were obtained on the extensor which showed a clear-cut triple innervation. In opener preparations most often a single axon stained but in some a clear picture of double innervation was obtained. In a few cases there seemed to be three axons present for small distances. Thus it remains completely uncertain whether one or two inhibitors are present in the opener.

Spirontocaris marmoratus. Only the first long chelate legs of the males were used for the investigation. The gross anatomical findings correspond to those of *Stenopus*. Here also, three muscles are present in the carpopodite. From investigations of a few other species, these three muscles seem to occur generally in the Natantia. Again two main nerve bundles occur and the distribution of the motor axons seems to be the same as for *Stenopus*. Preparation of single axons proved almost impossible but insofar as could be determined, the innervation is the same as that of *Stenopus*. Only two motor axons for the closer were obtained with complete certainty in the large bundle and in the small one only a single axon for the rotator. Inhibitory axons were never prepared and the presence of inhibition could be deduced only from the fact that stimulation of a nerve or bundle would give no visible contraction but that on further splitting contractions appeared.

Stomatopoda.

Of this group three forms were investigated, namely *Pseudosquilla ciliata* Miers, *Squilla oratoria* De Haan and *Lysiosquilla maculata* (Fabricius). Of the first species many specimens were used but the other two species were available only in limited numbers. In all of them the same difficulty was encountered; the muscular contractions which should result from nervous stimulation were either completely absent or, when present, disappeared after only very few stimuli. For this reason only few single axon preparations were obtained. From these we gained the impression that in all the species the same nervous distribution occurs. In almost all cases, only the subchelate second maxilliped was large enough to permit successful handling. In the larger *Lysiosquilla*, other maxillipeds and the small walking legs were used with but little success.

The number of muscles in the distal segments corresponds to that of the shrimps. The dactylopodite is moved by opener and closer; the propodite by bender, stretcher and rotator. Since the ischiopodite is short, no attempt was made to study the innervation of the four muscles in the meropodite. Two main nerve bundles are present in the meropodite. It is relatively easy to obtain single axons as they are of a fair size (40—70μ) and separate

easily. However in many preparations no contractions could be obtained at any stage in the operation. In others, particularly with opener bundles, contractions appeared after some splitting.

In all three species single motor axons have been obtained for each of the five most peripheral muscles. It is particularly noteworthy that opener and stretcher have separate motor axons and that there is no reason to believe that these are branches of a common axon. In *Pseudosquilla* and *Lysiosquilla* it has been possible to show, only for the closer, that a double motor innervation exists. In good preparations, contractions obtained by stimulating the larger axons to all the muscles were brisk whereas those caused by stimulation of the second closer axon were definitely slow in developing. This leaves no doubt that fast and slow systems do exist in the Stomatopoda. The motor axons for closer and bender are located in the thicker nerve bundle; those for the opener, stretcher and rotator in the thinner bundle. It is probable that two benders are present but the slow was never prepared as a single axon (Fig. 5).

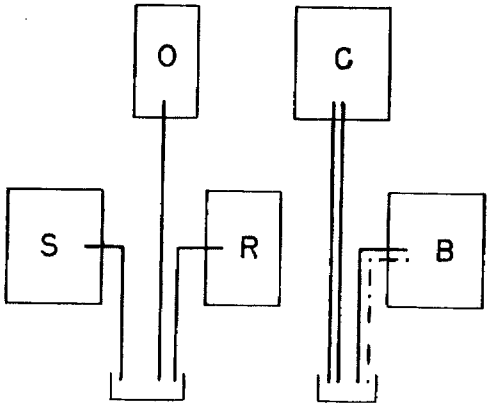

Fig. 5. *Stomatopoda.* Innervation of the five distal muscles of the second maxilliped. The broken line represents a motor axon whose presence has not been shown by axon isolation. Notation the same as for preceding figures.

Methylene blue staining was of little assistance, not so much because of failure of the stain but because the axons do not form regular, parallel arborizations on the surface of the muscle. Instead, they enter at the sides and are soon lost among the muscle fibres. The best preparations were obtained from muscles in the meropodite, which were not physiologically investigated. One extensor, for instance, showed a good triplotomic innervation. The closer most likely receives also three axons.

DISCUSSION.

The innervation schemes for the seven distal muscles of the limbs of the four Reptantia tribes may now be considered complete (Fig. 6), with one or two minor uncertainties. In all four tribes the motor innervation is identical but the inhibitory innervation varies considerably. Despite this variation between the tribes the inhibitory innervation is constant for all members of each tribe. The Anomura appear as an exception to this statement in that two inhibitory patterns are known to exist. *Birgus* and *Dardanus* share an identical innervation which must be considered typical for the tribe, but which differs from that of *Eupagurus*. The anatomical results for *Blepharipoda* (VAN HARREVELD, 1939) indicate that there are further differences in the innervation but as no physiological investigation has been made it is impossible to tell whether these differences are attributable solely to the inhibitory axons, or whether the motor innervation might also be involved.

It was previously considered that the Palinura had three inhibitory axons; common inhibitor, stretcher inhibitor and the opener-main-flexor inhibitor. It has now been shown that the common inhibitor and the opener-main-flexor inhibitor are branches of the same axon but that the point of dichotomisation between the two occurs at various levels in the limb, thus resulting in misleading data in some instances. There are in total only two inhibitory axons. A similar position may exist in the Astacura where at present four axons are known (WIERSMA, 1951*b*). It is possible, and indeed from a functional point of view seems probable, that all but the opener inhibitor are branches of a single axon. However, we have no definite evidence on this point.

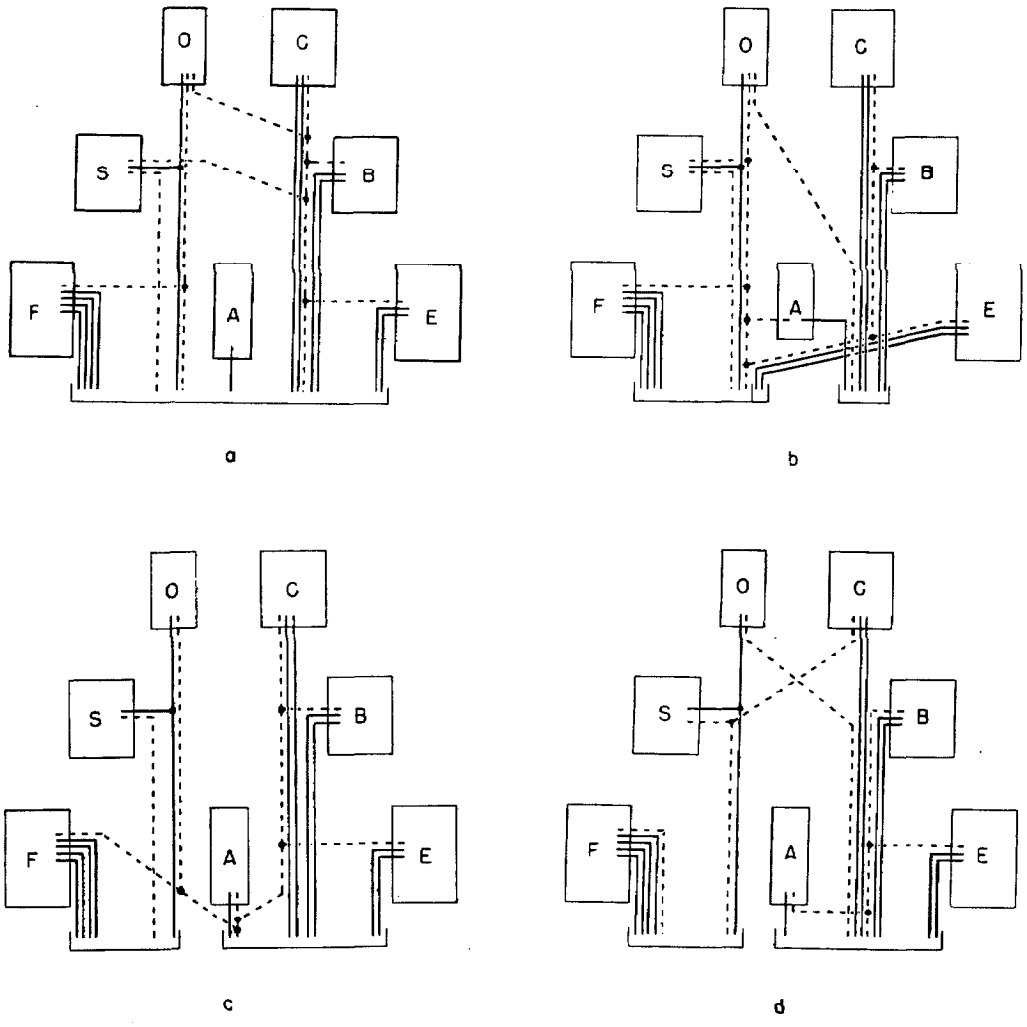

Fig. 6. Innervation schemes for the distal muscles in the thoracic limbs of the four Decapoda Reptantia tribes. a-Brachyura; b-Anomura; c-Palinura; d-Astacura. In the Anomura the common inhibitor is variable in position and may occur in the large nerve bundle. O opener; C closer; S-stretcher; B-bender; F-main flexor; A-accessory flexor; E-extensor. Solid lines represent single motor axons; broken lines — inhibitory axons; square brackets distribution of axons between nerve bundles, the largest bundle being on the right.

That the Scyllaridae must be considered members of the Palinura is beyond doubt, in view of the exact correspondence of the peripheral innervation schemes in the two groups. Some authors have separated the two groups on the basis of the general compression of the body and the accompanying modifications of the antennae and eye orbits but in our opinion this criterion does not justify taxonomic separation from the Palinura and should be considered as a habitat modification.

The Brachyura have presented a completely uniform picture. Even in the functionally modified swimming legs of the Portunidae, the motor axon distribution is completely in accord with the general scheme. The double inhibitory innervation of opener and stretcher is proof positive that the three known inhibitory axons are completely independent of one another.

Our knowledge of the Natantia is as yet incomplete. However, it should be noted that this group exhibits many characteristics which are found in the Reptantia. The opener and stretcher have a common motor axon; fast and slow contractions are brought about by single axons and inhibitory axons are present. At least in *Stenopus* the fast system of the closer has reached its maximal expression, i.e., a single stimulatory shock results in a maximal twitch of the muscle. The only other closer muscle in which this is known to occur is that of the crayfishes, *Cambarus* and *Astacus*. Even in the lobster, *Homarus vulgaris*, more than one impulse is necessary for complete closure of the claw. It may well be that this represents a specialization of this particular shrimp as the claws are much better developed than in the majority of the Natantia.

A third muscle is present in all species of the Natantia so far examined. Its contraction results in a bending and rotation of the propodite. The muscle has an independent motor innervation and has apparently been lost in the Reptantia.

Although work on the Stomatopoda was performed with the second maxilliped it would seem, in the light of previous experience, that the innervation scheme would not differ fundamentally from that of the walking legs. Our information on these animals is incomplete but the separate innervation of opener and stretcher is noteworthy. Both fast and slow contractions have been well established. Although we have seen many indications of inhibition, it would be premature to claim its existence.

The following hypothesis is based on our knowledge of the innervation patterns of the Crustacea. It will appear obvious to the reader that more data are required before the hypothesis can be given a firmer footing. We wish to interpret the innervation schemes from a functional point of view but in doing so it would be well to point out some of the more obvious difficulties and limitations of such interpretation.

In the first instance we have no knowledge of the limitations the central nervous system imposes on the peripheral axons. There may be certain combinations of axons which are not permitted to discharge simultaneously and there may also be central connections between the axons which make simultaneous discharge obligatory. Data on this aspect of the problem are accumulating and we have hopes of being able to interpret the phenomena more completely in the not too distant future. It is essential not to lose sight of the fact that the inhibitors, with rare exception, are much more effective against the slow contractions. This means that even if a muscle is suffering under obligatory inhibition it may still be capable of a fast response although the slow transmission pathway is completely blocked. Lastly, it must be considered that the common inhibitors do not play a part in the normal locomotary movements. It has been suggested that their role during moulting is to block reflex contractions resulting from the sensory bombardment of the central nervous system at this critical time in the animals life. However, the individual inhibitors almost certainly play an integral part in locomotory movements and may subserve a secondary function in the moulting process.

From a general consideration of Arthropod innervation it is probable that basically the muscles were innervated by one fast and one slow axon and that in addition all the muscles on one side of a metamere were inhibited by a single common inhibitory axon. From this fundamental scheme there have been divergences, primarily in the motor innervation and

then as a result of this, modification of the inhibitory supply occurred. In the Reptantia a spectacular increase in the number of axons to the flexor has occurred. This large muscle is responsible for one of the functionally most important movements of the limb and in order to bring about delicate movements, fine gradations of contraction are necessary. It may be in response to such a demand that the graded quadruple innervation has arisen. Conversely some muscles which play a rather simple role have lost one of the motor axons. However the point in which we are most interested is the loss of the double motor innervation by the stretcher and opener and the innervation of these two muscles by a single common axon. It appears probable that this modification led to the various alterations in the inhibitory innervation. The common opener-stretcher axon must have arisen before the divergence of the four Reptantia tribes from the ancestral stock, as indicated by its presence in the Natantia. Subsequently each tribe solved the problems which arose from this modification, in various ways.

The reason for the functional fusion of the opener and stretcher is obscure but in the usual type of locomotory movements and the defence action of the chelae, independent movements of the two muscles would rarely be necessary. In the presence of a rotator, such as possessed by the Natantia, the common opener-stretcher might have constituted an advantage but with the subsequent loss of the rotator it became undesirable. At approximately the time of this loss the four tribes were diverging and in the solution to the opener-stretcher problem the characteristic inhibitory innervation patterns were developed.

The simplest, although not the most efficient, solution of the problem is shown in the Palinura where the stretcher has developed an individual inhibitory axon (Fig. 6*c*). This allows independent contraction of the opener but the animal could not bring about closing and stretching without involving the opener as this would necessitate activity of the common inhibitor with resultant inhibition in the whole limb. If such movements are ever performed, the closer must simply override the contraction of the much weaker opener.

The Astacura (Fig. 6*d*) have an independent opener inhibitor but the stretcher and closer share a common inhibitor. This represents a much better solution for the regaining of independent control of both the opener and stretcher. Although inhibition of the stretcher would involve the closer, the animal can obviously have no need for simultaneous contraction of opener and closer. The functional aspect is further improved by the fact that even the slow closer inhibition is very ineffective in some members of the tribe. Inhibition of the opener allows independent action of the stretcher. The Astacura have then in effect inhibited antagonists whereas the Palinura have inhibited protagonists.

The Anomura (Fig. 6*b*) certainly have the most flexible system in terms of peripheral expression. The individual inhibitors of both opener and stretcher have reinstated independent movement of both these muscles and in addition the common inhibitor has retained branches to all the muscles.

The Brachyura (Fig. 6*a*) have essentially the same solution as the Palinura, i.e. a stretcher inhibitor, but in addition there is a common opener-flexor inhibitor. Their position is therefore slightly superior to that of the Palinura, as inhibition of the opener involves only the flexor and not all the muscles of the limb. The common inhibitor has retained branches to the opener and stretcher. This would indicate that the individual inhibitors have not arisen from the common inhibitor. However, to decide the issue of whether there was originally a common inhibitor or individual inhibitors, data on the lower Crustacea would be of great value.

SUMMARY.

1. By the method of axon isolation and electrical stimulation together with staining of the axons on the muscle surface with methylene blue, the efferent innervation of the muscles of the limbs of various Decapod Crustacea, both Reptantia and Natantia, and of Stomatopoda, has been studied. Innervation schemes for the four Decapod tribes, together with incomplete schemes for the Natantia and Stomatopoda are presented.

2. Limited investigations on the brachyurans, *Portunus sanguinolentus* (Herbst), *Calappa hepatica* (L.) and *Dromidiopsis dormia* (L.) confirm the scheme previously presented for the Brachyura. Although *Portunus* has the fifth legs modified for swimming the innervation of the most distal muscles is unchanged.

3. In the Anomura, the hermit crab *Dardanus asper* (De Haan) was studied. The innervation corresponds to that previously reported for *Birgus latro* L. and differs from that of *Eupagurus bernhardus* (L.).

4. Palinurans used were *Panulirus japonicus* (De Siebold), *P. penicillatus* (Olivier), *Paribaccus antarcticus* (Lund) and *Scyllarides squammosus* (Milne Edwards). The innervation of these four species confirmed that previously established for *Panulirus interruptus* (Randall) in all but one point. It was shown that there are two, and not three inhibitors participating in the innervation of all seven leg muscles investigated.

5. For the Decapoda Natantia it is shown that in *Stenopus hispidus* (Olivier) and probably in *Spirontocaris marmoratus* (Olivier), the innervation scheme resembles that of the Reptantia in many respects. In *Stenopus* double motor innervation was found for the closer, bender, extensor and flexor. The motor axon for opener and stretcher is common. An inhibitor for closer and bender was isolated. The Natantia have an additional muscle in the carpopodite which mediates rotation of the propodite. This muscle, and the accessory flexor were each shown to have a single motor axon.

6. The differences in musculature and innervation in the legs of the Decapod Crustacea support the division of this group into Natantia and Reptantia, with a subdivision of the latter into four tribes: Palinura, Astacura, Anomura and Brachyura as opposed to other classifications which have been used.

7. Owing to limited material and anatomical and physiological peculiarities, little evidence was obtained from the Stomatopoda. *Pseudosquilla ciliata* Miers, *Squilla oratoria* De Haan and *Lysiosquilla maculata* (Fabricius) were studied. In the large, subchelate second maxilliped double motor innervation was demonstrated for some of the muscles. Inhibitory axons, although probably present, could not be isolated. Like the Natantia, the Stomatopoda possess a rotator in the carpopodite.

8. In the light of the present data on the inhibitory innervation of the higher Crustacea a tentative evolutionary hypothesis for the development and the divergences of the innervation schemes is presented.

ZUSAMMENFASSUNG.

1. Mittels Isolierung einzelner Axone und elektrischer Reizung derselben, sowie durch Färbung der Axone auf der Oberfläche der Muskeln, wurde die Innervation der Beine verschiedener Krustaceen bestimmt. Sowohl Reptantia und Natantia Decapoda wie Stomatopoda wurden untersucht. Für die vier Gruppen der Reptantia konnten vollständige, für die Natantia und die Stomatopoda unvollständige Innervationsschemata gegeben werden.

2. Beschränkte Untersuchungen über die Brachyurenarten, *Portunus sanguinolentus* (Herbst), *Calappa hepatica* (L.) und *Dromidiopsis dormia* (L.) bestätigten das Schema, das vorher für die Brachyura gefunden worden war. Obwohl das fünfte Beinpaar von *Portunus* wegen seiner Funktion beim Schwimmen stark verändert ist, so ist doch die Innervation der distalen Muskeln die selbe geblieben.

3. Von den Anomura kam der Einsiedlerkrebs *Dardanus asper* (De Haan) zur Untersuchung. Die Innervation stimmt mit derjenigen von *Birgus latro* L. überein, wie dies schon früher bestimmt wurde. Sie ist von der von *Eupagurus bernhardus* (L.) verschieden.

4. Von den Palinura wurden vier Arten benutzt: *Panulirus japonicus* (De Siebold), *P. penicillatus* (Olivier), *Paribaccus antarcticus* (Lund) und *Scyllarides squammosus* (Milne Edwards). Ihre Innervation stimmt bis auf eine Abweichung mit derjenigen überein, die für *Panulirus interruptus* (Randall) bekannt war. Es wurde gezeigt, dasz nur zwei, anstatt drei

hemmende Fasern an der hemmenden Innervation der sieben peripheren Beinmuskeln beteiligt sind.

5. Für die Dekapoda Natantia wurde gezeigt, dasz bei *Stenopus hispidus* (Olivier) und wahrscheinlich auch bei *Spirontocaris marmoratus* (Olivier) das Innervationsschema dem der Reptantia in vieler Hinsicht gleicht. Bei *Stenopus* wurde für Schlieszer ,Beuger, Extensor und Flexor eine doppelte motorische Innervation gefunden. Öffner und Strecker haben ein gemeinsames motorisches Axon. Eine hemmende Faser für Schlieszer und Beuger konnte isoliert werden. Die Natantia haben einen zusätzlichen Muskel im Carpopoditen, der eine Rotation des Propoditen ermöglicht.Es konnte gezeigt werden, dasz dieser Muskel und der Accessorische Flexor je ein einzelnes motorisches Axon besitzen.

6. Die Differenzen in der Muskulatur und den Innervationsschemata der Beine der Decapoden stehen in Einklang mit einer Einteilung in Natantia und Reptantia, und der weiteren Unterteilung der Reptantia in vier Gruppen: Palinura, Astacura, Anomura und Brachyura, und sprechen gegen andere benutzte Klassifikationen.

7. Wegen des beschränkten Materials, sowie anatomischer und physiologischer Eigentümlichkeiten, wurden nur wenige Tatsachen für das Innervationsschema der Stomatopoden aufgefunden. Untersucht wurden die groszen zweiten Maxillipeden von *Pseudosquilla ciliata* Miers, *Squilla oratoria* De Haan und *Lysiosquilla maculata* (Fabricius). Für verschiedene Muskeln wurde eine doppelte motorische Innervation nachgewiesen. Hemmende Fasern konnten nicht einzeln isoliert werden, obgleich eine periphere Hemmung wahrscheinlich ist. Wie die Natantia haben die Stomatopoda einen Rotator-Muskel im Carpopoditen.

8. Auf Grund der presentierten Daten wird eine vorläufige Hypothese über die phylogenetische Entwicklung und Divergierung der Innervationsschemata aufgestellt.

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PART II.

THE EFFECT OF SPACED STIMULATION OF EXCITATORY AND INHIBITORY AXONS OF THE CRAYFISH

by

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INTRODUCTION

WIERSMA & ADAMS (1950) investigated the effects of intercalated shocks and of 'spaced' [2]) stimuli on motor axons in the legs of a number of decapod Crustacea. They demonstrated that in most fast neuromuscular systems 'spaced' stimuli resulted in a considerably greater contraction of the muscle than was caused by ordinary stimulation. In most slow systems, on the other hand, no obvious differences were obtained between those two methods of stimulation. It was concluded that the enhanced effect of spaced impulses can occur at these fast nerve endings only when, in addition to the facilitatory processes in the transmission between nerve impulse and muscular contractile substance, another property is present. It is therefore possible to distinguish two types of endings, those with and those without this property, which where named the 'pattern sensitive' and 'pattern insensitive' endings.

In the monaxonically motor innervated opener muscles of the legs of various Decapoda, spacing of stimuli usually had an intermediate effect. This is in agreement with the observation that these systems often have properties which combine those of biaxonically motor innervated muscles. This is clearly demonstrated by the opener of the claw of *Eupagurus bernhardus* (L). WIERSMA (1951) came to the conclusion that at least in this case, both types of endings must be present although they are served by a single axon. On the other hand it is known that in certain opener muscles, all endings must be insensitive to pattern, e.g. in the opener of *Pachygrapsus crassipes* Randall, which shows no fast properties.

From these experiments it follows that in pattern sensitive systems the same number of impulses per second can result in contractions of quite different magnitudes, depending upon the specific spacing of the shocks. The present investigation was undertaken in order to show that this phenomenon does not depend on any mechanical properties of the muscle

[1]) Rockefeller Scholar (Union of South Africa); Institute Scholar (California Institute of Technology) sponsored by the Institute of International Education.

[2]) A full explanation of this term will be found under *Methods*.

substance but is really located in the myo-neural junction. Advantage was taken of the fact that inhibition of contraction is possible. Determinations of the inhibitability of spaced and non-spaced contractions of the opener muscle (abductor of dactylopodite) of the claw of *Cambarus clarkii* Girard, were made. Since inhibition, like excitation, is subject to facilitation (MARMONT & WIERSMA, 1938; WIERSMA & HELFER, 1941) it was found possible to study, in addition, the effects of spacing the inhibitory impulses.

METHODS

Single axon preparations of the inhibitory and excitatory axons of the opener muscle were made in the manner described by WIERSMA (1941). The closer (adductor of dactylopodite) innervation was removed and as the opener motor axon is common to both opener and stretcher (extensor of propodite), the limb was clamped at the carpo-propodite articulation so that only the dactylopodite was free to move. The mero podite containing the exposed nerve bundles was placed in physiological saline solution (VAN HARREVELD, 1936). Prior to stimulation, the axons were lifted to the surface of the saline on fine platinum wire electrodes wielded in micro-manipulators.

In preparation of single axons induction coils were used but during the experiment electronic oscillators were employed. These oscillators deliver a rectilinear wave and allow independent control of the recurrence frequency, pulse duration and amplitude. For obtaining spaced stimuli the signal from one of the oscillators was fed into an electronic device (see KEIGHLEY in WIERSMA & HELFER, 1941) which takes advantage of the sharp rise and fall of the rectilinear wave to generate two pulses of the same polarity and of short duration (0.3 millisec.). As there are thus two spikes for each rectilinear wave, the effective stimulating frequency will be twice that of the oscillator frequency. By alteration of the pulse length of the rectilinear wave, the time interval between the members of a pair of shocks can be adjusted.

The instrument thus furnishes pairs of shocks which are repeated at a constant rate. We will call the frequency with which these pairs of shocks recur every second, the *repetition rate*; the interval between the first and second shocks of a pair (Fig. 1a'b') the *spacing interval. Equal spacing* is obtained when the spacing interval, a'b', is half the *repetition interval*, a'a''. In all experiments, the strengths of the first and second shocks of each pair were equal and slightly above threshold.

Records of contractions of the muscle were made on a kymograph with a light isotonic lever, the tip of the dactylopodite being used as reference point. For the observation of minute movements of the dactylopodite, such as in some inhibitory observations at low frequencies of stimulation, a binocular microscope was used. There is no apparent difference in response of the two sexes and both males and females have been used indiscriminately.

The increase in contraction height (spacing effect) was found to be greatest when the spacing interval is short. However, if this interval becomes less than the relative refractory period of the preceding shock the stimulation frequency is effectively halved and the contraction is correspondingly reduced. The maximum contraction occurred at spacing intervals of approximately 3 millisec. With longer spacing intervals the spacing effect gradually declines, until at about 12 millisec., it is no longer discernible. These results are represented graphically in Fig. 2. In this graph the repetition rate is 15 per second, so that at equal spacing the effective frequency is 30 shocks per second. The height of the contraction attained in 5 seconds at equal spacing is taken as unity. The height attained in the same period but with various spacing intervals is expressed as a percentage of this, and these percentages are plotted along the the ordinate $\left(\frac{H_s}{H_e}\%\right)$. The abscissa shows the spacing interval in milli-

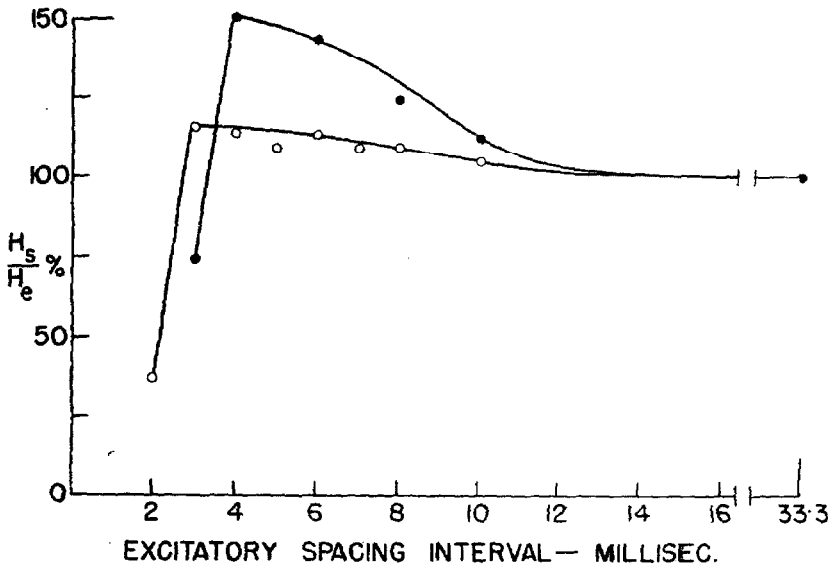

Fig. 2. The influence of the spacing interval upon the size of the muscular contraction. The two curves are from different experiments. The upper curve shows approximately the characteristic spacing effect whereas the lower shows very little increase in the contraction over the control obtained with the same frequency of equally spaced shocks (100 % at 33.3 millisec.). In both experiments the excitatory repetition rate was 15 per sec. (i.e. 30 shocks per sec.). The contraction height attained with shocks spaced at 5 millisec. (H_s) is expressed as a percentage of the height attained with equally spaced stimuli (H_e) and is plotted on the ordinate. At the shortest spacing intervals the contractions are much reduced due to the second shock of a pair falling in the relative refractory period of the first and thus effectively halving the number of shocks per second.

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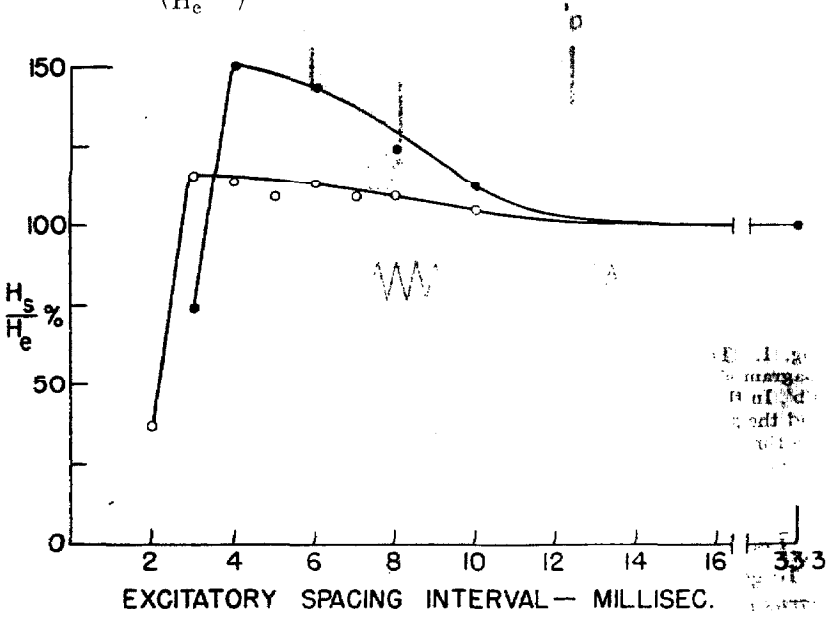

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seconds. In the upper curve the maximum spacing effect is 50 %, i.e. the spaced contraction is 1.5 times as large as the equally spaced contraction. For different preparations of the opener muscle the effectiveness of spacing varies, as is demonstrated by the lower curve of fig. 2, in which the increase was only 15 %. In a large number of preparations with stimuli spaced at 5 millisec. the spaced contraction was from 0—120 % greater than the control with an average value of + 54 %. In a very few cases a small negative spacing effect was observed; the spaced contraction being somewhat smaller than the control.

To study the influence of repetition rate on the spacing effect, the preparation was stimulated at a constant spacing interval of 5 millisec. over a range of frequencies. Five milliseconds was chosen as at this interval the spacing effect is still near its maximum but is more constant than with shorter intervals. The greater response at shorter intervals is often variable, due to the proximity of the second shock to the end of the relative refractory period of the first one, so that slight fluctuations caused by fatigue result in uneveness of the contraction. The range of repetition rates extended from 10 to 100 per second. At very low repetition rates

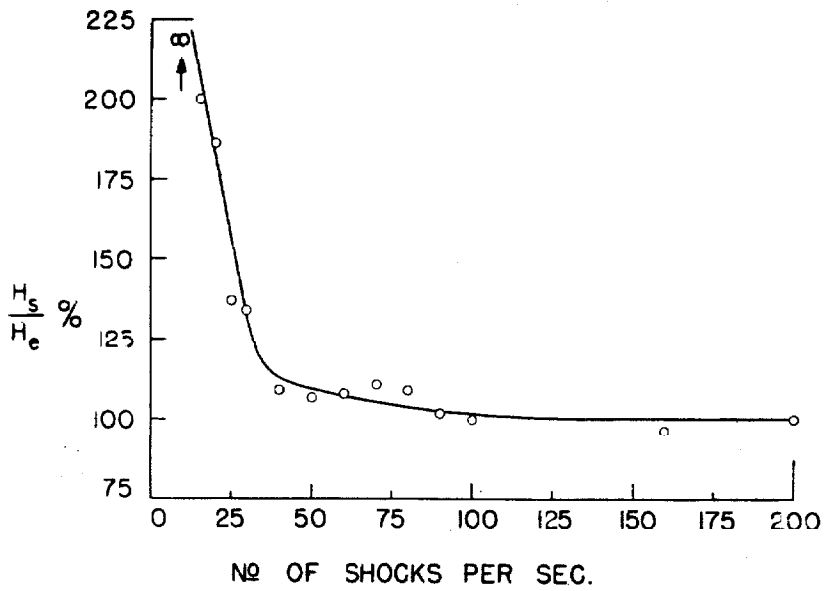

Fig. 3. The relation between the excitatory repetition rate and the spacing effect. The spacing interval is 5 millisec. throughout. The height of the contraction resulting from spaced stimuli (Hs) is expressed as a percentage of the contraction arising from the same number of equally spaced shocks per second (He). The curve becomes asymptotic to the ordinate at those frequencies at which closely spaced, but not equally spaced shocks, give rise to contraction.

seconds. In the upper curve the maximum spacing effect is 50 %, i.e. the spaced contraction is 1.5 times as large as the equally spaced contraction. For different preparations of the opener muscle the effectiveness of spacing varies, as is demonstrated by the lower curve of fig. 2, in which the increase was only 15 %. In a large number of preparations with stimuli spaced at 5 millisec. the spaced contraction was from 0—120 % greater than the control with an average value of + 54 %. In a very few cases a small negative spacing effect was observed; the spaced contraction being somewhat smaller than the control.

To study the influence of repetition rate on the spacing effect, the preparation was stimulated at a constant spacing interval of 5 millisec. over a range of frequencies. Five milliseconds was chosen as at this interval the spacing effect is still near its maximum but is more constant than with shorter intervals. The greater response at shorter intervals is often variable, due to the proximity of the second shock to the end of the relative refractory period of the first one, so that slight fluctuations caused by fatigue result in uneveness of the contraction. The range of repetition rates extended from 10 to 100 per second. At very low repetition rates

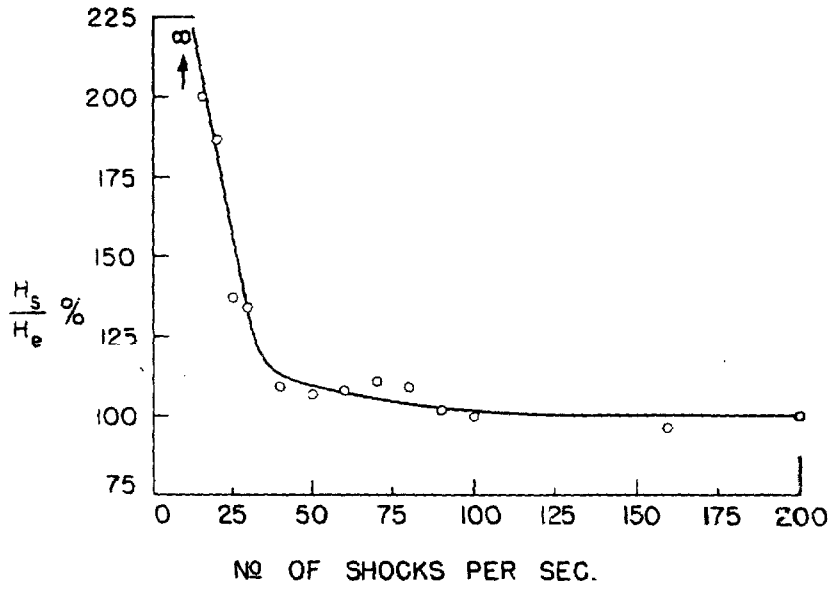

Fig. 3. The relation between the excitatory repetition rate and the spacing effect. The spacing interval is 5 millisec. throughout. The height of the contraction resulting from spaced stimuli (Hs) is expressed as a percentage of the contraction arising from the same number of equally spaced shocks per second (He). The curve becomes asymptotic to the ordinate at those frequencies at which closely spaced, but not equally spaced shocks, give rise to contraction.

In this investigation the *Rc* values of the control contractions (at equal spacing) were found to be much less constant than in the previous investigations. They varied from 0.40 to as high as 0.80 (average 0.5) in different preparations and in some cases showed considerable dependance upon the frequency of excitation. The method was rigorously checked for sources of error and although slight variation could be attributed to these, they were not sufficient to account for the overall picture obtained. We are at a loss to explain the discrepancy between these results and those obtained in the earlier investigations, the more so because of the variation from one preparation to another.

In comparing the effects of inhibition on closely spaced and equally spaced contractions, it was therefore necessary to take into account the variation of the *Rc* with the repetition rate of the equally spaced excitation. By expressing the results as $\frac{\Delta i}{i}$ (where Δi is the difference between the inhibitoty frequency necessary for suppression of a spaced contraction, and that necessary for suppression of an equally spaced contraction obtained at the same repetition rate; i is the inhibitory frequency necessary for the suppression of the equally spaced contraction) these discrepancies can be eliminated and the data from different experiments legitimately compared.

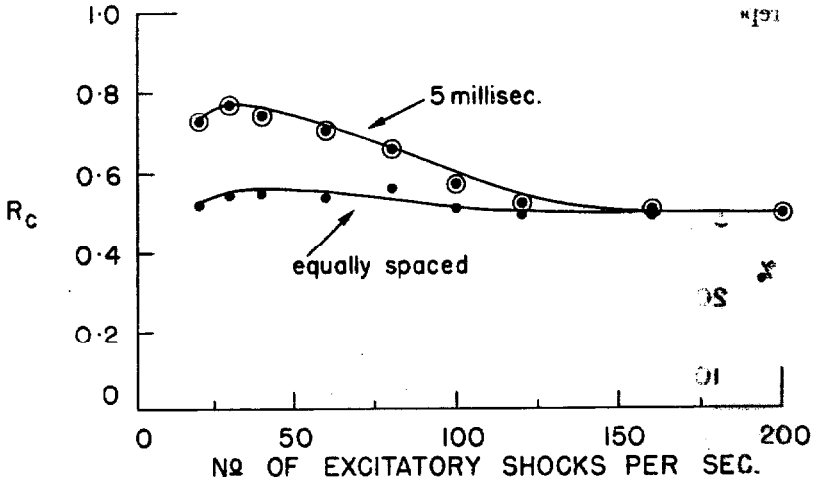

Fig. 4. Comparison between *Rc*'s from closely spaced and equally spaced excitatory stimulation. The upper curve shows the increase in *Rc* with decrease in the repetition rate of the excitatory stimulus spaced at 5 millisec. The lower curve shows very little variation in *Rc* with the excitatory shocks equally spaced. Both curves are from one preparation. The inhibitory stimuli were equally spaced throughout.

In Fig. 5 the results from another preparation are expressed in this form and are plotted as a function of the excitatory frequency. The dependance of $\frac{\Delta i}{i}$ upon the repetition rate is obvious and serves to emphasize the fact that the spacing effect is dependent upon the difference between the repetition interval and the spacing interval. As the difference in these two time intervals decreases towards the point of equal spacing, the muscular expression of spacing becomes less. From the results in Fig. 5 the greater effectiveness of spaced excitation can be estimated. At a repetition rate of 10 per second with 5 millisec. spacing, about 40 % more inhibitory impulses are needed to suppress the contraction than are necessary for the suppression of the control contraction. Considering the low *Rc* value for this muscle, this represents a very large functional difference.

3. *The Effectiveness of Spaced Inhibition in Suppression of Contraction.*

Inhibitory phenomena can be expressed only as functions of their effect upon excitation as they produce no measurable mechanical effect other than prevention or reduction of contraction. The best way to observe the effect of spacing the inhibitory impulses is to choose an inhibitory repetition rate which reduces a given contraction considerably, but not completely. Let the excitor axon be stimulated at a frequency of 60 shocks per second. The inhibitor then needs to be stimulated with about 10 repetitions per second to obtain considerable but partial inhibition.

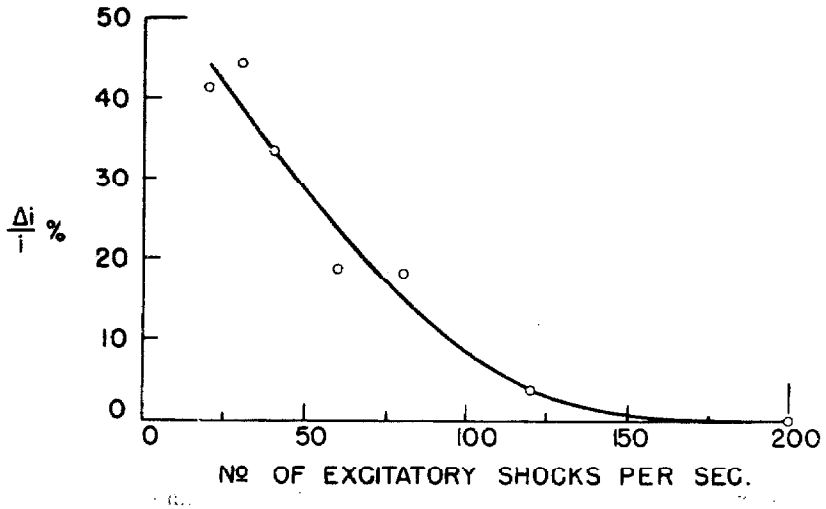

Fig. 5. Graph showing the percentage increase in the number of inhibitory stimuli necessary to compensate for the enhanced effect of spaced excitation at various frequencies under *Rc* conditions. Note that the spaced excitation is most effective at the lowest excitatory frequencies. For experimental details see text.

In Fig. 6 an isotonic kymograph record of such an experiment is presented. The first response is an uninhibited contraction obtained with a frequency of 60 per sec. Responses b and d are this same contraction during simultaneously initiated inhibitory stimulation at 10 repetitions per sec. with equal spacing. Response c is in all respects similar to b and d, but for the fact that the spacing is now 5 millisec. The considerably greater effectiveness of this spaced inhibition is apparent in this preparation. This difference in effectiveness between spaced and non-spaced inhibition varies from preparation to preparation, and corresponds to similar variation in the effects of spaced excitatory stimulation.

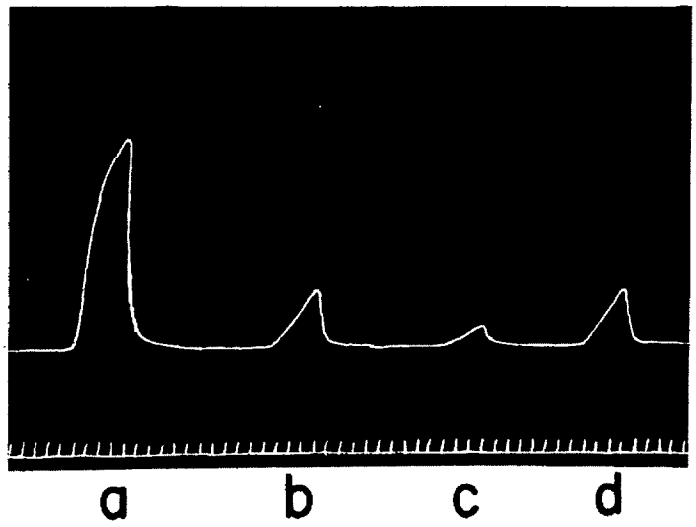

Fig. 6. Isotonic record of opener muscle contractions showing the effect of spacing the inhibitory impulses. a — excitation at 60 shocks per sec.; b and d — excitation at 60 plus inhibitory stimulation with 20 equally spaced shocks per sec.; c — excitation at 60 plus inhibitory stimulation with 20 shocks per sec. spaced at 5 millisec. Time record in seconds. Note that the spaced inhibitory stimulus (c) is much more effective in reducing the contraction than are the equally spaced inhibitory stimuli.

The results from an experiment with spaced and non-spaced inhibition are presented graphically in Fig. 7. The effectiveness of the inhibitory stimulus is expressed as $\frac{(H_{30}-H_i)}{H_{30}}$ %, where H_{30} is the height attained in 5 sec. by a contraction resulting from excitation at 30 shocks per sec.; H_i is the height attained in 5 sec. by a contraction where the excitor is stimulated at 30 shocks per sec. with simultaneous inhibitory stimulation at a certain repetition rate. This latter repetition rate is plotted along the abscissa. In the upper curve the inhibitory stimulus was spaced at 5 millisec.; in the lower curve it was equally spaced. It may be seen that the

spacing effect increases as the inhibitory stimulus approaches the value for complete suppression of the contraction. The range of inhibitory repetition rates over which one can make observations is of necessity limited, due to the low *Rc* ratio for this muscle and also by the fact that very low repetition rates produce such small amounts of inhibition that they are overshadowed by the errors inherent in the method. The inhibitory spacing effect is never as great as that observed with excitation.

As was shown for the excitor system, the shorter the spacing interval the greater is the effect at a given repetition rate, provided that the interval be longer than the relative refractory period of the axon under consideration. The effect is illustrated in Fig. 8 for which the data were obtained by stimulating the inhibitor at a repetition rate of 6.5 per sec. This rate of stimulation is insufficient to completely suppress a contraction elicited by stimulation at a repetition rate of 15 per sec. with equal spacing even when it is maximally efficient. The heights of the contrac-

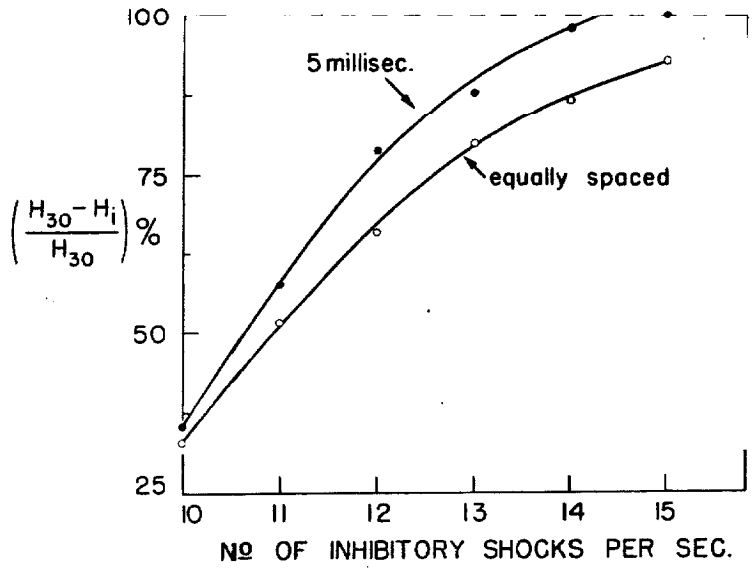

Fig. 7. Comparison of the effectiveness of closely spaced and equally spaced inhibitory stimuli in suppressing a contraction and the variation of this effectiveness as a function of the inhibitory repetition rate. The excitor is stimulated throughout with 30 equally spaced shocks per sec. and the contraction attains a certain height (H_{30}). This contraction is then inhibited by equally spaced stimuli (lower curve) and stimuli spaced at 5 millisec. (upper curve). The height of the inhibited contraction (H_i) is reduced. The amount of reduction of the contraction by the inhibitory stimuli, expressed as $\frac{(H_{30}-H_i)}{H_{30}}$ %, is plotted on the ordinate. On this axis 100 % represents complete suppression of the contraction.

tions, in arbitrary units, obtained over a range of inhibitory spacing intervals, are plotted against these intervals (in millisec.). The influence of increasing the spacing intervals stepwise is very much like that seen in excitation. With less than 2 millisec. spacing interval, inhibition is very ineffective as only one of the shocks of each pair reaches the muscle and the repetition rate of the inhibitory stimulus is thus effectively halved. With 3 millisec. spacing, maximum efficiency is reached and then gradually decreases until, with a spacing interval longer than 15 millisec. there is practically no difference in response to closely spaced and equally spaced stimuli.

Expressed in terms of the *Rc* ratio, spaced inhibition will give a lower value than is obtained with equally spaced inhibition. Experiments were conducted in which the repetition rate of the equally spaced inhibitory stimuli was held constant and the *Rc* determined by adjustment of the frequency of excitation. Using the same rate of inhibitory stimulation, but spaced at 5 millisec., the excitatory frequency was again adjusted to give *Rc* conditions. In many cases there was little spacing effect and the adjustment in excitatory frequency necessary to re-establish the *Rc* conditions, was small. It is inadvisable to stimulate an excitatory axon repe-

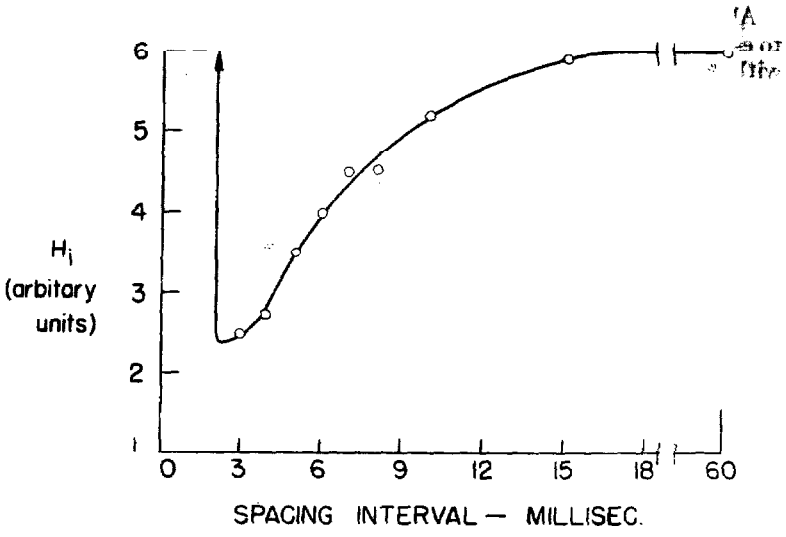

Fig. 8. Increase in effectiveness of spaced inhibitory stimuli as the spacing interval is decreased. The excitor axon was stimulated throughout at 30 shocks per second and the inhibitor at 13 shocks per second over a range of spacing intervals. The height of the inhibited contraction (Hi), in arbitrary units, is plotted on the ordinate; the inhibitory spacing interval in milliseconds, along the abscissa. At about 2 millisec. the second shock of each pair falls in the relative refractory period of the first and is consequently ineffective. This accounts for the sharp rise of the curve to a value of 16.5 units (not shown).

titvely at more than 150 shocks per sec. as fatigue sets in fast. At very low frequencies of stimulation, less than about 20 shocks per sec., the contraction is so small that *Rc* determinations are very inaccurate. If the *Rc* is of the order of 0.4, then the maximum range of inhibitory frequencies for *Rc* determinations which cover the working range of excitatory frequencies, is from 8 shocks per sec. to 60 per sec. Under experimental conditions this range is too small to allow the full effect of shortened repetition intervals to be expressed. In Table 1, the variation between *Rc* determined with closely spaced and equally spaced inhibition is shown for the possible experimental range.

Table 1. *The variation in the Rc determined with spaced inhibition as the inhibitory frequency is increased.*

Frequency of Inhibition (repetitions per sec.)	7.5	10	15	20	30
Non-spaced inhibition *Rc*.	.42	.40	.40	.39	.38
Spaced inhibition *Rc*.	.34	.37	.37	.35	.33[5]

Although small, the spacing effect is indeed present and serves further to establish the similarity between the excitatory and inhibitory processes with regard to the spaced impulses. If the inhibitory frequency range could be extended, it is highly probable that a decrease in spacing effect with increased frequency would be observed.

DISCUSSION

The point of greatest interest which emerges from this investigation is that the spacing effect occurs in the inhibitory transmission process on stimulation of the inhibitory axon with the appropriate stimuli. Several implications arise from this fact. It means that the spacing effect observed in the motor system is a real junctional effect occurring at the postulated facilitatory loci between the nerve impulse and the muscular contraction. That the effect is merely some mechanical action at the level of the muscular contraction must be ruled out. We shall return to this point later in the discussion. WIERSMA & ADAMS (1950), in studies on the effect of spaced stimuli on crustacean motor systems, came to the conclusion that the various systems could be classified as 'pattern sensitive' and 'pattern insensitive'. The present investigation has shown that the inhibitory system of the opener muscle in *Cambarus* can be added to the former class. As these authors pointed out, the spacing effect in the motor systems was always attended by a high degree of facilitation. Since the inhibitory system also exhibits the spacing effect, it follows that its facilitation must be relatively large. This confirms conclusions drawn from the fact that the *Rc* ratio remains almost constant over a wide range of

excitatory frequencies notwithstanding the relatively low frequency of the inhibitory stimulation under these conditions. The motor endings can, in many ways, be shown to exhibit facilitation and from the constancy of the *Rc* it must be concluded that the rate of facilitation of the inhibitor keeps pace with that of the excitor in order to maintain this constancy.

The spacing effect in either excitor or inhibitor is dependent upon the number of stimuli per second. It appears therefore that the difference between the spacing interval and the repetition interval is the governing factor. If stimuli are spaced at 5 milliseconds and the repetition rate is, say 15 per second, then the difference between the two intervals is large and the spacing effect marked. As the repetition rate is increased, this time difference diminishes until eventually the repetition interval is only twice the length of the spacing interval; in other words the impulses are equally spaced. At this point there can obviously be no spacing effect. There is therefore, of necessity, a gradual decline in the magnitude of the effect with increase in the stimulatory frequency, by virtue of the very nature of the stimulus. However, we can obtain a more accurate estimate of what the differences between these two time intervals must be to produce a measurable effect. When stimuli are spaced at 5 milliseconds, spacing becomes almost ineffective with a repetition rate of 65 per second. The repetition interval is approximately 15 milliseconds; the spacing interval 5 milliseconds and the difference therefore 10 milliseconds. Even if the spacing interval is slightly greater or smaller, the figure of about 10 milliseconds between the pairs of shocks appears to represent the lower limit for the expression of the spacing effect. In the final analysis this may assist in indicating the temporal characteristics of the facilitatory process.

At present the site and mode of action of the spacing effect is still uncertain. Since we have shown in this investigation that the spacing effect is not due to mechanical effects at the muscular level, we may draw further conclusions. The phenomenon is manifestly closely associated with the facilitatory processes. In some preliminary work, continuous records were made of the action potentials from a muscle contracting in response to closely spaced or equally spaced stimuli. It was noted that a mechanical spacing effect was invariably accompanied by peculiarities in facilitation of the action potentials. The first action potentials of successive pairs are all approximately the same size as the corresponding potentials from the muscle when stimulated with equally spaced shocks. The second shocks of the pairs show much larger facilitation than that of the corresponding potentials elicited with equally spaced shocks. Their size is over and above that to be expected if they merely summed with the first shock of each pair. On the scheme proposed by WIERSMA (1941) there are two loci of facilitation in the transmission processes of both the motor and inhibitory axon endings. Action potential facilitation occurs at the first locus. It is therefore obvious that the first locus is involved in the spacing phenomenon.

The degree of facilitation remaining after a muscular contraction has been elicited can be demonstrated by stimulation of the neuromuscular preparation a short time after it has relaxed from a previous contraction. WIERSMA & ADAMS (1950) found with a number of crustacean preparations, including the opener of *Cambarus*, that there is no greater lasting-facilitation resulting from a previous spaced contraction than there is from an equally spaced contraction. This is surprising as the height attained by the spaced contraction was much greater than that of the equally spaced contraction. However, examination of the results obtained from continuous records of the muscle action potentials when spaced stimuli were used, show that there is agreement between the two facts. If the first muscle action potential from a pair of stimuli shows no more facilitation than the corresponding potential from the muscle with equally spaced stimuli, it is obvious that there can be no greater degree of lasting-facilitation, at least at the first locus. That this is borne out by the absence of lasting-facilitation in the experiments of WIERSMA and ADAMS implies that there is no lasting-facilitation at either locus. The spacing effect may well depend upon the very large facilitation of the muscle action potential produced by the *second* shock of each pair. The growth of this second action potential is a complex phenomenon which will have to be studied further.

The above rather meager conclusions as to the site and mechanism of the spacing effect shed no light on the problem of why some systems showing a large degree of facilitation should be pattern sensitive and others not. In 1937 WIERSMA & VAN HARREVELD demonstrated in some fast systems that facilitation itself could be facilitated. In view of the fact that this facilitated facilitation occurs in exactly the muscles where we would expect to find the spacing effect very pronounced, it appears that this process might determine pattern sensitivity. It seems possible that in facilitation of facilitation may lie the answer to the problem of why the motor system of the opener muscle shows more constant and larger spacing effects than the inhibitor although the two systems exhibit the same degree of facilitation, as evidenced by the constancy of the *Rc* with increasing frequency of stimulation.

What, if any, is the significance of this spacing phenomenon in the functioning of the animal as a neuromuscular machine? Is the observed phenomenon merely the fortuitous result of the combination of a number of parameters concerned with unrelated neuromuscular mechanisms or has it any functional significance? It is theoretically possible for an animal to use such a principle in maintaining maximum economy of the neuromuscular apparatus. For example, four spaced impulses could be quite as effective as six, or even more, equally spaced impulses. It has been rather casually observed that if a muscle is brought to the same degree of contraction, on the one hand by equally spaced stimuli and on the other by closely spaced stimuli, then the rate of fatigue is less with the latter stimulus. This observation has been carried no further, yet it

suggests some justification for postulating the natural occurrence of spaced nervous impulses.

It is possible to visualize an economy at a higher level of analysis. If a single axon innervated two muscles, one of which was 'pattern sensitive' and the other 'pattern insensitive' then, by the appropriate temporal sequence of nerve impulses, it would be possible to have both muscles contract, the pattern sensitive one only, or the pattern insensitive one. One might expect such a situation to arise in the opener-stretcher system of the Crustacea Decapoda where there is a common motor axon but as far as is known both these muscles are equally pattern sensitive. Both WIERSMA & ADAMS (1950) and DONNER (1950) have visualized mechanisms which apply to the central interpretation of afferent sensory discharges coming from a number of sensory endings and transmitted in one axon. The mammalian eye is a good example of the need for such mechanisms as the number of retinal sensory elements is far in excess of the optic nerve fibers. Pattern sensitive and pattern insensitive synapses might conceivably be responsible for the 'unscrambling' of complex information arriving in one sensory axon.

In the Crustacea at least, a point which militates against the view that naturally occurring spaced motor impulses play a functional role is the variability encountered between individual animals in the extent of the spacing effect. Some specimens exhibit almost no spacing effect in either excitor or inhibitor and others show very large mechanical and inhibitory effects. If the phenomenon was of much functional significance such variability could hardly be tolerated.

SUMMARY

1. Isolated inhibitory and motor axons of the opener muscle (abductor of the dactylopodite) of *Cambarus clarkii* were stimulated with pairs of shocks repeated at regular intervals. The contraction of the muscle was recorded on a kymograph with a light isotonic lever attached to the tip of the dactylopodite.

2. Stimulation of the motor axon with these spaced shocks gives rise to a contraction which is greater than that arising from stimulation with equally spaced shocks. The maximum spacing effect occurs when the interval between the shocks of a pair is between 2 and 3 millisec. Maximum observed values show an increase of 120 % in the contraction height. The average value is 54 %.

3. As the spacing interval is increased the spacing effect decreases and with an interval of 12 to 15 millisec. is no longer discernible. As the repetition rate of the pairs of shocks is increased the spacing effect declines and eventually coincides with the value obtained with equally spaced shocks. At a stimulatory frequency of 130 shocks per second and a spacing interval of 5 millisec. the spacing effect is no longer measurable.

4. For comparative purposes the effect of spaced excitation upon the *Rc* ratio has been studied. Spaced motor stimulation caused a rise in the

Rc as a greater number of inhibitory stimuli are necessary to suppress the spaced motor stimulation than are necessary to suppress the equally spaced excitation. The effect is greatest at low frequencies (25 per sec.) and disappears at about 150 per sec.

5. A spaced inhibitory stimulus leads to greater suppression of a contraction than does an equally spaced stimulus. The enhanced effectiveness is greatest as the inhibitory frequency approaches the value necessary for complete suppression of the contraction.

6. If a contraction be partially suppressed by spaced inhibitory stimulation it is then found that as the spacing interval is decreased from about 15 millisec. to about 3 millisec., the amount of suppression of the contraction increases. At less than 2 millisec. the second shock of a pair falls in the relative refractory period left by the preceeding shock and the stimulatory frequency is effectively halved.

7. Spaced inhibitory stimulation results in a lower *Rc* than is obtained with equally spaced stimuli. Fewer closely spaced than equally spaced inhibitory shocks are necessary to completely suppress a given contraction.

8. The possible experimental range in repetition rate over which the inhibitor can be tested is limited by characteristics of the preparation. For this reason decline in the effectiveness of spaced inhibition, with increase in the stimulatory frequency, was not observed.

9. It is concluded that the spacing effect is a true junctional phenomenon, and not a mechanical effect at the level of the contraction.

ZUSAMMENFASSUNG

1. Isolierte hemmende sowie motorische Nervenfasern des Öffnermuskels (Abductor des Dactylopoditen) von *Cambarus clarkii* wurden mit Paaren von Reizen, die gleichmässig wiederholt wurden, gereizt. Die Muskelkontraktion wurde mittels eines leichten isotonischen Hebels, der an der Spitze des Daktylopoditen befestigt war, am Kymographion registriert.

2. Reizung des motorische Axons mit derartig gepaarten Reizen verursacht eine Zuckungskurve, die höher ist als eine, die durch gleichmässige Reizung hervorgerufen ist. Der maximale Effekt der Reizpaarung erfolgt, wenn das Zeitintervall der Reize eines Paares 2—3 Millisekunden beträgt. Die grösste beobachtete Höhenzunahme betrug 120 %, der Durchschnitt ist 54 %.

3. Mit Zunahme der Zeit zwischen zwei Reizen eines Paares nimmt der Effekt der Paarung ab und ist bei einem Wert von 12—15 Millisekunden nicht mehr wahrnehmbar. Mit zunehmender Wiederholungsfrequenz der Paare nimmt der Effekt der Paarung ab und bei einer Reizfrequenz von 130 Reizen per Sekunde und einem Abstand der Reizpaare von 5 Millisekunden ist kein Paarungseffekt mehr messbar.

4. Für vergleichende Zwecke wurde der Einfluss der Reizpaarung auf den *Rc*-wert studiert (*Rc* ist das Verhältnis zwischen Hemmungsfrequenz und Erregungsfrequenz das gerade zur Unterdrückung der Kontraktion

führt). Paarung führt zu einer Vergrösserung von *Rc*, da eine grössere Anzahl von hemmenden Impulsen nötig ist um die erfolgende grössere Kontraktion zu unterdrücken. Das Resultat ist am grössten bei niedrigen Reizfrequenzen (25 per Sek.) und verschwindet oberhalb etwa 150 per Sek.

5. Gepaarte Reizung eines hemmenden Axons führt zu einer verstärkten Hemmung einer Kontraktion als normale Reizung. Die Verstärkung wird umso grösser, je näher die Hemmungfrequenz dem Wert kommt, der zur vollständigen Aufhebung der Kontraktion führt.

6. Wenn eine Kontraktion nur teilweise von einer gegebenen Hemmungsfrequenz unterdrückt wird, findet man, dass mit der Abnahme des Abstandes zwischen den Reizen eines Paares von 15 zu 3 Millisekunden die Hemmung zunimmt. Mit einem Intervall von 2 Millisekunden und weniger ist die entstehene Kontraktion wieder grösser, da der zweite Reiz jedes Paares in die Refraktärperiode des ersten fällt, die effektive Frequenz also halbiert ist.

7. Gepaarte hemmende Reizung gibt einen niedrigeren *Rc*-Wert als gleichmässige Reizung. Weniger eng gepaarte Reize sind notwendig, um eine Kontraktion zu hemmen.

8. Das experimentell mögliche Frequenzgebiet der Wiederholungsfrequenz mit der das hemmende Axon gereizt wird, ist durch die Eigenschaften des Präparates beschränkt. Es ist daher nicht möglich, die erwartete Abnahme des Paarungseffektes mit Grösserwerden der Wiederholungsfrequenz zu beobachten.

9. Es wird gefolgert, dass der Effekt der Reizpaarung ein echtes Synapsen-phenomen darstellt und dass es sich nicht um einen mechanischen Effekt in der kontraktilen Substanz handelt.

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PART III.

NEUROMUSCULAR MECHANISMS IN THE GRASSHOPPER, ROMALEA MICROPTERA (BEAUV.)

I. INTRODUCTION AND SURVEY OF THE LITERATURE.

Perhaps the most striking difference between the world's two most successful groups of animals, the Arthropoda and the Vertebrata, is their several solutions to the same problems of locomotion imposed by the common environment. Superficially the solutions arrived at by both groups have much in common on the land, in water and in the air. But immediately one looks deeper at the organization of the locomotory systems large differences emerge. Then again when the systems are resolved at a still more fundamental level it is apparent that both great groups are making use of essentially the same principles of contractile muscle cells and neuronal message conduction. It is at the level of the organization of the muscle and nerve units where the pronounced differences lie.

The vertebrate myo-neural system has remained essentially unspecialized and pliable. Extroception and muscular action and coordination are accomplished in all cases by enormous duplication and multiplicity of nerve and muscle cells. In both sensory and motor systems of the vertebrates an outstanding feature is this multiplicity and complexity. Advantages of such systems are obvious, as they give potentialities for more exact extroception, more accurate control of muscular reactions and larger safety factors with less demand on individual units. It is with some awe then, that one finds comparable

degrees of accuracy and control among the arthropods, where the same functions are fulfilled with a minimum of the building units. Their solution has been coloured by their small size. A gnat has to see, smell, hear, know the position of its legs and accurately control the delicate flight mechanism but has no room in its puny body for thousands upon thousands of nerve fibres. It has been forced to economize with as little detriment to control as possible. Thus delicate and ingenious organs of sense and motor control have arisen and allow the mosquito with its puny handful of axons, to elude the man with his countless thousands of axons and sense organs.

Among the Arthropoda, the Crustacea have been most extensively studied from the point of view of the neuromuscular physiology. The Insecta, representing the most advanced Arthropoda, have received a great deal of preliminary rather superficial attention but until recently little more than the cataloguing of anatomy and complex reflex behaviour had been done. This may be attributed almost entirely to two causes: the small size, which renders usual techniques of dissection and isolation very difficult; and the system of respiratory tubes, the trachea, which invest most cells of the body. With the advent of modern electronic equipment for the recording of minute electrical and mechanical events and such devices as the micromanipulator and microelectrodes, the subject has found new life and a few physiologists are again turning attention to the problems of neuromuscular organization and function in terms of the more modern concepts.

(i) Histology.

The European anatomists of the late 1800's and early 1900's made

meticulously careful studies of the musculature of a large assortment of insects but gave less attention to the peripheral nervous system. This was due largely to lack of suitable histological stains which would differentiate the fine axon branches and lack of appreciation of what are today widely recognized problems. Even at present there is no really satisfactory stain for nerve terminations in the invertebrates. A great many of the early works were devoted to the nature of muscle and particularly its cross striations and their role in contraction. Many bizarre concepts were expressed and a great deal of misinterpretation was made due to fixation artifacts. However, as early as 1880 Foettinger (1) presented evidence on the nature of the nerve terminations on muscle fibres which compare very favourably with much later work. He placed various live Coleoptera in alcohol so that muscle was fixed in varying degrees of contraction. From such material stained with osmic acid he concluded that the muscle fibres were innervated as many as nine times per millimeter. The nerve endings were shown to be arranged along one side of certain muscle fibres but in others they were on opposite sides or scattered over the surface in random fashion. He noted the presence of a sarcolemma and maintained that it was continuous with the membrane of the nerve termination. The finest branches arose from the axon in the Doyere's cone and are shown as continuous with the cross-striations of the muscle fibre. Only this latter observation has not been confirmed. His observation of "local" contractions starting under the nerve terminations is precisely in agreement with views currently held! Jordan (2) studied isolated muscle fibres of the wasp and did much to clarify the disposition of the various striation bands in fibres in the contracted and relaxed

state. He realized that fixation artifacts were responsible for a great deal of the diversity in results obtained by various workers. Nevertheless, he claimed that in fibrous muscle the content of the sarcosomes contributed material to the anisotropic band during contraction of the muscle and that on relaxation the anisotropic material re-entered the sarcosome. As these observations were made on fixed material they must be taken with reserve.

The presence of two distinct types of insect muscle, tubular and fibrous, has long been recognized. The tubular muscle is very similar to vertebrate skeletal muscle. In the fibrous flight muscle there is a problem as to what constitutes the muscle fibre. Schaffer (3) in 1891 reported that the structures usually considered as fibrils were possessed of a "sarcolemma" but this has never been confirmed by later workers. This question is of considerable interest in terms of modern concepts of muscle function which dictate the presence of a membrane. Its specific locality or absence in the flight muscles, particularly those of Diptera and Hymenoptera which appear to have specialized action, may prove of considerable importance to an understanding of its function.

Rollet (4) in 1891 is one of the earliest students of the motor nerve endings. He depicts the nerve endings on the muscles of a Chrysomelid beetle. His impression of a termination much like the end-plate of the mammal has persisted in some of the most modern studies but in 1905 Mangold (5) was of the opinion that there was really no such specific end organ and that the so-called end-plate and Doyere's cone were caused by slight stretching and pulling on the terminal membranes and connective caused either by shrinkage during

fixation or by handling of the muscle. His observations are based on both fresh and fixed material and appear well founded. By intra-vitam staining of various beetle and caterpiller muscles he was the first to show a number of axons running parallel to each other in the nerve and its branches on the surface of the muscle. In Coleoptera, Lepidoptera larvae and Orthoptera he shows a diplotomic innervation similar to that characteristic of certain crustacean muscles. By metallic impregnation methods he was able to demonstrate the diplotomic nature of some of the finest branches and their penetration of the sarcolemma. In many cases the diplotomic innervation does not extend to the finest branches but in some cases it is continued even after penetration of the sarcolemma. This is the only direct evidence that in insect muscles innervated by more than one axon, at least some of the muscle fibres are innervated by all the axons. In musculature of a caterpiller quadruplotomic innervation is shown and the depressor tibia of the locust, Decticus, has a diplotomic innervation. Beneath the sarcolemma the endings are depicted as a row of small swellings which do not penetrate any distance into the sarcoplasm.

In both Crustacea and Insecta not only do a number of separate axons innervate the muscle fibres but each axon, by its many branches, innervates each muscle fibre many times along its length. As opposed to the terms diplotomic, triplotomic etc. which have been coined for the Crustacea to describe the former type of innervation, the latter type, where one or many axons each innervate the fibre many times, will be termed multiterminal innervation. In the Crustacea the muscle fibres always have multiterminal innervation and characteristically triplotomic as well. In these terms then, Mangold observed diplotomic and quadruplotomic multiterminal innervation in insects and this is

confirmed both histologically and physiologically in this work on the locust. His diagrams also indicate that the axon terminations occur not on one, but on many sides of the fibres. Montalenti (6) in 1927 observed the branchings of tracheoles within muscle fibres and reports diplotomic and triplotomic innervation in Coleoptera.

Zwarzin (7) made an observation in 1924 which relates to a physiological paper to be discussed later. He made a detailed study of the non-paired median ventral nerve in the nymph of the dragonfly, Aeschna, and shows four axons of which he considers two sensory and two motor. He did not trace the axons to their peripheral terminations but states that Tichomirow (8) found them innervating the spiracles and associated muscles in Bombyx. He concluded that they are not "sympathetic". His pupil Orlov (9), made observations on sensory and motor endings in the gut. He depicts Doyere's cones on the musculature and many sensory endings innervated by one axon.

Hilton (10) confirms that the non-paired nerves are associated with the spiracular mechanism and "...are not sympathetic in function." In a later paper (11), by using intra-vitam methylene blue staining on a small transparent beetle larva he was able to observe numerous different nerve endings on muscle. He concludes that the motor endings have many different forms varying from simple scattered endings to more complex structures resembling mammalian end plates and Doyere's cones. The muscle fibres are innervated numerous times in their length. In some instances he observed diplotomic endings and also penetration of the axons beneath the sarcolemma. His optical sections of the fibres show the fine nerve endings fairly evenly distributed throughout the sarcoplasm and entering from many sides. Without giving the evidence he

concludes that, "It was also determined that at least ten nerve cells supply each muscle fibre of the larger size." Hilton et al. have accumulated impressive amounts of data on the gross anatomy of the central nervous systems and main nerve trunks in a wide variety of insects. For reference to these data his paper of 1942 (12) should be consulted. Morison (13) and (14) produced a very comprehensive work on the musculature of the honey bee Apis mellifera. For detail of the muscular anatomy this ranks with Snodgrass' (15) monograph on Melanoplus, Carbonell's (16) for the cockroach, Periplaneta americana, Kraemer's (17) on Lucanus (Coleoptera), Heidermann's (18) and Cremer's (19) on Aeschna (Odonata). His descriptions of the histology of the "tubular" and "fibrous" muscle are very extensive. Tubular muscle is restricted to locomotion and movements of mouth parts, antenna, etc. but excludes the indirect flight muscles. The fibres of tubular muscle closely resemble those of vertebrate skeletal muscle in the presence of a sarcolemma, the cross striations and the radial arrangement of fibrils about the central axis. These fibrils are 3 μ in diameter and are divided into sarcomeres by the "Z" band or zygophragma. The zygophragmata unite the fibrils to each other and to the sarcolemma. The central axis of the fibre is penetrated by a canal in which lie numerous nuclei. In this latter point the bee differs from many other insects. Various authors show the nuclei lying largely around the periphery of the fibre with a few interspersed between the fibrils. In fibrous muscle Morison states that the "fibrils" are not organized rigidly into fibres but are very easily split apart and appear to have no limiting membrane. They are organized into large bundles which are covered by a dense meshwork of trachea appearing to hold them together.

These fibrils are striated in the same manner as are those of the tubular muscle, and appear to be united by the "Z" bands. Nuclei are dispersed between the fibrils in orderly rows as are also the sarcosomes. He observed cytochrome in these bodies and a recent paper by Wanatabe and Williams (20) has demonstrated a high concentration of the cytochrome oxidase system. Along with Jordan (2) he believes that the sarcosome material enters the fibrils during contraction and leaves with relaxation. As his observations were also all made on fixed material in various stages of "contraction" this must be received with much reserve. Finally he makes an observation which again relates to the problem of what composes the physiological unit in the fibrous muscle. At the ends of fibrils, where they attach to the exoskeleton, as many as eight "tonofibrils" can be seen. These are fine subdivisions of the fibril and he ventures the idea that they may be the true fibrils and the fibre proper may correspond to what is usually regarded as a fibril. He believes that there is a nerve plexus in the fibrous muscle but this is denied by numerous other histologists.

The results of Marcu (21) on nerve endings in Coleoptera, Diptera and Orthoptera must be mentioned. The chief criticism in this work lies in the fact that he used various gold and silver impregnation methods which are known to stain tracheoles even in preference to axons and he makes no mention whatsoever of the tracheoles or that care had been taken to distinguish between the two structures. In Diptera and Coleoptera he reports that the nerve endings penetrate the sarcolemma and splay out into a brush-like terminal often forming a little hillock or Doyere's cone on the surface of the muscle fibre.

These cones occur largely on one side of the fibres and many of them appear in the length of the fibre as reported by Foettinger (1), Hilton (10) and others. In Orthoptera the axons were not observed penetrating the fibres but branched profusely on the surface. In one diagram there appears to be a diplotomic innervation. His illustrations look so similar to the intra-cellular tracheoles in muscle that one cannot be convinced that he distinguished between them and axons.

The position then with regard to the motor terminations on or in the muscle fibres is not entirely clear. Some authors have reported penetration of the sarcolemma and others not. The objects being studied in these preparations are very near to the limit of optical resolution and this, combined with their erratic and generally poor staining properties, has left the situation clouded. The general structure of the muscle is known but a problem exists in the placement of the sarcolemma in fibrous muscle. This could more probably be solved by micro-electrode technique than by histological means. Apart from Mangold's excellent observations and the more casual reports by Hilton, little is known histologically of the number of axons innervating the muscles. Unfortunately in various physiological studies where a multiple innervation has been indicated the confirmatory histological studies have not been made. With a polychrome stain, serial sections of the muscles show clearly at least the interfibre nerve twigs and a great deal more of this type of analysis is needed. It is laborious and has not attracted many of the recent physiologists.

(ii) Physiology.

Early physiological observations on myo-neural mechanisms were

concerned largely with the general nature of the contraction and the parameters affecting it. The majority of these studies were performed around 1930 when techniques of electrical stimulation and recording were neither as accurate nor as widespread as they are today. The pioneer physiological work on the innervation of crustacean muscles had not been published and little attention was paid to the possibility of there being only a few axons, each with a different function, innervating a whole muscle. Research centered around the muscles of the legs and wings and in none of these works was the stimulation of such a nature as to make the results entirely acceptable. The small size of insects made dissection difficult and most workers resorted to either "direct" muscle stimulation or to thoracic stimulation with high current strengths. More often than not, the nerve in question remained attached to the ganglion, thus introducing complex central effects. However, the papers are worth considering not only from an historical viewpoint but also because they have led to some of the more recent concepts and methods. The papers on insect flight will be dealt with largely in a separate section.

Solf (22) studied the effects of temperature, stimulus strength and fatigue on the response of tibial muscles. Five species of Orthoptera were used, Tettigonia, Phasgonura, Gryllotalpa, Orthocanthacris and Prionotropis, and in all cases the femur was isolated and the muscles stimulated by pin electrodes in the femur. Isotonic records of contraction were made from the apodeme of the extensor. He used high current strengths and his records show indications of double discharge of the nerve and tonus-producing injury in the muscle. From his results, particularly those on the extensor in Tettigonia (=Decticus), he concluded

that all the muscle fibres had the same threshold and that fibre groups were recruited by summation of subthreshold stimuli. The muscles responded with twitches at low stimulation frequency. After long periods of twitch contractions, three stepwise decreases in contraction height occurred. Because of his "direct" muscle stimulation these may be due simply to fatigue and altered threshold of the nerve twigs. All the muscles he studied responded to every shock and the fusion frequency was 36-50 per sec. at 24° and 10 per sec. at low temperature. The curves from his data relating temperature to rate of relaxation, show a sharp inflection point at about 10°, suggesting that there are two different temperature dependent processes. Tettigonia extensor tibia developed a tension of 5.95 Kg./sq. cm. of cross-sectional area, and the rate of contraction depended upon the frequency of stimulation, temperature and loading.

Experiments on the "directly" stimulated flight muscle of the dragonfly, Aeschna, were performed by Heidermanns (18) in 1931. In these animals alone among the Pterygota, the wings are motivated by direct muscles so it is more appropriate to deal with this paper here rather than in the section on flight. Contraction records were made isotonically from the apodeme and the muscle was stimulated by large electrodes on its surface. Shocks were delivered by an induction coil. It is not clear how much the nerve was damaged during preparation of the muscle but it appears probable that it was pulled out or cut away from the surface of the muscle. Many of the data can be satisfactorily explained on the basis, not of direct muscle stimulation as Heidermanns supposes, but of stimulation of the isolated nerve twigs within the muscle.

The isolated muscle preparation was shown to respond with an incomplete tetanus at a frequency of stimulation which corresponded to the normal flight frequency of the wing beats. The fusion frequency of the muscle at room temperature was 45 per sec. Maximum rate of contraction was obtained with high loading. He concluded from these observations that in flight these muscles respond with incomplete tetanus under nearly optimal loading conditions.

With increasing strength of stimulation, the tension in the muscle increased in an apparently smooth fashion. This suggests that the muscle fibres were directly stimulated and nerve elements were not involved. However, the method of recording the contraction could not have resolved very small stepwise increases in tension and the explanation probably lies in the stimulation of increasing numbers of isolated nerve twigs as the current strength increased throughout the muscle. Other records show gradual increase in the response at constant current strength. This cannot be muscular facilitation as he states that slight decrease of the current strength resulted in a corresponding decrease in the contraction height. Heidermanns believed this increase was evidence for summation. A more probable explanation is that threshold changes as the muscle contracted,* caused more nerve twigs to respond. His records suggest that the muscle may be innervated by more than one axon but the poor experimental technique makes interpretation of many experiments impossible and direct muscle stimulation cannot be ruled out a priori. His anatomical descriptions of the musculature are excellent.

Kraemer (17 and 23) introduced the neon discharge stimulator to insect myo-neural physiology. He studied the complex extensor

trochanteris muscle in Dytiscus and one of the coxal muscles of Lucanus. Stimulation was applied either in the thorax with the innervation intact, or directly to the muscle. Unfortunately in many of the experiments he does not indicate which of the two stimulation methods was used so that evaluation is difficult. As the load on the muscle was increased an optimum value for contraction and relaxation rates was reached. Observations were also made on the effects of temperature on the contraction and relaxation rates. With thoracic stimulation, a stepwise decrease in the contraction height occurred with very strong stimulation. This he interprets as ganglionic inhibition. It could however have been due to anelectrotonic effects in a number of axons. With the electrodes placed over the muscle, gradually increasing stimulus strength elicited at least four, and probably six, stepwise increases in the contraction height. He believes that the different heights of contraction can be identified with the muscle flags but the results are characteristic of the multiple axon innervation to be described in this thesis. The descriptions of Lucanus musculature are good.

Studies on the flight musculature of a different species of Aeschna were performed by Cremer (19). His preparation was essentially the same as that of Heidermanns' (18), but the nerve was stimulated by pin-electrodes in the thorax. He found very high optimal loading for maximum contraction and relaxation rates. Such tensions might well be developed in the animal during flight. It is particularly interesting that he was unable to obtain contraction of the nymphal flight muscles although histological studies showed that they were completely developed at least with respect to the fibrils. Nymphal leg muscles exhibited very

slow contraction to single shocks delivered to the muscle and almost no relaxation. The same slow relaxation was observed in some cases after response to direct "make" shock stimulation of the wing flexors but on "break" shock relaxation was much more rapid. In many of his experiments apparently anomolous results can probably be accounted for by the high current strengths employed. His histological results show that the flight muscles are of the "tubular" type with central nuclei and radially arranged fibrils. In development, the fibrils appear first at the periphery of the fibre.

In the studies of Rijlant (24) we find the first recordings of electrical events in the muscle. Reflex preparations of a number of Arthropoda, among them the beetle, Hydrophilus and the common fly, were observed. Electrodes were placed in one of the leg muscles and the electric responses were recorded during "tonic" inactivity and active contraction. He found a tonic discharge rate of 30-40 per sec. in both the flexor and extensor tibia. Manual flexion of the tibia increased the rate in the extensor and decreased that in the flexor. This was interpreted as evidence for a Sherringtonian-like reciprocal inhibition mechanism. During active contraction much larger spike potentials were observed, and it was concluded that there is a double innervation with a slow "tonic" fibre and a fast fibre for "voluntary" contraction. No facilitation of the potentials can be observed in his records but it appears that there are more than two action potential types from the muscles. However the preparation was complicated by the three muscles in the femur and a certain amount of pick-up from adjacent muscles may have occurred.

Friedrich (25) claimed to have observed peripheral inhibition in Dixippus. Using isolated limbs with both "direct" and indirect stimulation, and recording from a split tibia so that extension and flexion could be recorded simultaneously, he showed that the nerves to the muscles have different thresholds. Using "direct" and indirect stimulation simultaneously, and recording from an intact tibia, so called inhibition of the extensor was shown. The indirect stimulation elicited extension and the direct stimulation flexion. Simultaneous stimulation caused flexion. He believes this demonstrates inhibition of the extensor, but as the flexor is the more powerful muscle the results indicate only that it can overcome the extensor contraction. He states that after a contraction the muscle relaxes more rapidly if a stimulus is applied which is sub-threshold for contraction. His published records do not substantiate this claim. In the double muscle system, increase in the stimulus strength caused an increase in the flexor contraction. Friedrich considered that this was due to inhibition of the extensor but it might equally well have been due to excitation of another flexor axon resulting in stronger contraction. From the results it cannot be concluded that an inhibitory mechanism is present.

Ludany and Wolsky (26) compared the tension developed in the extensor tibia of the males and females of winged and wingless grasshoppers. Differences in muscular power and body weight between the sexes in winged, but not wingless forms, agree with Borelli's law.

In 1939, Pringle (27) reported the first study of the electrical and mechanical events in an isolated myo-neural preparation. Although Mangold and Rijlant had produced evidence for at least diplotomic innervation, Pringle's work put the earlier observations on a much

firmer footing. Pringle examined the extensor tibia in the cockroach, Periplaneta. By cutting the branches of a certain nerve in the thorax and severing the nerve from the ganglion, the extensor alone could be induced to respond. Muscle action potentials were recorded with a Matthews oscilloscope. The electrodes were inserted in each end of the femur. Stimulation was delivered by a neon discharge circuit. Mechanical contraction was also recorded on the camera by a mirror attached to the tibia. In some of the experiments movement of the tibia was resisted by a spring so that its excursion was small. In other experiments the tibia moved freely.

Two distinct sizes of action potential were obtained from the muscle. The axon which gave rise to the smaller potentials had the higher threshold so that it could be studied only after the nerve had been treated so as to raise the threshold of the axon associated with the large potentials. The small potentials increased to about six times their initial size after some seconds of stimulation. They were associated with a detectable contraction only when the frequency of stimulation was in excess of 30 per sec. The contraction, as recorded from free tibial movement, was smooth and slow in developing and the tension was much less than that elicited by the fast axon in the same time. As the frequency of stimulation was increased up to and above 300 per sec. the tension resulting from slow axon stimulation likewise increased.

The muscle potentials elicited by fast axon stimulation showed almost no increase in size and were associated with a fast developing tension. The contractions fused at 70 per second and below that frequency incomplete tetanus occurred. Pringle regards the slow

contraction as a posture maintaining mechanism. The fast response occurred in bursts at about 75 per second in a reflex preparation and is believed to be responsible for the movements executed in running. As both contraction types develop equivalent maximum tensions, he believes that the two axons innervate every muscle fibre. This, of course, does not necessarily follow. His statement that observation of the contracting muscle fibres showed that the same fibres contracted on stimulation of either axon, must be accepted with much reserve. With the slow contraction he opined that at low frequencies only a few fibres contracted and that as facilitation progressed, more fibres were recruited and their potentials summed. With the fast contraction on the other hand, all the muscle fibres were involved simultaneously. Thus a maximal twitch would occur and the potentials would not grow in size. His results, however, show that there is appreciable enhancement of the electrical response associated with the fast twitch. This might be due to increasing resistance of the muscle as it contracted within the limiting femoral exoskeleton. The same might apply to at least part of the increase in height of the small potentials. Objections can be raised to his concept of recruitment on the grounds that if a few fibres in the muscle contracted maximally, the nerve twigs might be damaged by the tensions applied to them. van Harreveld (28) has produced evidence for this in muscles of the crayfish.

Pringle's evidence for two contraction types is good but because of the method of recording the slow contraction there is reasonable doubt as to its exact nature. By allowing the tibia to move freely and recording muscle action by this movement, serious errors in

interpretation are involved because of the special leverage factors at the femoro-tibial articulation. Neither facilitation nor slow contraction have been reported by more recent workers and they are in need of verification.

By acoustic observation of muscle potentials and visual study of the movements of the limb in a reflex preparation subjected to nicotine stimulation in the thorax, Pringle developed a rough map of the number of axons supplying the leg muscles. For many of these muscles he claimed various multiple combinations of fast and slow axons. This acoustic method is rather inexact and his results require confirmation. Unfortunately no histological study of the nerves and muscles was made. Rates of discharge determined acoustically were as high as 800 per sec. in slow axons. This figure appears exorbitant and errors could easily have been made in the determinations.

Hughes (29) subjected the thoracic ganglia of Periplaneta to various ascending and descending direct currents and recorded the "reflex" activity in both the nerves and the muscles of the limbs. Such currents, depending on their direction, evoked flexion in the limb on one side and extension on the opposite side and vice versa when the current was reversed. Interpretation of results from such a complex preparation is not possible. Records from the muscles contracting under these conditions show a number of action potential types which add a few axons to the innervation schema presented by Pringle.

Measurements of chronaxie in the leg and wing muscles of various insects were made by Lapique (30). He stimulated intra-thoracically and found that decapitation resulted in a 50% increase in chronaxie. Apparently removal of the head decreased some form of central excitation.

In butterflies the chronaxie of the wing muscles varied directly as the wing area and the rate of wing beat in free flight.

A more detailed study of the electrical and mechanical events in the cockroach muscle was made by Roeder and Weiant (31 and 32). These authors studied the tergal remotor of Periplaneta (muscle 162 of Carbonell (16)) in situ but with the nerve freed from the ganglion and stimulated with a square wave oscillator. Muscle and nerve potentials were led off with pairs of electrodes on the muscle surface or with one electrode on the muscle and an "indifferent" electrode surrounding the preparation. The onset of contraction was visualized on the oscilloscope screen by means of a piezoelectric crystal with a glass stylus touching the muscle.

Only a single axon of the fast type could be demonstrated. A single shock to the nerve resulted in a twitch contraction which was accompanied by a monophasic positive muscle potential. Under these experimental conditions the nerve potential was diphasic and had initial negative sign. During activity the point of nerve entry into the muscle and the immediately adjacent region was always positive with respect to any other region on the muscle and was usually positive to an indifferent electrode. Considerable variation in the sign of the potential with respect to an indifferent electrode was observed. Nerve latency was 0.5 millisec., the muscle potential occurred 1-1.3 millisec. after the nerve potential and had a duration of 4-5 millisec. As an exploring electrode was moved along the muscle, away from the point of nerve entry, both nerve and muscle potentials declined in size. There was no change in the nerve latency but the muscle potential latency increased. They take this to mean that the nerve

potential was always led off from its point of entry into the muscle. From the delay of the muscle potential they estimate the conduction velocity in the muscle as 5-6 meters per sec. This conduction is believed to be in the nerve twigs and not in the muscle fibres. No indication of facilitation or summation was found. Using pairs of shocks the relative refractory period of the preparation was shown to be about 10 millisec.

The picture presented by them is one of local potentials and contractions at nerve endings with the excitation being conducted in both directions from the central point of nerve entry by nerve branches. This differs considerably from the concept entertained by Pringle in which muscle fibre response would be all or none with gradations brought about by nerve facilitation and recruitment of fibres. Roeder and Weiant pointed out that the leading-off conditions were complicated by the presence of the exoskeleton and the situation of the muscle in the thorax and for this reason an attempt to explain the positivity of the muscle potential was not made.

After severing nerve trunks to the limbs of living cockroaches they found that the nerves degenerated in 5 days and the muscle could not be electrically excited. This they believe is additional evidence that conduction of the excitation in the muscle is due to nerve twigs and that direct muscular stimulation is not possible. Apart from the data on the indirect flight muscles, this paper, together with those of Pringle and Rijlant, form the backbone of the information pertaining to insect neuromuscular physiology.

Neither Pringle nor Roeder and Weiant found any sign of peripheral inhibition. Except for the inconclusive studies of Friedrich , inhibition

has been reported by Ripley and Ewer (33) in the levator tarsus of Locusta. Nerve stimulation at 20 shocks per second at an intensity slightly in excess of threshold, resulted in smooth contraction. Muscle action was recorded isotonically from the movement of the tarsus. Antagonistic muscles were freed from their attachments so that they did not influence tarsal movement. When stimulation intensity was increased to three times threshold value, a marked decrease in contraction size occurred. The threshold value was initially very low. This response was observed only with the levator tarsus muscle in gregaria-phase locusts. Other muscles of the animal did not show the phenomenon at intensities greater than 20 times the threshold current intensity. The pulse duration in the experiments was 1.3 millisec. and the polarity was not considered in placing the electrodes on the nerve in each case, so that both ascending and descending currents were probably used. From their report it is not possible to say a priori that the decrease in contraction height could not have been due to anelectronic effects at the point of nerve stimulation although it appears improbable. Until more extensive studies are made the results cannot be considered conclusive. However, one must not lose sight of the possibility of an inhibitor being present in any of the preparations used in the recent studies as it could put a very different complexion on the interpretation of certain results.

I have not discussed the numerous papers dealing with the biochemical and metabolic studies of insect muscle but finally in this section I must mention one such study performed by Buddenbrock (34). In 1920 he measured the oxygen consumption of Dixippus during two different "cataleptic" stances. In one case the animal lay on its back with the

legs folded close to the venter. In the other it stood on a platform with its legs extended and body lifted clear of the substrate. In both cases the oxygen consumption of the whole animal was the same. Bruddenbrock concluded that there must be a "tonus" mechanism in the muscles but he assumed that when the animal was lying on its back the leg muscles were relaxed. This may not be the case as was pointed out by Pringle (27). Had these muscles been contracted then the conclusion drawn by Buddenbrock would be incorrect.

The present status of our knowledge of the insect myo-neural events is very meagre and although the recent observations have made steps in the right direction the possible complexity of the preparations with respect to multiple excitatory and inhibitory axons must be taken into account. Until a satisfactory single axon preparation can be made the issues are likely to remain clouded. Variations of muscular action and innervation patterns between the orders and species and even between muscles of the same species, prove a further drawback to integration of results, and generalizations at this stage are not possible. Many questions remain to be answered before these studies can be placed on as firm a footing as that enjoyed by the crustacean researches.

(iii) Flight.

For many years flight has attracted experimentalists because of its many spectacular properties. Rates of wing beat, energy output, control and structure were among the earliest observations. I will deal only with those papers which relate specifically to modern concepts of the problems. Flying insects can be divided, as far as is known, into

three groups, viz. the Odonata in which the wings are activated by direct flight muscles; the "fast" flyers comprising the Diptera, Hymenoptera and Coleoptera in which the wing muscles activate the wings indirectly through the exoskeletal structures and in which the beat frequency is higher than the frequency of the observed muscle potentials; the "slow" flyers in which the wing muscles are indirect but the muscle behaves in a formal way so that there is an action potential for every wing beat. This last group includes all the orders of pterygote insects other than the Odonata and fast flyers.

Wingbeat frequencies were studied exhaustively by Sotavalta (35). In small Diptera the beat frequency is greater than 1000 per second. This observation stimulated much speculation as to the mechanism of the wing flapping, as times allowed for reflex activity appear much too short. Studies on the effects of altering the wing load were initiated by Roch (36) in 1922. In Diptera and Hymenoptera he observed that halving one wing had no effect on the flight frequency but when both wings were clipped the frequency increased markedly. He concluded that the frequency depends upon the loading and that the wings are linked through the ganglion so that the most heavily loaded wing is the limiting factor. This effect need not be ganglionic at all as the wing linking is through the mechanical structure of the thorax. More recently Sotavalta (37), Roeder (38), Williams (39), Chadwick (40 and 41) have studied the effect of loading the wings by various methods. There is general agreement that in the "fast" flyers decreased loading gives rise to an increase in the response frequency. With these insects recordings of the large potentials in the thoracic musculature (Pringle (42), Roeder (43)) showed that no increase, and

often a decrease, in their frequency occurs with the increased beat frequency. It is concluded that in the fast flyers the increase in beat frequency is not reflexly controlled but is a peripheral effect due to loading. In the slow flyers, decreased loading results in little increase of beat frequency and flight potentials maintain a 1:1 relation to beat frequency. In this case control appears to be of a central reflex nature. Loading of the isolated Odonata flight muscles was studied by Heidermanns (18) and Cremer (19) and has been discussed above.

The high frequency of flight in insects together with the long sustained periods for which flight is possible has led to a number of biochemical observations. Jongbloed and Wiersma (44) Kalmus (45), Krogh and Weis-Fogh (46), Chadwick (47) and Chadwick and Gilmour (48) and Weis-Fogh (49) are among those who have studied respiratory and metabolic factors of flying insects. Apparently different insects use different metabolic fuel during flight. Osborne (50) has considered flight from an aerodynamic aspect. A more complete list of these miscellaneous works can be found in Roeder's paper in 1951 (43).

The recent theories and observations of the flight mechanism were triggered by Pringle (42) in 1949. He presented evidence from Calliphora wing and haltere muscles which suggest neuro-muscular principles heretofore unobserved. In a bisected fly from which the thoracic ganglia have been removed, the haltere, which is activated by a single muscle, will show sustained vibrations if it is once flicked from the rest position. The vibrations can be stopped by gently stopping and releasing the haltere. When a fly is partially anaesthetized it will fly steadily and the rate of wing beat is increased if the wings are

clipped. Reflex activity of the legs is absent indicating that the increased flight frequency cannot be reflex. In sagittally bisected flies electric stimulation evokes no contraction from the muscles. This has been confirmed by Roeder (43), Boettiger and Furshpan (50) and Wiersma and Ripley (unpublished). Cremer (19) noted the same thing in flight muscles of last instar dragonfly nymphs and de Wilde (52) in the striated alary muscles in various caterpillers and Hydrophilus.

Pringle (42) further observed that the beat frequency is independent of the large action potentials observed in the thorax. These potentials start before wing motion and end before the flight stops. During flight they maintain a constant frequency of about one-tenth of the flight frequency. This observation has been confirmed by Roeder (38 and 43) and extended to other Diptera and Hymenoptera. Similar confirmation has come from unpublished work in this laboratory. In Roeder's studies the beat frequency was ingeniously recorded by suspending the insect from a glass stylus attached to a phonograph pick-up arm. His records show that the wing beat starts and stops by gradual increase or decrease in the amplitude of beat but without frequency changes. Both these authors conclude that the flight mechanism depends not on a reflex mechanism in the fast flyers but that a low rate of discharge from the ganglion which is presumably associated with the large spikes observed, enhances the excitability of the muscle so that it responds with a fast twitch to a stretch stimulus. This twitch stimulates the antagonistic muscles which respond likewise and thus, provided both the stretch and neural factors are present, the system remains active. The beat frequency is controlled by loading factors on the wings.

I would like to draw attention to a number of points which throw doubt on this hypothesis. In both Pringle's and Roeder's electrical records, in addition to the large potentials there are almost always much smaller potentials which have a 1:1 relation to the beat frequency. These are not incorporated in their hypothesis and Roeder suggests that they may arise as vibration artifacts in the muscle. In results that I will present for the locust limb, potentials many times smaller than the maximal potential can be recorded and are associated with muscular contraction. It appears possible in the fast flyers that these small potentials are associated directly with the flight muscles and are small possibly due to the fact that they are the algebraic sum of potentials in antagonistic muscles contracting alternately to each other. Secondly, in Pringle's experiments with the haltere, continuous activity for prolonged periods is observed in the denervated muscle. The stretch factor is there but no neural factor is present as the ganglion was removed. How is this to be accounted for? Roeder's results show that in one case after a specimen of Eristalis had spontaneously stopped flying, flight was again started with approximately the same beat amplitude as that at which it stopped. In this case there were no accompanying large potentials, the last one having occurred about 0.2 sec. before. If it is argued that there is sufficient neural factor remaining for the initiation of flight the second time why did flight stop spontaneously in the first instance? The muscular stretch factor could not have been altered. This particular record is almost proof positive that their hypothesis is either incorrect or incomplete.

Boettiger and Furshpan (51, 53, 54, 55) have expressed a different hypothesis to explain the flight mechanism of Diptera. They describe a 'click' mechanism in the mechanical structure of the wing articulation which captures the wing near the end of the up and the down stroke. This mechanism is such that, together with the scutellar lever it forms a mechanism for sudden release of the tension of alternately the longitudinal and vertical indirect flight muscles. The direct muscles would, according to their theory adjust leverage and articulation factors such that the 'click' mechanism could operate to various extents. McEnroe (64) has recorded the tension in a single vertical muscle freed only from the tergum. During flight this vertical muscle develops a constant tension under the conditions of the experiment. It is postulated that all the muscles are responding tetanically. The 'click' mechanism stores energy and is such that quick release and redevelopment of tension in antagonists occur and result in beating of the wings.

The position is at the moment unclear as there are a number of points pro and con both hypotheses. In terms of the observed phenomena it is possible to state a third equally plausible hypothesis which is more in keeping with formal neuro-muscular physiology. This will be taken up again in the discussion.

In the slow flyers the position appears much simpler. There is a 1:1 correspondence between wing beat and muscle potential in each of the numerous muscles involved. Voskressenskaya (56) and Ripley and Ewer (57) have shown that the flight muscles of locusts will follow the frequency of nervous stimulation up to about 20 per sec. At higher frequencies, provided the strength of stimulus is not much above

threshold, the wing muscles continue to beat at about 20 per sec. which is the normal frequency in free flight. At high frequency, if the intensity is increased, a tetanus results. Voskressenskaya believed the non-paired nerve from the ganglia was responsible for the flight which occurs some seconds after strong stimulation of the ganglion. Apart from the numerous other criticisms which can be brought to bear on this study the non-paired nerve, as has been previously stated, is associated only with the spiracular mechanisms.

Ripley and Ewer (57) showed that the low frequency of the beat was due to the long refractory period of the nerve, so that stimuli falling in its refractory period were not effective unless they were much above threshold. Ripley and Wiersma (unpublished data) showed by electrical recording from various muscles in the thorax of the sphingid, Celerio, that the antagonistic vertical and longitudinal muscles responded alternately as has long been inferred from considerations of the anatomy. Phase shifts in the time of contraction of these antagonists modify the amplitude of wing beat. In the sphingid it has also been shown with the isolated nerve muscle preparation of the longitudinal flight muscles that a number of twitch contraction types are evoked in response to increasing stimulus strength. A number of axons appear to be involved and there is some indication that they may fire sequentially, as the amplitude of reflex contraction of the muscle can only be approached with nerve stimulation if pairs of shocks a few millisecs. apart are given. These axons also have a long refractory period.

II. MATERIALS AND METHODS.

The material used in these studies was the large lubber grasshopper, Romalea microptera (Beauv.) (Rhomaleum micropterum of older authors), which occurs in the southeastern states at very circumscribed times of the year. Live adult specimens were purchased from the Carolina Biological Supply Company and arrived in reasonably good condition. The animal does not live well in captivity and feeds only very erratically, dying in most cases, apparently from starvation. One batch was infected with a disease which produced small lumps of dark material in the muscles and connective tissue of the thorax, and which resulted in high mortality. Tachinid parasites also took fairly heavy toll of the animals. Animals were kept in a large cage at room temperature (ca. 26°), fed on corn plants, and watered twice daily.

Romalea was used for the majority of the experiments but certain studies were made with the locally common locust, Schistocerca sp. This animal survives extremely well in the laboratory at temperatures of ca. 30° and feeds well on tomato plants. Specimens have been kept for over four months. Unfortunately the animal is much smaller than Romalea, and consequently experiments are more difficult to perform.

The lack of abundant supplies of insects suitable for myo-neural studies has greatly hindered the pursuit of these investigations.

(i) Histology.

Gross dissection of the thoracic nerves and their branches in the pro-, meso- and metathoracic limbs have been made. Live material is much more satisfactory for this purpose than fixed material, as nerve and tracheal tissue is more easily differentiated in the former. In

the preserved material the nerve and trachea adhere to the other tissues in masses of congealed haemolymph, rendering fine dissection almost impossible. Intra vitam staining with methylene blue is unsatisfactory, as the axons rarely stain, whereas the nerve sheath and nuclei stain heavily. In a few cases reduced methylene blue (Pantin (58)) proved reasonably successful in staining the axons ramifying on the surface of the femoral muscles. Although the staining is short-lived and very erratic, some observations could be made. In some cases gross staining with concentrated solutions of methylene blue in physiological saline made it easier to distinguish the nerve from the other tissues simply by the general toning effects. By this method microdissection can be extended to the very fine intramuscular nerve twigs.

Most histological determinations of the number and disposition of the axons and muscle fibres were made from serial sections of the limbs. Previous experience had shown that shrinkage during fixation was one of the chief causes of failure to observe the axons accurately. With this in mind most of the material was fixed with a 4% solution of formaldehyde (Merck reagent) in physiological saline solution (Carlson (59)). The living animals had the pretarsi of all the limbs removed and were then injected intra-abdominally with 10-15 ml. of the solution. Removal of the pretarsi allows flow of the solution through the limb and results in much better fixation. Injection of large amounts of the solution irrigates the entire animal and removes most of the haemolymph cells and fluids. The animals were left for some 10 minutes and then heads, wings, abdomens and the visceral content of the thorax were removed. Specimens were submerged in more

of the formol solution and subjected to reduced pressure (ca. 12 mm. Hg.) for 5 hours with occasional tapping to loosen trapped air bubbles. This procedure removes the air from the tracheal system and greatly improves fixation and subsequent embedding in paraffin. The reduced pressure causes a loss of formaldehyde from the solution; therefore, after this treatment specimens were placed in fresh solution before being removed for subsequent dissection and sectioning at intervals up to two months thereafter.

Because of the hardness of the exoskeleton and the extreme difficulty of cutting good sections of the soft muscle tissue in its presence, the contents of the femur were dissected free of the chitin. This was a slow process and great care had to be exercised in order not to damage the muscles or the fine intra-muscular nerve twigs. The chitin was sliced away with a sharp scalpel and the muscle gradually and gently detached from each piece. This operation was carried out in water, as fixation was already complete.

In the normal alcoholic dehydration process prior to clearing and embedding, the remaining formalin was removed from the tissue. When the specimens were in xylol they were again subjected to reduced pressure to remove air bubbles, and then again when they were in a xylol-paraffin solution at 50°. Thus at the final embedding in paraffin (Tissuemat, M.P. 52°) all air had been removed from the tracheal system. This is most important, as improper filling of the trachea with wax is the commonest cause for poor sections. Even with this rigorous treatment ragged sections frequently occurred owing to the toughness of the large apodemes of the levator and depressor muscles.

Staining of insect axons has long presented a problem. The usual silver stains are occasionally successful, but much more frequently the silver is deposited in the trachea and tracheoles. This greatly confuses the picture, as the finest tracheoles are easily mistaken for axons. A number of authors report that gold chloride techniques give the best results, but these methods are very time-consuming, and with the prospect of having to stain some 10,000 sections the method was deemed unsuitable. A faster, less expensive method presented itself in the form of lead haematoxylin as introduced by MacConaill (60). He found that this stain was taken up well by invertebrate axons. His method was satisfactory in the grasshopper provided the axoplasm was not badly shrunken. However, muscles and most of the other tissues and nuclei also stained to varying degrees and a fine crystalline precipitate was deposited on the slide and defied removal. After various attempts to modify the method met with no success, the stain was rejected.

Some years ago I made similar sections and found that Mallory's triple stain gave fair results if the sections were overstained in the aniline blue/orange G solution. Fixation in this instance had been with Bouin's solution. Application of this method to the formalin fixed Romalea tissue gave very much better results and the method was therefore adopted. The axoplasm and axon sheaths stained deep blue, the muscles bright red, connective tissue orange and mauve and chitin either red or purple according to its nature. The strong colouration makes differentiation easy and is a great asset in observing large numbers of sections. The method as described by Pantin (58) was used in a slightly modified manner.

Below are a list of the reagents with the manufacturers' names.

Acid Fuchsin (National Aniline Division)
Orange G (National Aniline Division)
Aniline blue - Water Soluble (National Aniline Division)
(Above three stains certified by the Commission of Standardization of Biological Stains.)
Phosphomolybdic Acid (Merck Reagent)
Xylol (U.S.P. Mefford Chemical Corporation)
Absolute Alcohol (U.S. Industrial Corporation)

All solutions were made up in distilled water. When tap water was specifically called for the distilled water was made slightly alkaline with a drop or so of NaOH. This is necessary due to the high chlorine content of the local water.

Serial sections were cut 8 μ thick and the nerve and its branches were traced throughout the whole series. Particular attention was paid to the branching, the size and number of the axons in the branches, the endings of the axons on the muscle fibres, the site of innervation of the fibres and number of times a muscle fibre is innervated. Attempts to determine whether the same axons innervated every muscle fibre were not successful due to the minute diameter of the terminal axons (less than 1.5 μ) and the inability to trace the number and disposition of axons when the nerve twigs were cut obliquely or longitudinally. The sensory nerves, their endings, and sense organs were also noted.

This method gave few data as to the nature of the axon endings on, or in the muscle fibres. Both transverse and longitudinal sections were therefore stained with various silver stains. Pictures resembling very closely the "nerve endings" described by various authors could be obtained in this manner. Fresh, dissected muscle stained in aqueous solution of acid fuchsin and not subjected to reduced pressure, was examined in glycerine. With the right lighting conditions it is easy to see the finest air-filled branches of the tracheoles within

the muscle cells and it was concluded that the fine intracellular fibrils seen in the silver stained sections were tracheoles and not axons.

Sausage-like organs which lie between the muscle fibres and which are presumed to be either sensory or in the nature of sarcosomes, were cursorily studied in dissected muscle stained with carmine or fuchsin. This showed only the gross anatomy and the finer structure was determined from the Mallory-stained serial sections.

(ii) Physiology.

In choosing an insect suitable for myo-neural studies such as these one looks for a number of features. Large size, inherent hardiness, slowly clotting haemolymph and widely spaced limbs are very advantageous. Devising a good physiological saline solution is time-consuming, and a choice of animal which will survive well in one of the few established solutions is desirable. Fortunately Romalea meets all these specifications, thus making it an excellent study object. The drawbacks are the poor survival in captivity and the short season during which it is available.

The preparation from which most of the physiological results were derived allows stimulation of nerve trunks in the thorax, recording of the nerve potentials in the nerve branch to the relevant muscle, isometric recording of contraction from the apodeme of one of the limb muscles and electrical recording of the muscle action potentials. These may all be done simultaneously. In addition the tracheal supply of the animal is left intact and this prolongs the life of a preparation by many hours and maintains the muscle in excellent condition.

In essence the preparation is simple, but the operative techniques are delicate, and considerable experience is needed before they can be performed satisfactorily. The insect is placed on its back in a shallow petri dish, the bottom of which has been covered with Plasticine*. Wads of plasticine pinion the legs in a spread-eagled fashion. This secures the animal very firmly and is far superior to fixing it down with pins. A large cube of plasticine is used to secure the femur of the experimental leg firmly to the dish (Fig. 1).

Trouble is encountered in autotomy of the metathoracic limbs when the thorax is incised. This may be obviated by outward rotation of the tibia and cephaled compression of the coxo-femoral articulation. Unless this is done the limb usually autotomises at the trochantinal-femoral line. The pro- and mesothoracic limbs are never autotomised.

The lateral half of the metathoracic sternite on the side homolateral to the experimental limb is removed and the body cavity immediately flooded with ice cold saline (Fig. 2). This prevents clotting of the haemolymph and the low temperature partially narcotizes the animal, making operation easier. Moribund animals can be restored to activity by this inundation with saline, the composition of which is as follows (Carlson (59)):

NaCl	7.0 g.	
KCl	0.2 g.	
$CaCl_2$	0.2 g.	
$MgCl_2$	0.1 g.	(C.P. reagents used throughout)
Dextrose	8.0 g.	
NaH_2PO_4	0.2 g.	
$NaHCO_3$	0.05 g.	

pH 6.5

Distilled water to make up to 1000 ml. solution.

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*This is a commercial trade name and is the only one of a number of such modelling clays which has suitable plastic properties and is non-toxic.

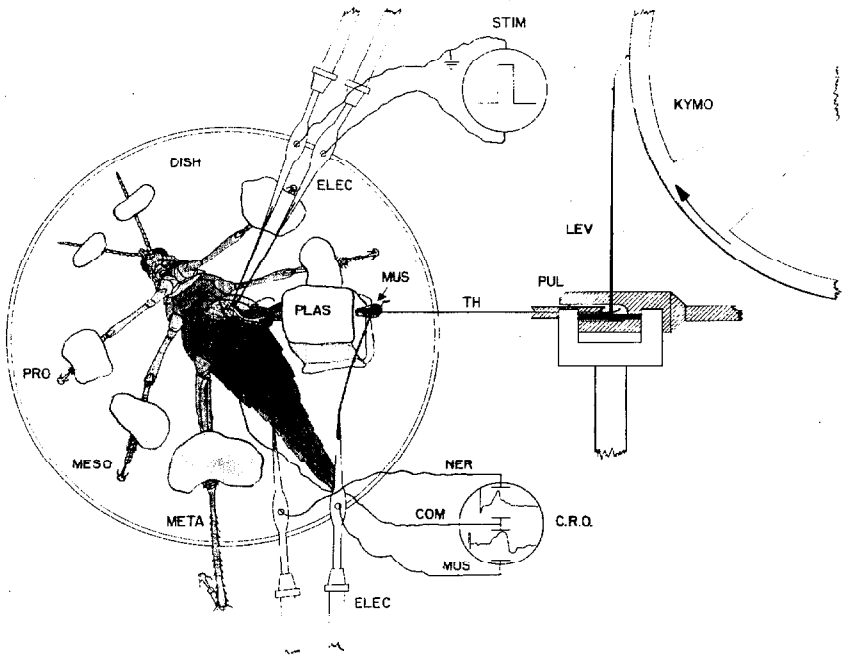

Fig. 1. Romalea microptera. Myo-neural preparation of the femoral muscles of the metathoracic limb with stimulating and recording electrodes in position. The muscular apodeme is attached to an isometric lever via a pulley and inscribes responses on a smoked drum. COM - common electrode on thoracic cotton pad; CRO - double beam oscilloscope registering nerve and muscle potentials; DISH - shallow petri dish; ELEC - micro-manipulated stimulating electrodes (upper) and recording electrodes (lower); KYMO - kymograph drum; LEV - isometric lever; MESO - meso-thoracic limb; META - metathoracic limb; MUS - (upper) ligated end of femoral muscle, (lower) connection from electrode on muscle; NER - connection from electrode on nerve; PLAS - plasticine cube fixing femur; PRO - prothoracic limb; PUL - pulley; STIM - square wave stimulator; TH - thread from muscle to lever.

> The chemicals are dissolved in the order given except that $NaHCO_3$ is dissolved in ca. 500 ml. water and then slowly added to the rest of the solution. This prevents the precipitation of Ca^{++} as $CaCO_3$. Stock solution may be made up without the dextrose which is added just before use.

The use of glucose in the solution is largely responsible for recovery of moribund animals.

The thoracic air sacs and trachea may now be seen bulging rhythmically as the animal breathes. For good survival of the preparation it is essential not to break the trachea to the limb muscles. It is possible to pull the large air sacs aside and lay them on the exoskeleton. They adhere in this position and the air circulation is not seriously impaired. Below these air sacs the main tracheal trunk to the metathoracic limb is seen. Lying almost directly below it and extending obliquely across the thorax from the metathoracic ganglion to the coxa, is the nerve trunk which supplies many of the coxal muscles and all the muscles and sensory structures of the leg peripheral to its base. It is the only nerve which enters the limb and is easily identified in the thorax by its posterior origin from the ganglion and large diameter. In all except the reflex preparation, this nerve must be severed close to the ganglion and it is at this moment that the greatest danger of autotomy occurs.

The nerve trunk is invested by a loose, yellow, spongy sheath below which lies the true epineurium which is transparent and very tough. The outer sheath has to be slightly damaged when the three branches to the coxal muscles are severed. Muscles in the lower part of the thorax may now be removed without damage to the main tracheal vessel. Severed trachea may be prevented from leaking by pinching their ends firmly with forceps. Now the nerve trunk and

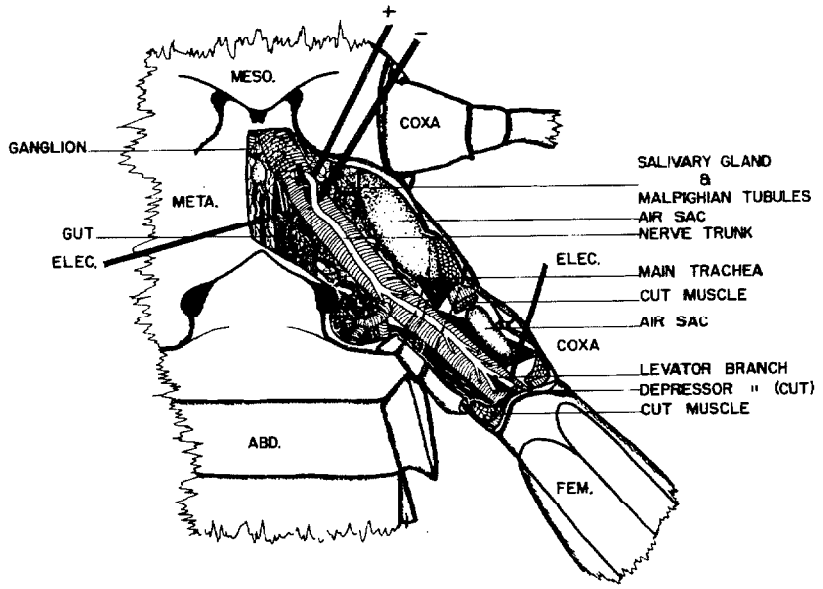

Fig. 2. Dissected metathorax of Romalea microptera. Right half of thorax from ventral aspect with electrodes in position for stimulating and recording nerve potentials. ABD - first abdominal segment; ELEC - (left) common electrode in position on cotton pad (not shown), (right) recording electrode on nerve to femoral muscle; FEM - base of femur of metathoracic limb; MESO - caudal end of mesothorax; META - incised strenite of metathorax; - and +: stimulating electrodes near cut end of nerve trunk.

main trachea are exposed as far as the coxa. If at this stage respiratory movements are too violent, the oesophageal commissures on either side of the neck are severed. This usually reduces respiration sufficiently. If further reduction is required the last three nerves which run posteriorly from the metathoracic ganglion may be cut. The metathoracic ganglion is really a compound structure of metathoracic and first abdominal ganglion and these three nerves are the motor nerves to respiratory muscles in the first abdominal segment. Even greater reduction of respiration can be procured by cutting the ventral nerve cord posterior to the metathoracic ganglion. This is seldom necessary. In most preparations all the homolateral nerves of the metathoracic ganglion were cut to eliminate untoward muscular activity.

In earlier preparations no further operation was performed on the thorax or leg base. The muscles in the femur which were not required for the experiment were plucked out at the femoro-tibial articulation. This procedure is not entirely successful with the metathoracic limb as there is damage to the tracheal system and the apodeme often breaks so that considerable portions of muscle remain and are frequently active. For this reason a method was devised for isolating the unwanted muscles by severing the relevant nerve branch in the distal end of the coxa.

The chitin bridge on the median ventral aspect of the coxal articulation is removed together with the attached muscles. The ventral surface of the coxa is incised with two longitudinal cuts at the boundaries of its internal muscles and the cuts extended peripherally to the femoral boundary. Removing the flap of chitin so formed gives access to the coxa and trochantin. This is the most delicate of all the operations as damage to the nerve and trachea can very easily

result. If the coxal muscles are cut while gaining access to the coxa it becomes impossible to free them cleanly at their origins and insertions, with the result that locating and severing one of the nerve branches to the femoral muscles is not possible. After removing the coxal muscles and their nerve supply, the main nerve is seen to split into two branches of unequal size. The larger of the two branches (Fig. 3. Main $Nerve_1$) innervates the levator tibia, depressor pretarsus and both tarsal muscles and sense organs. The smaller branch (Fig. 3, before split of Main Nerves 2 and 3) innervates the depressor tibia and sensory structures in the femur. After raising one of these branches on a micromanipulated hook it may be easily severed, leaving the other branch intact. Thus either the levator or depressor tibia muscles may be left with intact innervation. If Main $Nerve_1$ is severed, the depressor will be the only muscle to respond. If the other branch is severed, the levator tibia together with the tarsal muscles will remain innervated. The next step is to isolate the apodeme of either the levator or depressor at the femoro-tibial articulation for recording of contraction. Operative procedure differs only slightly depending on whether the depressor or levator is to be prepared. In either case the ventral articular membrane is removed, exposing the levator apodeme where it attaches to the tibia. If this muscle is to be studied, its apodeme is ligated with a fine thread and its attachment to the tibia severed. If the depressor is required, the levator apodeme is simply severed and allowed to retract into the body of the femur. Below the levator apodeme lie air sacs, trachea and two large nerves, all of which are severed. A fine transparent apodeme extends from the insertion of the depressor pretarsus

high up in the femur (Fig. 3, apodeme (upper)) through tibia and tarsus and is inserted on the unguitractor plate of the pretarsus. With either preparation this apodeme is grasped firmly and the depressor pretarsus gently pulled out. It almost always comes out in its entirety and without damage to either the innervation or tracheal supply of the main muscles. In the case of the depressor preparation it is not necessary to remove the depressor pretarsus but as a matter of routine this was almost invariably done.

One other structure is always removed from the limb. At first it was thought that this structure was an accessory levator muscle as it is described by Snodgrass (61), but at least in Romalea it is now recognized as some sort of sense organ which probably indicates the position of the tibia with respect to the femur. This organ has a fine apodeme attached to the tibia at the outer edge, close to the depressor tibia attachment (Fig. 3, sense organ). The apodeme can be grasped and the organ removed.

If the levator apodeme had been previously ligated then the depressor apodeme, which is the most dorsal structure in the articulation, is cut and the tibia removed. If a depressor preparation is required, the depressor apodeme is ligated before removal of the tibia. The thread from the ligated muscle is attached to an isometric lever for recording the contractions. Moist cotton pads are maintained over the operated sites and the preparation is ready for placing of recording and stimulating electrodes (Fig. 1).

Stimulating electrodes were fine platinum/iridium alloy wires carried in a Zeiss micromanipulator mounted to the left of the

dissection microscope on which the preparation rested. To the right of the microscope a similar micromanipulator wielded the fine hooked platinum recording electrodes. Stimulating electrodes, about 0.5 mm. apart were placed under the nerve trunk in the thorax, and the nerve was lifted just clear of the underlying fluid and tissue. Occasional lowering prevented its drying out. The negative electrode was always nearest the muscle. Recording electrodes were placed in a variety of positions according to the experiment being performed. In many instances a common electrode was placed on the thoracic cotton pad central to the stimulating electrodes. The electrode recording nerve potentials was placed under the intact nerve branch in the coxa and the nerve lifted to the surface of the fluid. Muscle potentials were recorded by an electrode in the moist tissue of the distal end of the femur (Fig. 1).

The point of attachment of the thread from the apodeme to the lever was less than 1 mm. from the fulcrum. This approximates closely the actual conditions under which the muscle contracts in the intact animal. The lever inscribes muscle contraction on a smoked drum. The arm of the lever was light and rigid and has little tendency to vibrate at its natural frequency.

Stimulating current was supplied by a square-wave electronic oscillator which allowed independent control of recurrence frequency, pulse duration and amplitude. The oscillator has one output terminal grounded and delivers a potential which is negative with respect to ground. In some cases a device was employed which produces two short shocks (0.2 millisec. duration) separated by a variable time interval and recurring at various frequencies. Electrical recording was made

either with a single beam DuMont 247 oscilloscope or a DuMont 279 double beam. With both instruments the sweep was triggered by the stimulus generator. Amplification was obtained with various condenser-coupled, high-gain preamplifiers with differential input. These amplifiers have a linear output from about 1 cycle per second to a few kilocycles per second. Permanent records of oscillograms were made with single frame cameras. For continuous records of moderate duration a Matthews (62) oscillograph was used. Records were made on bromide paper with an optical lever supplying the final stage of amplification.

In cases where reflex activity of the muscles was studied, the preparation was essentially the same as that described above except that the nerve remained attached to the ganglion and the commissures in the neck were never cut. Reflex activity was elicited in various ways. Tapping the cephalad portions of the body elicits levator activity and the caudal portions depressor activity. A draft of air on the head is very satisfactory when high amplification is necessary for examining nerve potentials. Stimulation methods involving touching the animal cause extraneous potential deflections and are not suitable.

In all studies the preparation remained on the microscope stage. The contractions of the muscles could thus be observed as movements of the apodeme. Some contractions are so small that the resistance, damping and inertia of the lever system prevented recording them, and microscopical observation was the only indication of their occurrence.

For study of muscles of the mesothoracic and prothoracic limbs, the operative technique and recording was essentially the same. The nerve trunks to these limbs originate from the meso- and prothoracic ganglia respectively so that obviously meso- or prothoracic sternites

had to be removed. The main nerve does not branch conveniently in the coxa of these limbs but some distance within the femur. In consequence the muscles could not be isolated by severing the relevant nerve branches. The depressor pretarsus was pulled out from the tibial end of the femur and either of the other muscles pulled out likewise or dissected away from the femur. Removal of the muscles by traction is much easier and is more successful with these limbs than with the meta-thoracic limb.

In a few cases the tarsal muscles were studied. The nerve was stimulated either in the thorax or in the femoro-tibial articulation. In the tibio-tarsal articulation either of the muscle apodemes was ligated and the other muscle plucked out. The tarsus was removed and the pretarsal apodeme plus its muscle flags in the tibia was pulled out through the proximal end of the tibia. Although these techniques are rather crude, the tracheal supply remains effective and the action of the experimental muscle and its nerve is not impaired. Muscle potentials were led off either from the two ends of the tibia or from the thoracic cotton pad and the distal end of the tibia.

In an attempt to resolve some of the problems of the distribution of the axons to the muscle fibres, it was desirable to watch the surface muscle fibres of the contracting muscle. To effect this, the inactive muscle together with its chitinous attachment was dissected away. Careful cutting of nerve branches prevented damage to the innervation of the experimental muscle and the preparation responded normally for hours. Observations were made under magnifications of up to 60 X, but it was not possible to decide whether all or only certain muscle fibres were contracting to a given stimulus.

A considerable length of nerve is required to determine conduction velocities and related phenomena. The main nerve trunk to the meta-thoracic limb was dissected free of the leg as far as half way down the tibia. Only the mixed branch which supplies the tarsal muscles was retained (Fig. 3, Main Nerve $_1$). The nerve was placed in the narrow slot formed between two glass plates resting on a third and was moistened with saline. It was stimulated a short distance from the thoracic end and the action potential led off by two electrodes, one at the distal end on a dry portion of the nerve above the surface of the saline so that the actual point from which the potential was led off was the saline surface, and the other about half way along the nerve just below the surface of the saline.

As has been shown by Wiersma (63), it is possible to isolate single motor axons in crustacean limbs by gradually splitting the nerve into finer and finer bundles and rejecting those which do not give the desired response. It was felt that the same procedure might meet with success in the insect. In a few cases it was indeed possible to isolate single axons, but this was after many hours of very trying dissection and neither the preparation nor the investigator were in condition for further experimentation. There are a number of difficulties. The first is the necessity of allowing the animal to breathe while at the same time avoiding even minute movements in the thorax which make dissection virtually impossible. To this end a trough was designed which held a large volume of saline in which the thorax and legs could be submerged while the abdomen projected through a water-tight membrane into the air. This proved very satisfactory, and it was with this apparatus that the few single axons were isolated.

Other difficulties are anatomical. The epineurium is nearly transparent and extremely tough and the greatest difficulty is experienced in removing it without damaging the axons. Once it has been removed the axons are found to be completely transparent, very delicate, of a diameter not exceeding 20 μ and bound together by relatively tougher tracheoles. Thus manipulations have to be performed almost blind. Only very short lengths of axon bundles can be isolated, and the close proximity of the stimulating electrodes to the bundles which are not being tested make it very difficult to prevent cross stimulation. However, the few successes which I have had suggest that with a suitable insect and a nerve with fewer axons and improved instruments, the method may yet prove rewarding.

III. RESULTS.

(i) Anatomy.

The femur of Romalea houses three muscles: depressor tibia, levator tibia and depressor pretarsus. In the terminology of Snodgrass (61) these are levator tibia, depressor tibia and depressor pretarsus, respectively. The largest muscle (depressor tibia of this thesis) is the muscle used by the animal in jumping. It extends the tibia so that tibia and femur come to lie in a straight line. It might better be called the extensor tibia and the smaller antagonistic muscle which folds the tibia against the ventral surface of the femur, the retractor tibia. I shall however continue to use levator and depressor in the opposite sense to that used by Snodgrass.

The femur is about 30 mm. long and about 7 mm. at the widest proximal part. The depressor tibia takes origin along almost the entire

dorso-lateral wall on the outer side of the limb. Bundles of fibres are attached to striations on the chitin and are inserted on a broad, flat apodeme which narrows and thickens towards the tibia and is finally inserted on the dorsal lip of the tibia at the articulation. The levator tibia lies on the inner ventro-lateral aspect, its muscle bundles taking origin on the chitin of these sides and being inserted on an apodeme which is shorter and narrower than that of the depressor. The muscle tapers towards the tibia and is finally attached to the ventral edge of the tibial end. Both muscles have small flags of fibres close to the articulation whose function it is to guide the apodemes in the articulation during contraction. Those of the depressor are short fibres originating on the dorsal aspect of the femur close to the articulation and are inserted on the apodeme so that their action is almost at right angles to it. The levator has two flags which pass on either side of the depressor apodeme and also originate on the dorsal surface of the femur near the articulation. During contraction these fibres pull the levator apodeme towards the centre of the limb (Fig. 3).

The depressor pretarsus lies in the proximal half of the femur. It is much smaller than the other two muscles and is unlike them in that its fibres are almost parallel and all originate from one point on the proximal ventral head of the femur. The fibres are inserted on a very fine apodeme which passes into the tibia where it receives a number of oblique muscle flags before passing on through the tarsus to be finally inserted on the unguitractor plate of the pretarsus. It serves to depress the claws and pulvillus ventrally.

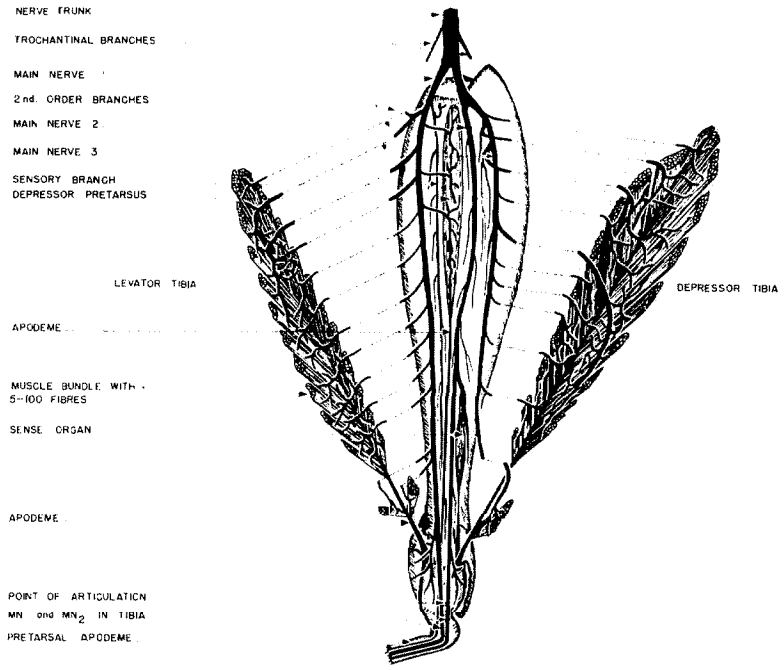

Fig. 3. The dissected femur of the metathoracic limb. Note the recurrent nerve branches and the division of the muscles into bundles. For the sake of clarity the tracheal system has been omitted. 2.5X natural size. Correction: 'MUSCLE BUNDLE WITH 5-100 FIBRES' should read, 'MUSCLE BUNDLE WITH 5-20 FIBRES.'

The depressor and levator of the tarsus are situated in the distal end of the tibia; the depressor originates ventrally and the levator dorsally. Both muscles exhibit a characteristic fan-shaped arrangement of the fibres which are inserted on short apodemes terminating respectively on the ventral and dorsal lips of the proximal tarsal end.

In the first and second limbs, the muscles are arranged in essentially the same way. Their respective sizes are altered, however. In these limbs which are used in dragging the body forward, instead of pushing it as does the third limb, the levator muscles are the largest and the depressors tibiae considerably reduced. The fibres of the muscles tend to be arranged more nearly parallel than those of the third limb but still exhibit the same general character.

It is necessary to appreciate the geometry and disposition of the muscle bundles in the femoral muscles in order to interpret the electrical events. Figs. 3 and 4 indicate the way in which the muscles are broken up into discrete bundles. In the levator each bundle has some 5-20 fibres. In the depressor the bundles are larger with about 15-80 fibres. Although these bundles splay out in three directions from the apodeme, the muscle fibres in each bundle lie essentially parallel to each other. The bundles are also arranged in an overlapping fashion so that origins and insertions occur serially down the length of the muscle. The muscle fibres vary in length, as do the bundles, from ca. 4-10 mm. Each fibre is striated and is bounded by a sarcolemma. The myofibrils are arranged more or less radially about the longitudinal axis of the fibres. Ovoid nuclei are numerous at the periphery and occasionally are found between the fibrils. This disposition of the nuclei is at variance with that described for tubular muscle in

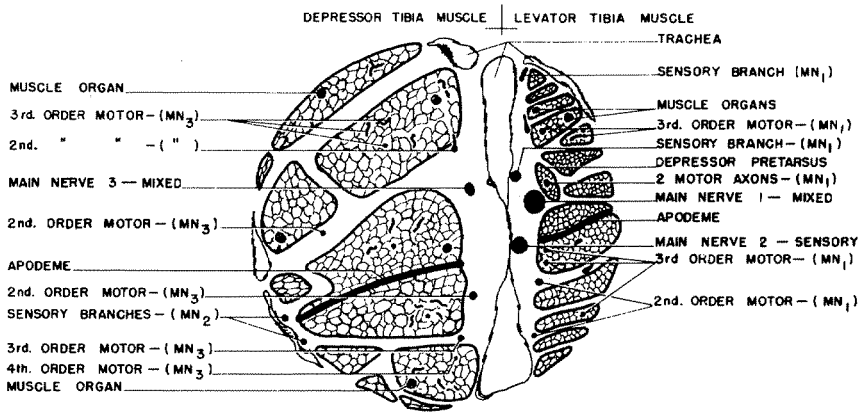

Fig. 4. Diagrammatic cross-section at approximately the mid-point of the femur. MN_{1-3} in parentheses indicate the parent nerves from which the branches arise. The organs of dubious nature between the muscle fibres have been designated 'Muscle Organs'. The external skeleton was removed from the limb before sectioning. For this reason the muscle bundles are separated more than in the intact limb. Tracheal branches have been omitted. About 10X natural size.

many insects. Trachea enmesh each fibre and penetrate between the fibrils.

Only one nerve trunk enters the limb. Having branched to muscles and sensory structures in the coxa and trochantin, it enters the femur as two thick branches, one smaller than the other. Figs. 3, 4 and 5 indicate the detailed innervation. These maps were made in part by microdissection but largely as reconstructions from the serial sections. The three main nerves proceed down the length of the femur. Of these, Main Nerve$_2$ (MN_2) is purely sensory and concerns us little (Fig. 7). Towards the distal end of the femur at least one half of its axons innervate the large sense organ whose apodeme attaches to the tibia (Figs. 3 and 5). Sections show clearly that this organ is not muscular but is probably some form of stretch receptor. In one instance I managed to record from the isolated nerve to the organ and on extending the tibia, bursts of activity were observed in the sensory nerve. Unfortunately the stain used in the sections was not suitable for observing the detailed structure of this organ. MN_2 proceeds into the tibia and the tarsus where it receives axons from numerous sensory structures.

The largest branch, Main Nerve $_1$ (MN_1), innervates both sensory and motor structures. The motor axons for the levator tibia, depressor pretarsus, levator and depressor tarsus, are carried by it. Its largest axons, of which there are about 30, vary from 8-16 μ in diameter (Fig. 7). In addition, it receives axons from various cuticular, tracheal and intramuscular sensory structures. The motor supply to the levator tibia is our chief concern and it is shown in Figs. 3 and 5. For the sake of convenience in classifying data from

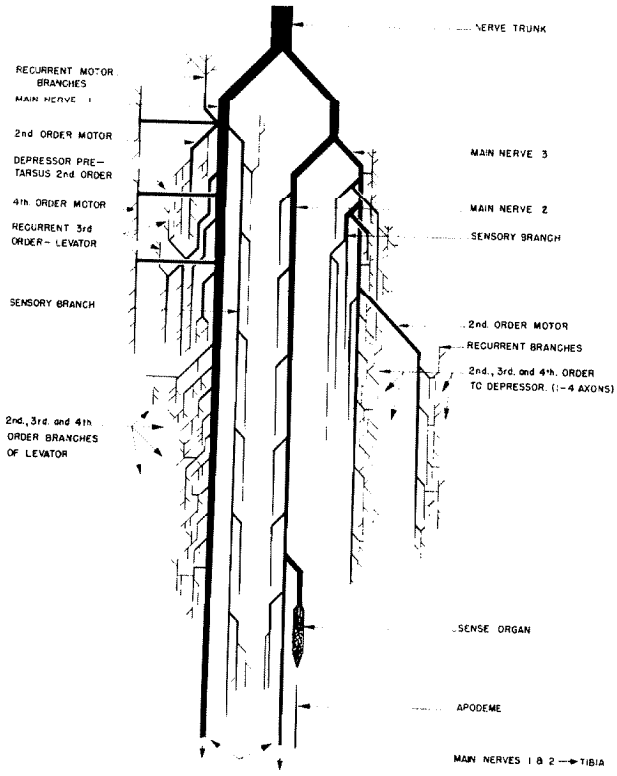

Fig. 5. The nerves of the metathoracic femur. The finer sensory branches have been omitted. Depressor pretarsus innervation on the extreme left. Levator tibia innervation on left of Main Nerve$_1$ and depressor tibia innervation on extreme right. Main Nerves$_{1 \text{ and } 2}$ continue into the tibia.

the serial sections, the nerve branches have been divided into a number of rather ill-defined, arbitrary categories (Fig. 6). The largest branches arising from MN1 and carrying axons, the largest of which vary between 4 and 8 μ in diameter, are designated 2nd. order branches. Third order branches carry axons, the largest of which are from 1.5-4 μ in diameter, and represent the distribution to the individual muscle bundles (Fig. 7). These branches usually proceed to the centre of each bundle where they branch into 4th. (axons less than 1.5 μ diameter) and 5th. order branches which supply the individual fibres. Due to the tortuous path taken by many branches, the nerve is cut obliquely and it is often not possible to determine the number of axons. The matter is further complicated by the small size of the axons and the variable number of sensory axons carried in the branches. Difficulties are readily appreciated when one realizes that in the sections the fine axons are actually small cylinders about 1.5 μ in diameter and 8 μ long. If these cylinders lie somewhat obliquely, then it is not possible to distinguish any detail. Shrinkage of the axons within the epineurium leaves spaces which can easily be mistaken for axons. Fine intra-nervous tracheoles are also easily confused with axons. Motor axons can be distinguished from sensory axons by the much greater thickness of their sheathes and generally by their larger size. In some of the photographs in Fig. 7 axon sheathes appear thicker than they are in reality because of the slight oblique tilt of the axon cylinders and the depth of focus of the microscope.

Invariably in 2nd. order branches in the levator, 5 large, well-defined axons can be distinguished. In the vast majority an additional

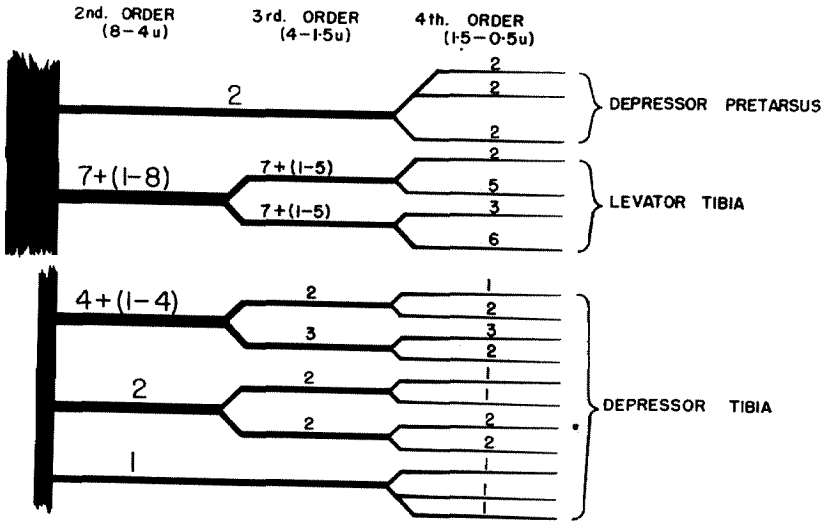

Fig. 6. Innervation scheme of the three femoral muscles and variation of the depressor tibia innervation. The figures immediately above each nerve branch represent the number of motor axons observed. Those figures in parentheses represent the observed variation in the number of sensory axons. The figures for axon number in the 4th. order nerves, especially those for the levator tibia, represent only the lower limits. Of the four large axons represented in the uppermost branch to the depressor tibia, at least one is probably sensory. 5th. order and sensory branches have been omitted.

2 smaller axons are visible, making a total of 7 (Fig. 7). In numerous branches, 7 well-defined axons and up to 8 thin-walled, less defined axons can be seen. These latter I believe to be sensory, due to their capricious occurrence and because they leave the main nerve in branches that do not appear to innervate the muscle fibres.

In 3rd. order branches accurate determination of axon numbers is more difficult. 5 or 6 axons are usually seen and in many cases a seventh but on the other hand, smaller numbers can often be counted. I believe this to be due to imperfections in staining and fixing the material which renders some of the finer axons invisible. With the 4th. order branches only rarely can numbers be determined and when they can it is doubtful that all the axons can be distinguished. From 2-6 axons have been seen and these approach the minimum size of optical resolution. A seventh is probably present. Finally the number of axons in terminal branches can never be determined although more than one axon is certainly present. The general course of these terminal branches and their contiguity to the sarcolemma can be seen but their penetration of this membrane has not been observed. Thus an important question, and one of the most difficult to answer either anatomically or physiologically, remains open. That is, does a branch of each of the six or seven motor axons repeatedly innervate each muscle fibre or are there categories of muscle fibres each innervated by only one or several of the axons? The latter seems improbable for a number of reasons only one of which will be stated now. From the histological data each muscle bundle appears to receive seven axons and yet in the smallest bundles there are less than seven muscle fibres. For this and other reasons the most probable situation in the levator is multiterminal,

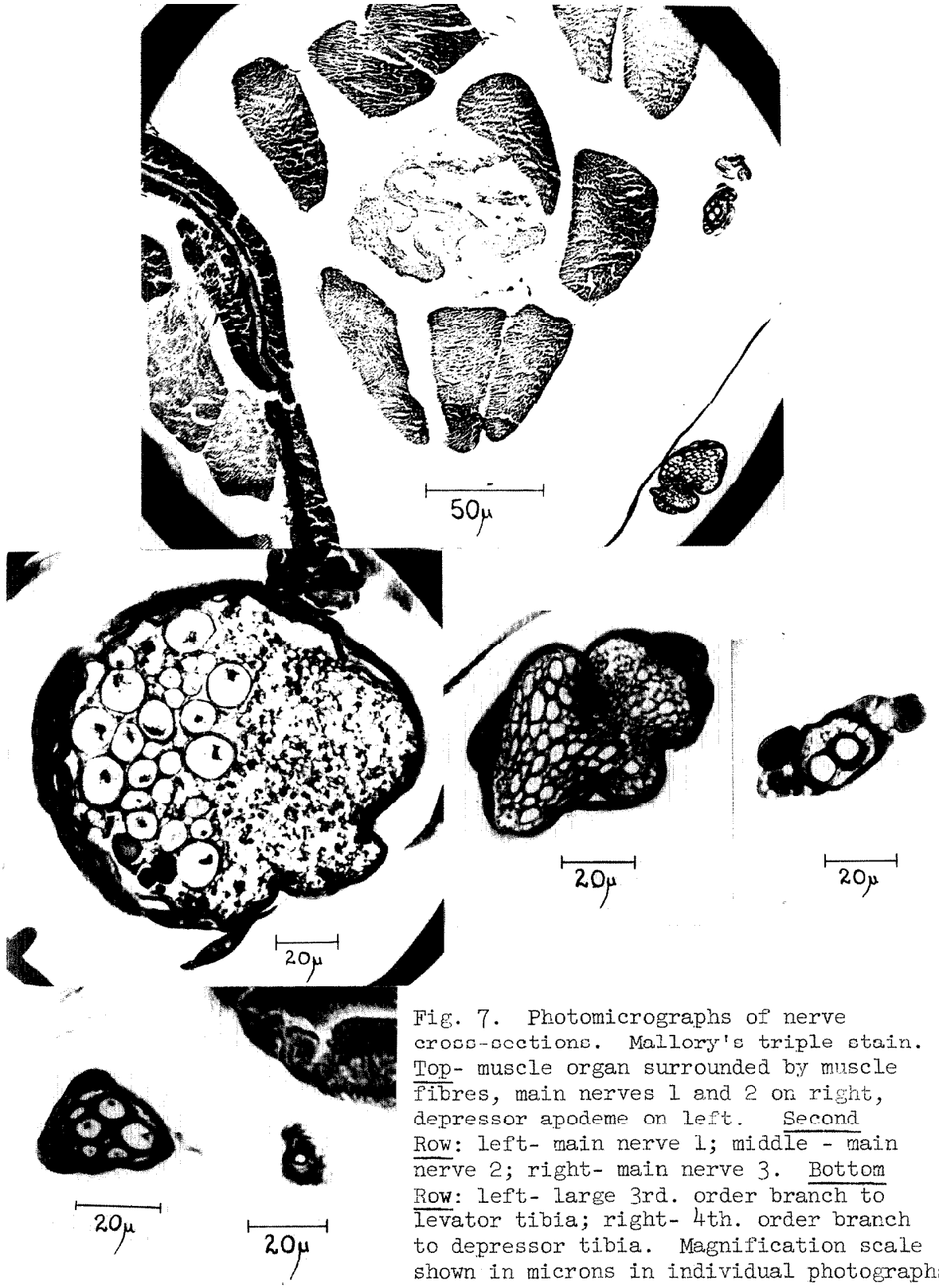

Fig. 7. Photomicrographs of nerve cross-sections. Mallory's triple stain. Top- muscle organ surrounded by muscle fibres, main nerves 1 and 2 on right, depressor apodeme on left. Second Row: left- main nerve 1; middle - main nerve 2; right- main nerve 3. Bottom Row: left- large 3rd. order branch to levator tibia; right- 4th. order branch to depressor tibia. Magnification scale shown in microns in individual photographs.

sexoplotomic or septuplotomic innervation of all muscle fibres.

Individual muscle fibres have been examined in every section over their entire length. Counts were made of the number of times that it could be decided with certainty that the fibres were innervated. Of four levator fibres followed serially, the innervation counts were 21, 18, 14, and 12. This represents the lower limits of the number of innervation sites. In the depressor the number of sites is less; counts were 6 and 8 in the two fibres traced.

The depressor pretarsus is innervated by three 2nd. order bundles from MN_1. In all cases there are two large axons of equal size which run in the muscle and give off very fine branches to the fibres. The 3rd. order category of branches does not exist. In many instances it appears that each axon innervates the fibres of one half of the muscle but this cannot be considered as established.

Main Nerve $_3$ (MN_3)is also a mixed nerve (Fig. 7). Its sensory branches come from tracheal and muscular sensory structures and from the outer dorsal aspect of the chitin. Throughout its length there are two large, well-defined axons and a third which is considerably smaller and has a thinner sheath. In the most proximal portion, a 4th. thin-sheathed, large axon is present, but it is probably sensory. The number of sensory axons is restricted to 25 or less and their diameter is very small. They are thin-sheathed and often poorly defined in the sections. Branches of the two larger axons can be seen leaving the main nerve to form 2nd. order branches either with or without a sensory component. However, most of the branches which leave the main nerve are extremely fine and carry either one or two axons. They again branch only when they have reached the muscle fibres. Hence 2nd. order branches are

frequently missing. The largest 2nd. order branch may be seen diagrammatically in Fig. 5, and crossing to the right of the muscle in Fig. 3. Axons of the 2nd. and 3rd. order branches are smaller than those to the levator and hence, although there are fewer of them, the data are not as conclusive as for the levator.

Three different arrangements of the axons may be observed in Fig. 6. In the cases where the 3rd. order branches carry 2 or 3 axons, the 4th. order branches have either 1, 2 or 3 although this third axon has been observed only once or twice. In the commonest case, where the branches leaving the main nerve have but one axon, the terminal branches must also have but one. Single, very fine axons can often be observed in the muscle (Fig. 7). In this case the muscle fibres at that particular level in the bundle must obviously share the same single innervation. At a different level they receive 3rd. order branches with two axons, and both single- and double-axon 4th. order branches can be observed. The muscle fibres might therefore be innervated by the two axons in a number of different combinations. Again the fate of the terminal branches once they reach the sarcolemma is uncertain. For the depressor tibia then, there appears to be a multiterminal, single or double innervation.

From the point of view of physiological interpretation, the nature of the nerve branching is important. In the levator, each branch innervating a muscle bundles carries the same number of motor axons. Its branches proceed not only distally in the muscle fibres but also proximally (Fig. 5). These recurrent branches are often quite long but never as long as those proceeding peripherally. Considering the length of the branches and the number of sites of innervation

of each fibre, there must be considerable overlap of the innervation, forming a feltwork within or on the fibres. If the muscle bundles are taken as the unit of structure, one can say that in the levator and the depressor pretarsus, each bundle receives an identical innervation. In the depressor tibia this may not be so; some bundles may receive branches of one axon, some branches of the other axon and some branches of both axons.

The second point that must be stressed is the site of innervation of the individual muscle fibres. In many crustacean muscles it is believed that nerve terminations occur only on one side of the muscle fibres throughout the muscle. With the grasshopper this is not so. The finest visible branches may innervate any side of the fibres. However, within a bundle, the vast majority of terminations are always towards the centre, so that a cylindrical structure is formed with the innervated sides of the fibres towards the centre of the cylinder. Mention has already been made of peculiar cells running parallel to the fibres in both muscles. These take the form of a cylinder constricted at regular intervals. In the non-constricted part they stain much as muscle tissue but have a more granular appearance. Quite suddenly the substance becomes divided into strands of coarsely granular tissue with very different staining properties. A few of these "muscle organs" occur in each muscle and continue throughout its entire length. The granular portion of one is shown in Fig. 7 but in photographic reproduction detail of the more delicate tissue has been lost. Their function is not known and a detailed study has not been attempted.

(ii) Physiology.

a. Frequency response.

The first general picture that is required is one of the overall frequency response of the muscle. It is not necessary to enter into details for each muscle as the responses are very similar, differing only in the speed, strength and fusion frequency of the contraction. As will be shown below, the muscular system is complicated by the fact that there is a multiple innervation. In this initial survey then, a stimulus was employed which was strong enough to stimulate all the axons. In later sections the response of the various axon-motor systems to frequency of stimulation will be considered in more detail. The following data will pertain only to the maximal response at each frequency.

Indirect stimulation of the muscles in any of the three limbs, and isometric recording from the muscle shows that a single shock invariably produces a brief twitch. The duration of the twitch in the fresh, unfatigued muscle varies between 30 and 60 millisec (Fig. 8A). With repetitive shocks up to about 10 per second, the responses remain as discrete twitches, relaxing to the baseline after each shock (Fig. 8B). At frequencies higher than this the muscle does not relax to the baseline between shocks (Fig. 8C) and the contractions increase both in rate and in tension (Fig. 8, G and H). The frequency at which the contractions fuse completely to form a smooth tetanus varies for the different muscles. The large depressor tibia of the third limb has the highest fusion frequency at ca. 80 shocks per second. The metathoracic levator tibia shows complete fusion at 70 per sec. In Fig. 8E, a record from this muscle at 60 per sec., the incomplete nature of the tetanus

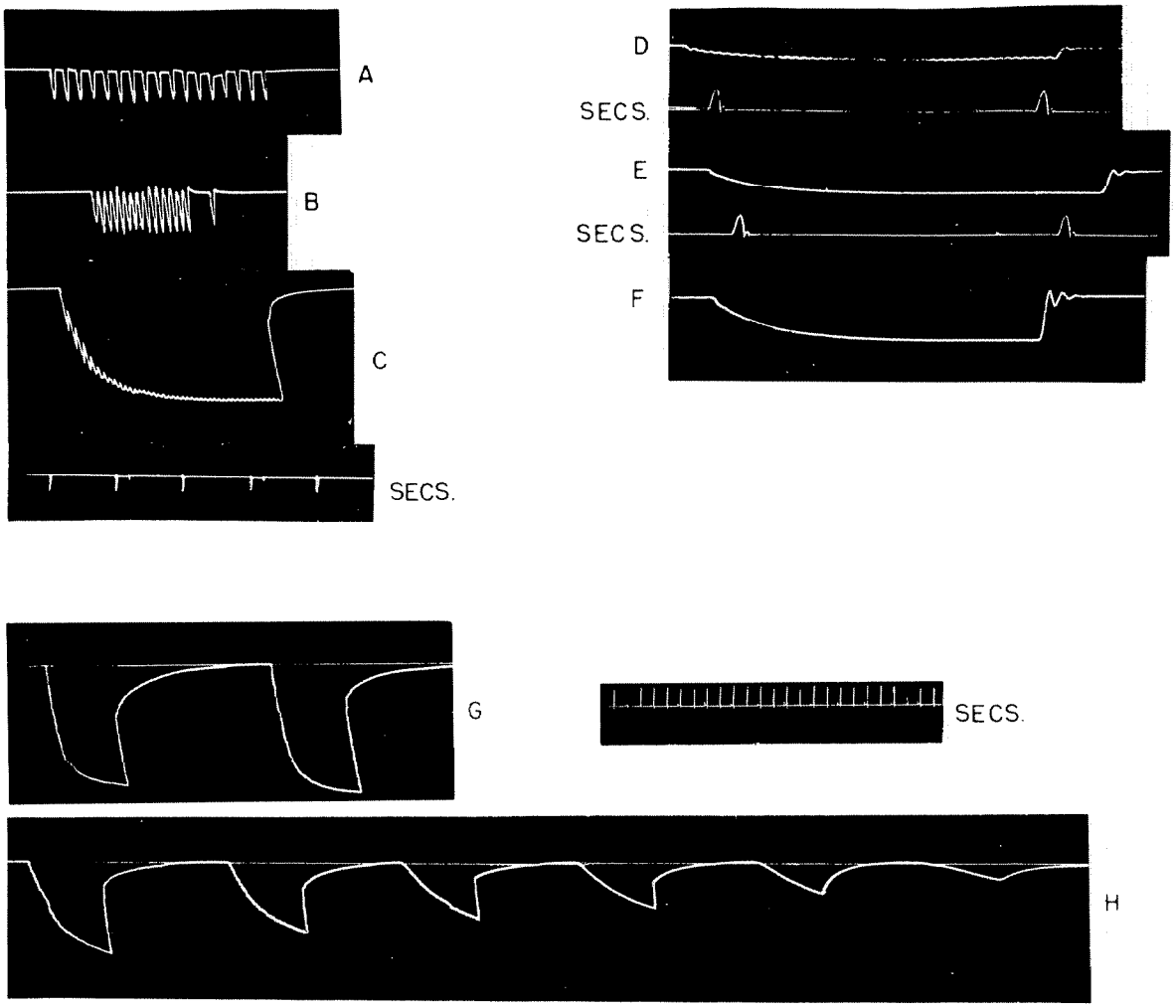

Fig. 8. Frequency responses of the limb muscles. All records were made isometrically with the lever attached to the apodemes. Time records in each case are in seconds. A, B and C - Metathoracic depressor tibia at 5, 10 and 15 shocks per second. D, E and F - Metathoracic levator tibia, (reading from top) 40, 60 and 70 shocks per second. Note that E shows incomplete tetanus. G and H - Mesothoracic levator tibia, (reading from left to right) in G, 300 and 200 shocks per second; in H, 80, 60, 50, 40, 30 and 20 shocks per second.

can just be discerned. Fusion frequency for the other muscles varies between 50 and 70 per sec. In all the muscles, both maximum rate of development and maximum tension attained, occur at approximately the same frequency which is somewhat higher than their fusion frequencies. The depressor tibia of the third limb attains its maximum at about 90 per sec. whereas the smaller depressor tibia muscle of the first limb, which is not specialized for leaping, reaches a maximum value at 160 per sec. At frequencies higher than these and up to 500 per sec. there is no increase in speed or tension of the contraction. At high frequency, fatigue occurs very rapidly. Relaxation may require 7 or 8 seconds for completion (Fig. 8G).

Estimates have been made for the tensions which the muscles of the third limb can develop. These compare fairly closely with the capabilities of vertebrate skeletal muscle. The estimates range between 6 and 9 Kg./sq. cm. of cross-sectional area. In the case of these muscles which taper sharply, an average cross-sectional area was estimated.

b. Mechanical responses to variations in intensity.

Stimulated by the work which has shown that crustacean muscles have multiple motor innervation and by the observations of Pringle (23) on the two contraction types in a muscle of the cockroach, studies were initiated to test the occurrence of similar multiple innervation in Romalea. The simplest, although not the most satisfactory method of testing such a postulate, is to stimulate the nerve with various strengths of shock in order to ascertain whether the axons may be differentiated by their thresholds. The possible occurrence of inhibitory innervation must not be overlooked. It is essential to

guard against "direct" stimulation of the muscle. This has been checked in many preparations by crushing the nerve distal to the stimulating electrodes. Mechanical and electrical activity in the muscle is completely abolished even at frequencies and current strengths much greater than are ever used for nerve stimulation. A second precaution must be taken. If the shocks are of long duration and of fairly high intensity there may be repetitive discharge of the axons. To this end pulse duration was always kept between 0.1 and 0.2 millisec. In a section to be discussed later checks were made for repetitive discharge which was absent even with pulse durations of 5 millisec. and high current strength.

With the levator tibia preparation of the third limb, as the strength of nerve stimulation is gradually increased from a sub-threshold value to values resulting in maximal response, the muscle responds in a stepwise fashion (Fig. 9). This same phenomenon may be seen with all the muscles except the metathoracic depressor tibia. In the latter only one contraction type can be distinguished. Due to the closeness of the thresholds for the various contraction types and to their variability, it is difficult to secure good mechanical records. The contraction types are readily distinguished by microscopical observation of the apodemal end of the muscles. With frequencies of 5-10 per sec., the contraction types are most readily seen, but at such low frequencies the first response is so small that it is not possible to record it mechanically with the relatively heavy lever made necessary by the strong maximal contraction. However, if its size is increased by a higher frequency of stimulation it then becomes practically impossible to separate the larger contraction types.

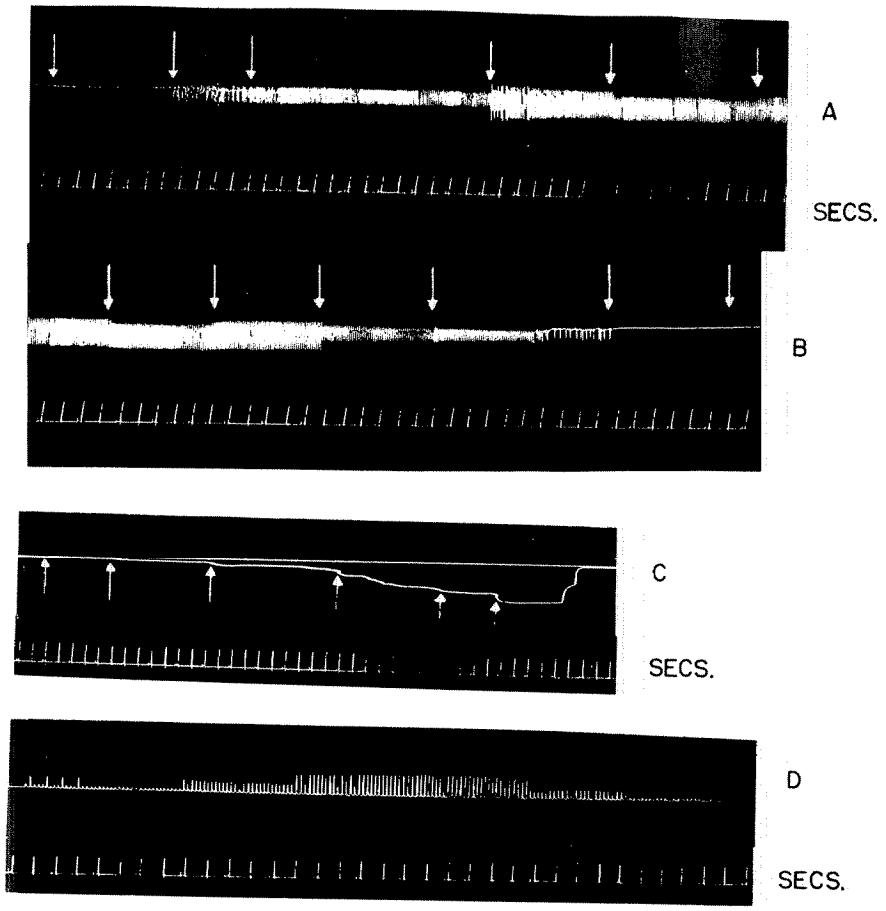

Fig. 9. Muscular response of the metathoracic levator tibia to variations in the stimulus strength. Records A-C are isometric. Record D is isotonic. A - ascending stimulus strength at 10 shocks per sec. From right, arrows indicate onset of contraction types 2, mixture of 3 and 4, 4, 5 (at first erratic), 6 and finally 5 again as intensity was decreased. B - part of same record as A but descending stimulus strength. From right, arrows indicate contraction types 6, 5, 4, 3 (erratic towards end), 2 and finally no response. C - from the same preparation as A and B. Frequency 100 per sec. and intensity ascending. From right, arrows indicate types 1, 2, 3 (erratic at end), 4 (with 5 coming in erratically), 5 and finally 6. The slow relaxation was interrupted by stopping the drum. D - isotonic record. Frequency 5 per sec. Intensity first ascending and then descending. Response 2 was too small to record. From right, first response is type 3 with four twitches of type 4 superimposed on it. Note that this does not increase the height of response 3. Next, type 4 appears and then 5, somewhat erratically. Contraction 6 can be seen as a very small plateau followed again by 5 as the stimulus strength was decreased. This is followed again by responses 4 and 3. In all cases the time record is in seconds.

From Fig. 9 it is evident that the steps may be obtained with increasing intensity and again in reverse order with decreasing intensity. At low frequencies each step is clear-cut and there is no gradation from one to another. At higher frequencies the records become obscured for a number of reasons. These are most clearly illustrated when dealing with the electrical events but an outline must be given here. At moderate frequencies the threshold values become more variable and lie closer together. At just threshold intensity for each contraction type, responses do not bear a 1:1 relation to the stimuli. As threshold is approached, a certain axon will respond to, say, every third shock, then every second and finally every one provided the frequency is moderate. An axon with a lower threshold will respond, at this intensity, to every shock while the one with the higher threshold is responding to only every other shock. Thus in effect we get a graded transition from one contraction type to the next due to this alternation of response, and the true contraction types are obscured (Fig. 9C).

Records of the muscle potentials of the metathoracic levator tibia, made at 1 shock per sec., showed that the first potential to appear was very small and apparently was not accompanied by a visible contraction. Assuming that this might be a system which responded actively only to higher frequencies, careful searches were made at frequencies around 50 per sec. With oscilloscope records of the active muscle as a check, it was observed that an apparently slow contraction of small strength and rate, does indeed occur (Figs. 9C and 10A). This has been shown only for the levators of the meso- and metathoracic limbs. The contraction differs from the other types in that it does

not appear at low stimulus frequency and the first observable response appears smooth. It has not been possible to determine the fusion frequency.

To recapitulate, as the stimulus intensity is increased, first a minute slow contraction is evoked and is followed by four fast contraction types. There is one more contraction type; the 6th. In Fig. 9A and B, it may be seen that with the highest amplitude of stimulation there is a dip in the baseline of the clonic response as though the whole response had been shifted down some little distance. It is not possible to determine the exact nature of this contraction but it appears to be smooth even at 10 shocks per sec. At 5 shocks per second it appears to be clonic (Fig. 9D). At high frequency it is difficult to distinguish this contraction from the preceding large 5th. contraction. However, if the action potentials are also observed its presence can be correlated with an increase in the tension (Fig. 9C). The tetanic nature of the contraction even at low frequency might be due either to a slow relaxation rate or, if the axon has a very small diameter, temporal spread of the excitation in the muscle might cause numerous individual twitches to be out of phase thus resulting in what appears to be a smooth contraction.

We may summarize the position as follows. From mechanical evidence there appear to be six contraction types in the meso- and metathoracic levator tibia muscles and at least four in the prothoracic levator and pro- and mesothoracic depressors. Gradation from one response to another does not occur and the strength of each contraction type differs from that of its fellows. In the meso- and metathoracic limbs, the 1st. levator contraction is smaller than the others and incomplete

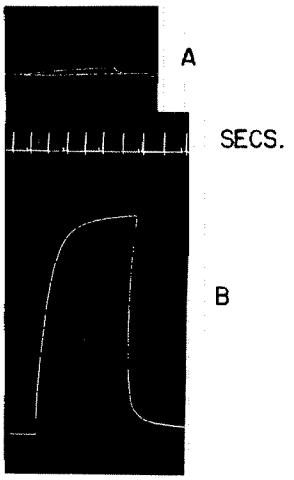

Fig. 10. Isotonic responses of the metathoracic levator tibia at 50 shocks per sec. A - Contraction 1 (verified by check of muscle potentials). Time record in secs. B - Maximum contraction at the same frequency for comparison.

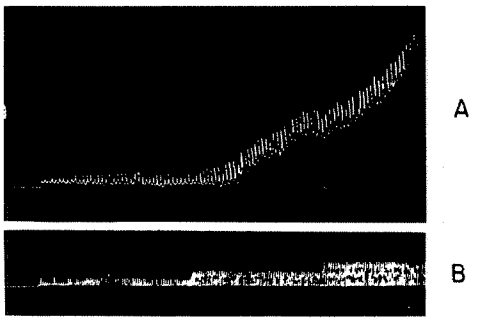

Fig. 11. Auxotonic and isotonic responses of the metathoracic levator tibia. A - Auxotonically recorded contraction at 5 shocks per sec. (lever attached to tibia). B - Isotonic record from same preparation at 10 shocks per sec. (lever attached to apodeme). The same isotonic lever was used in both cases.

tetanus has not been observed. In the metathoracic levator, the 6th. contraction responds to low frequency with a smooth contraction and develops considerable tension at higher frequencies. Contraction types 1 and 6 have not been found in the levator or depressor in the first limb nor in the depressor of the second or third limbs. In the large specialized 'jump' muscle, the depressor tibia of the third limb, only one fast contraction type can be mechanically distinguished.

It is possible to account for these contraction types in terms of what is known of arthropod neuro-muscular mechanisms, by either of two postulates. The one is that there is but one or a couple of axons innervating the muscle fibres. On increasing the strength of stimulus, first skipping of impulses occurs and then repetitive discharge by progressive steps. This could result in a series of steps in the contraction. From consideration of mechanical response to ascending intensity at frequencies of about 1 per sec. this appears improbable. There is no skipping of stimuli and yet contraction steps are evident from the beginning. The highest twitches are of only slightly longer duration than the smallest ones. This could hardly be expected if they were evoked by two or three discharges close together. As will be shown below, the nerve has a long relative refractory period which would prevent repetitive discharges occurring very close together. As was pointed out in an earlier section, the conditions of stimulation are most unfavourable to repetitive discharge. It has, in fact, not been possible to elicit repetitive discharge from the nerve except by damaging it irretrievably.

The second postulate is that for each of the contraction types observed, there are a number of axons each innervating part or all of the muscle.

From the histological data this appears very probable although the exact axon distribution is not known. Multiterminal innervation certainly occurs and septuplotomic innervation of each muscle fibre seems probable. The data from the mechanical recordings conform to the expectations of the second postulate. Only six contraction types have been observed although the histological data indicate that a 7th. axon is present. If this 7th. is a motor axon it is quite possible that because of its threshold relationship to the other axons, its activity could have remained obscured. On the other hand, it might be an inhibitory axon which again could easily have been overlooked.

In order to confirm and extend the observations on the contraction types, examination of the action potentials from the muscles was undertaken. Simultaneous recordings were also made from the nerve but have not proved very informative because of the large numbers of both motor and sensory axons present. With combined recording it was possible to correlate mechanical and electrical activity in the meta-thoracic levator tibia and to eliminate any doubt that the six con-traction types are due to multiple innervation. No sign of a 7th. muscle potential or of inhibition has been found in this muscle.

Before presenting the muscle potential data, I would like to digress in order to draw attention to a deceptive method of recording muscular data. This is important as it has been used by a number of workers in the field and leads to incorrect interpretation of such data. The method in question is that of affixing a lever to the tibia in order to record contractions of one of the tibial muscles. This leaves the femoro-tibia articulation in the system. The articulation, at least in the grasshoppers and locusts, is so constructed that the

leverage and mechanical advantage of the muscle changes according to the position of the tibia with respect to the femur. Consider the levator muscle. It has the least mechanical advantage when the tibia is either fully extended or fully retracted and the advantage is maximal when the tibia is at approximately right angles to the femur. Two responses from the same preparation are shown in Fig. 11. The upper record was made with an isotonic lever attached to the tibia. The other was made with the same isotonic lever attached directly to the apodeme of the muscle. The conclusions are self-evident. Similar results can be obtained with isometric levers although the differences are not as pronounced. Not only is the tension curve misrepresented even after corrections have been made for the leverage factors, but detail of small contractions is lost.

c. Correlation of the electrical and mechanical events.

The electrical and mechanical responses from a single muscle preparation were observed. In many of the experiments mechanical records were not made. Instead, the muscle end was observed microscopically and the potentials characteristic for each of the contraction types was photographed. The stimulus was allowed to trigger the oscilloscope sweep and the frequency was kept low so that photographs of single potentials could be made with ease.

After preliminary experiments with the recording electrodes disposed in various positions, the procedure was standardized. One electrode was placed in the moist part of the cut distal end of the femur. The other occupied a position on the saturated cotton pad on the thorax, central to the stimulating electrodes. In the levator tibia of the third limb, which was the object of the majority of the

studies, six characteristic action potentials may be observed. The potentials have a characteristic shape and with practice can be recognized one from another. In the initial parts of this section the sign and nature of the potentials will not be discussed.

At stimulus frequencies of 1 per sec., as the amplitude of stimulation is increased from below threshold, the first potential to appear is very small and evokes no mechanical response. It corresponds to the 1st. 'slow' contraction which can be demonstrated at higher frequencies. Potentials 2, 3, 4 and 5 are each of diagnostic shape and each one is larger than the one preceding it. The 6th. potential is small and occurs as a hump on the declining phase of the 5th. Fig. 12 represents the potentials and the corresponding contraction steps. Mechanical response 1 was made at 50 shocks per sec. with a very light isotonic lever. Responses 2-6 were made with a heavier isometric lever at 5 shocks per second. In Fig. 13 (bottom) the potentials have been enlarged and superimposed in order to show more clearly the differences between them.

The photographic records of the first 3 potentials do not differ greatly from one another. This is misleading. On the oscilloscope screen the first potential remains constant as the stimulus intensity is increased. At the threshold for appearance of the 2nd. potential, fluctuations occur in its latency so that it first appears erratically and well to the right of the first potential. When the axon responds to every shock the 2nd. potential moves closer to the first and is eventually superimposed upon it. Due to the erratic behaviour at the threshold, it is very difficult to make photographs without a continuously recording camera. If the intensity is increased somewhat more rapidly, the

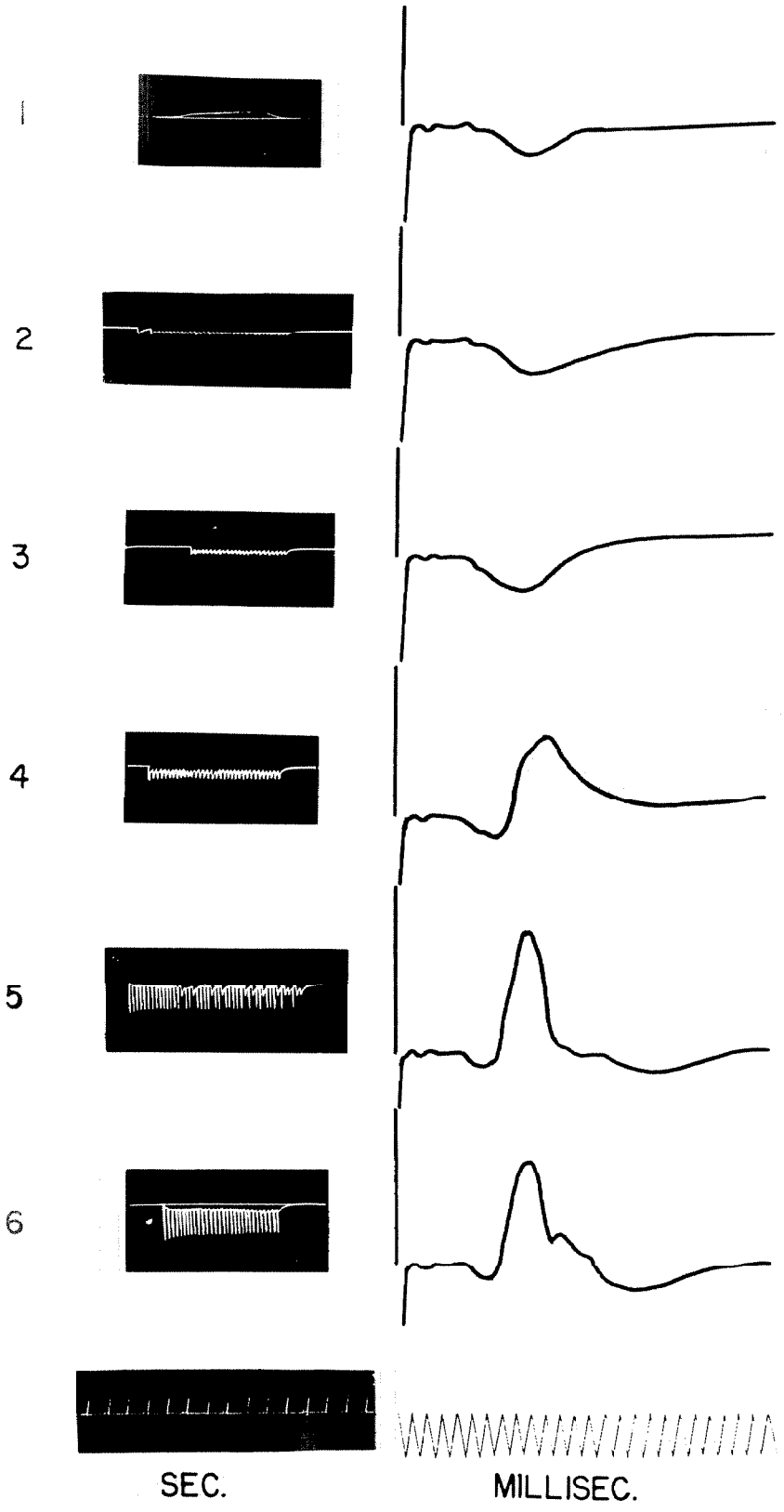

Fig. 12. Mechanical and electrical responses of the metathoracic levator tibia. Numerals show contraction types with corresponding potentials. Contraction 1 was made isotonically at 50 per sec. Contractions 2-6 are isometric at 5 shocks per sec. The stimulus triggered the sweep circuit of the oscilloscope. Time records in secs. and millisecs. Note nerve potential close to stimulus artifact.

erratic stage with increased latency is not observed and the 2nd. potential appears suddenly in its definitive position. This is easily observed as the sudden slight change in the shape and amplitude catches the eye immediately, although the final result is not obviously different. There is no doubt whatsoever that there are three of these small potentials, each one behaving in an all-or-none fashion depending on the stimulus intensity. There is a third factor that makes the recorded potentials appear smaller. When the latency is prolonged, the potential is seen to be considerably larger than when it is superimposed on the previous potential in its definitive position. In other words, when two axons are stimulated and the resulting muscle potentials are superimposed, this double potential does not represent the exact algebraic sum of the two but is somewhat smaller. With the large potentials 4 and 5, there is more nearly algebraic summation.

The levators of the first and second limbs and the corresponding depressors have been studied less extensively. In Fig. 13 superimposed muscle potentials from the mesothoracic levator tibia (upper) and the prothoracic depressor tibia (centre), are shown. In the former 7 potentials are shown. There is some doubt as to the existence of the potential labelled 4 and it has not been correlated with a contraction. The sign of the first three potentials is quite capricious with respect to the last three but usually their peaks all have the same sign. Limited supplies of animals made it necessary to concentrate on the muscles of the third limb. For this reason complete correlations between the muscle potentials and mechanical responses of the other limbs has not been made. It may be stated that all the muscles except the metathoracic depressor tibia apparently fall into the same category of

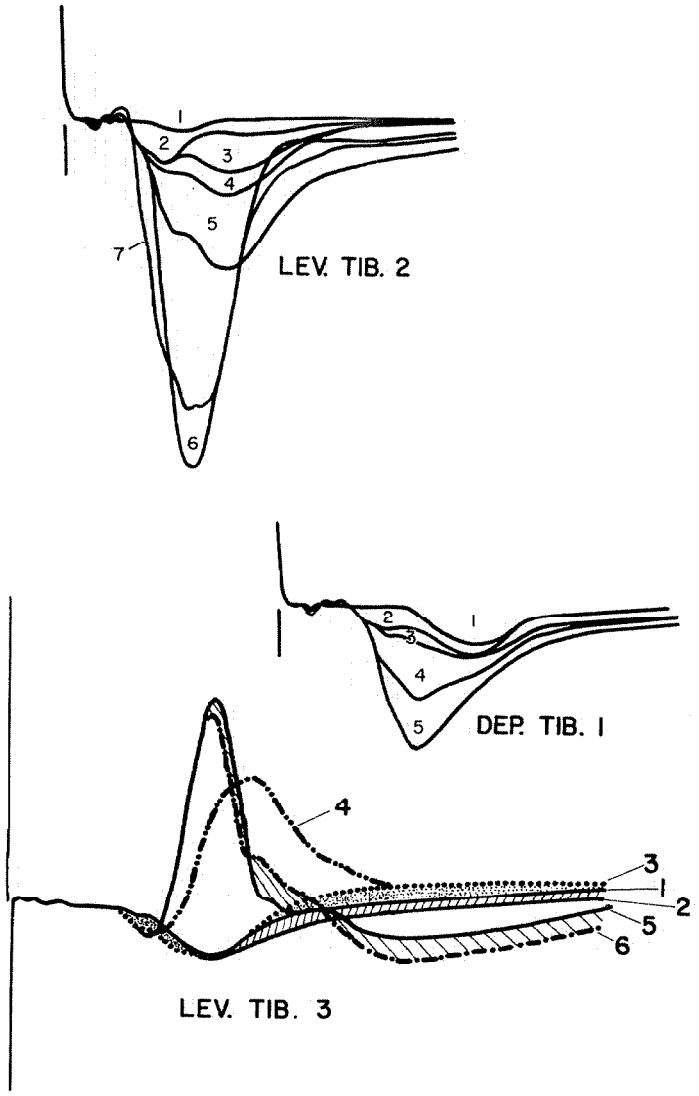

Fig. 13. Superimposed muscle potentials. Top - levator tibia of the 2nd. limb. Potential 4 is of a dubious nature and it has not been correlated with mechanical contraction. Centre - Depressor tibia of the 1st. limb. Bottom - Levator tibia of the 3rd. limb. These are the potentials 1-6 of Fig. 12. Shaded and stippled portions clarify potentials 2, 3 and 6. Potentials usually have the same sign at the peak as in the top and centre diagrams.

multiple innervation with graded fast contractions and the possibility of quasi-slow contractions.

Continuous records of the muscle potentials demonstrate most clearly the lack of growth of the potentials, the sudden appearance of each type and the skipping of shocks around the various threshold intensities (Fig. 14). Owing to the length of these Matthews (62) oscillograph records, selected pieces have been presented. In some cases, as an aid to photographic reproduction, the records have been darkened with a soft pencil. Both the general shape and the order of appearance of the potentials makes it simple to homologize them with potentials recorded on the cathode ray oscilloscope.

In Fig. 14 records A-E are from a continuous record of the metathoracic levator tibia response to increasing intensity of stimulation. Six action potential types are discernible. The heavy vertical line indicates where part of the record has been removed. In each case the part removed was from only one potential type and varied in duration from 1-2.5 secs. The abrupt transition from one type of potential to the next is readily seen. The first and last potentials of each type are the same size so that no marked facilitation is present. There is no evidence of fatigue in either mechanical or electrical response at these moderate frequencies. However, although facilitation of the potential is strongly contra-indicated it cannot be concluded from these experiments that it is altogether absent. If fatigue and facilitation had exactly the same time course then the potentials would remain the same size. That this balance could occur in numerous preparations over a wide range of frequencies and times, seems improbable.

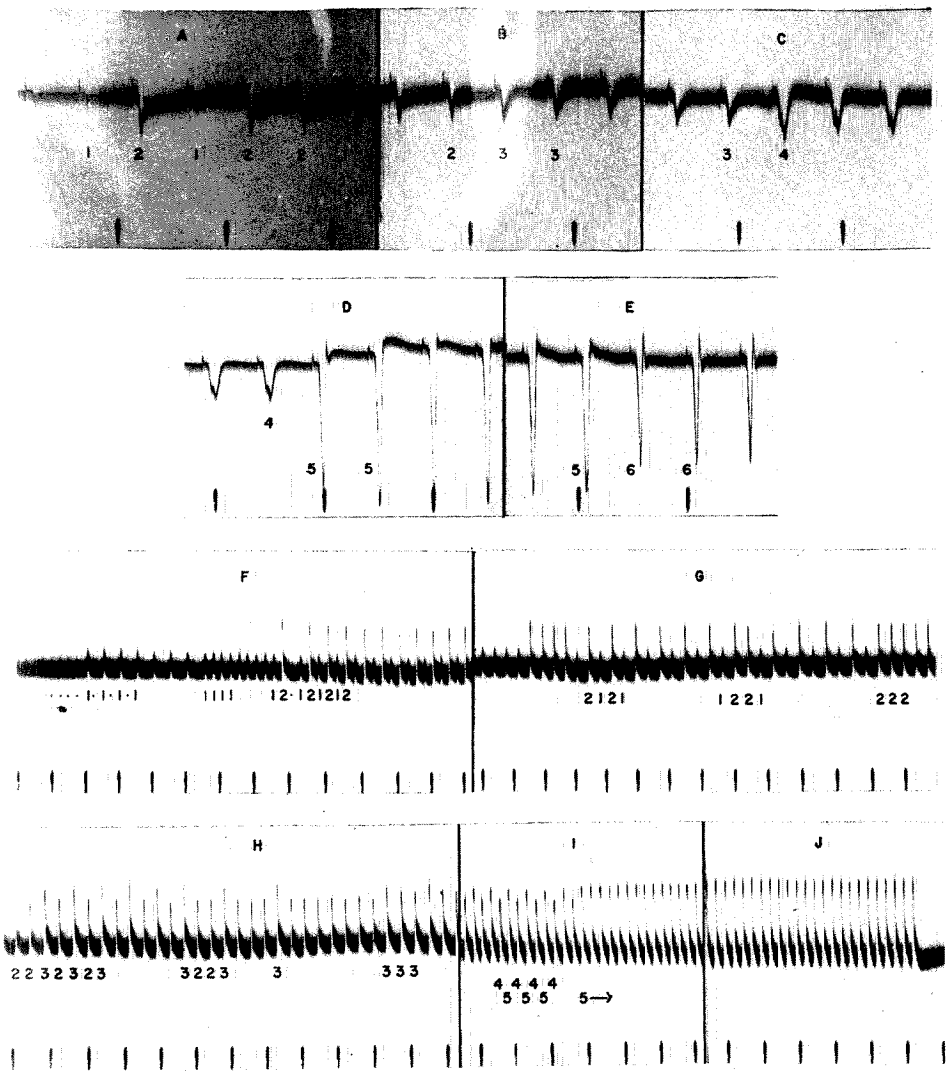

Fig. 14. Matthews oscillograph records of responses of the metathoracic levator tibia to increasing stimulus intensity. The numerals show the potential types. Time record in tenths of a second. For full description see text.

Records F-J in Fig. 14 are from the mesothoracic limb. The amplification of the response is the same in all cases although the individual portions were selected from three records made at different stimulation frequencies. F is from one record, G and H from another and I and J from a third. They have been chosen as they illustrate the alternation of potentials around the thresholds for the individual axons. In F the stimulus was first subliminal. Potential 1 then responded to every second shock and at a slightly higher intensity to every shock. After the first potential 2, neither axon responded and a little later 1 presumably responded on every shock and 2 on alternate shocks. In G, various combinations of potentials 1 and 2 are shown. In H potential 3 appeared. Towards the left it responded to every other shock but further to the right it missed seven shocks before responding to every one. Potential 2 responded to these seven shocks and it must be inferred that where alternation is occurring the axon with the lower threshold is responding to every shock and the one with the higher threshold to every other shock. There is no reason to believe that they are both firing alternately. If this were so, the size ratio would be different and when a number of axons were involved the record would become very much more complex and probably erratic. Also, the alternation occurs at an intensity higher than that at which the axon with the lower threshold responded to every shock.

Record I shows alternation of potential 5 and then constant response to every shock. J is taken from the same record 47 secs. later and shows that there is practically no increase of the potential size and also no appreciable fatigue.

Not only can electrical facilitation not be demonstrated but the contraction itself does not display facilitation. This can be shown by mechanical recording in the following type of experiment. After a stimulus at, say, 20 shocks per sec. for about 5 secs. the stimulus is turned off so that the muscle relaxes. It is then immediately turned on again and the rate and tension of the second contraction compared with that of the first. If facilitation were at all marked it would reflect in the greater rate of rise of the second contraction. With maximal stimulus this is never the case. Only rarely when the thresholds of the other axons are widely separated, can they be tested in this fashion. Hence it has been possible to test only the contractions associated with the third and fourth potentials. No sign of facilitation has been found. As these contractions result from stimulation of more than one axon it cannot be concluded that mechanical facilitation does not occur in the smaller contraction types.

Although the histological studies show either one or two axons innervating the metathoracic depressor tibia, only one large complex potential can be obtained. Neither variations of frequency, intensity, pulse duration nor various maltreatment of the nerve have succeeded in separating potentials or contraction types, if indeed more than one exists. The functions of the second and possibly third axons are not known. In reflex preparations activity in the jump muscle may be elicited by tapping the abdomen. One to three large potentials usually result and are followed by a characteristic fast extension of the tibia. The reflexly obtained potentials have the same general form as those elicited by electrical stimulation of the nerve although they show more variability (Fig. 15). Changes in the amplitude and exact shape

of the last phase of the potentials are usually observed. This could be due to a second smaller potential elicited at higher threshold but the very erratic nature makes it seem improbable. The variability is more likely due to fatigue of muscle fibres and changes in fluid distribution during contraction. In reflex preparations the second potential of a pair is frequently, but not invariably larger and of shorter duration than the first. The change in amplitude could be due to fluid changes causing increased resistance but the shorter duration is not easily explained. It may represent some facilitatory process in the system. Unfortunately there is a large sensory component in the nerve so that reflex responses from it show many potentials. However, in all cases the muscle potentials are preceded by very large single axon potentials which are easily distinguished from the background of smaller potentials. These must be from the axon responsible for the fast contraction. It is not possible to determine whether there are smaller axons also responding. Fig. 15 shows a selection of reflex and stimulation responses. The general similarity of the electrical events elicited in the two ways is apparent. Records 1 and 2 were made from the same preparation but differences in placement of the recording electrodes after severing the nerve are probably responsible for the slight differences in the responses. The sweep speed in record 2 is faster. In other preparations the potentials were almost identical. Muscle potentials elicited by stimulation show that the first of a series is only occasionally smaller than those following. The second potential is always of maximum size.

With different preparations there is wide variation in the exact shape of the depressor muscle potentials although they all have the same

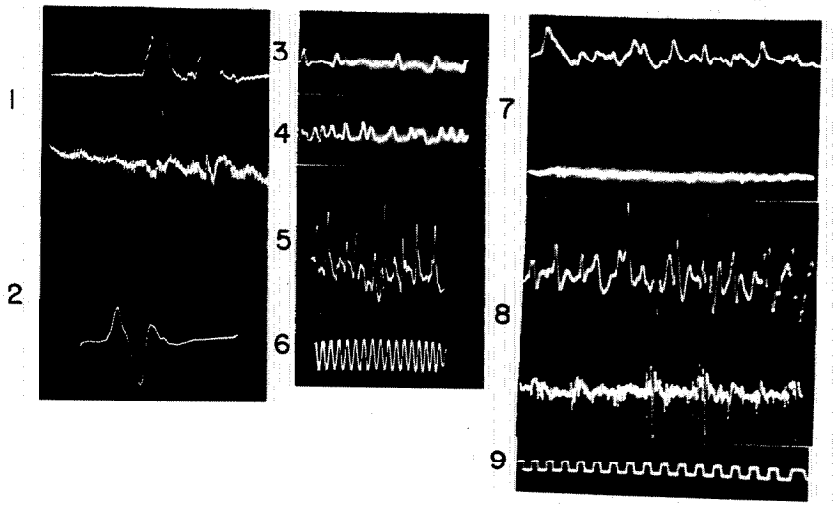

Fig. 15. Reflex nerve and muscle responses from the metathoracic limb. 1. Muscle (upper) and nerve (lower) potentials from the depressor tibia elicited by touching abdomen. Sweep for both beams is common. Amplification of lower beam is much higher than upper. Note large nerve potentials preceding muscle potentials and also larger size and shorter duration of second muscle potential. 2. A single muscle potential from the same preparation elicited by electrical stimulation of the nerve. Sweep speed faster than in 1. 3, 4 and 5. Muscular potentials from the levator tibia elicited by tapping the head. Amplification and sweep speed is the same in all cases. Note the different sizes and shapes of the potentials. 6. Time record, 60 c.p.s. 7 and 8. Nerve (lower) and muscle (upper) potentials from the levator tibia elicited by a jet of air directed at the head. Amplification of nerve potentials is much higher than is that of muscle potentials. Note summation of different potential types and complexity of nerve response. 9. Time record, 60 c.p.s.

complex triphasic shape. Continuous records show clearly the all-or-none nature of the response and the lack of facilitation (Fig. 16). In these records the intensity of stimulation was increased from a sub-threshold value during the record. Only the beginning of each record is shown. The corresponding contractions of the muscle appear above.

Few records have been made from the tarsal muscles. With stimulation of the nerve in the thorax or femoro-tibial articulation, the levator tarsus shows at least four potential types and probably a fifth. Mechanical recording is difficult with this small muscle. Microscopical observations of the apodemal end show four distinct contraction types. The muscle responds to every shock with a twitch and no facilitation is evident. Fusion frequency is lower than for the tibial muscles and the rate of development of tension is slower. All the potentials are distinctly monophasic. No evidence exists for slow contractions although complete correlation of electrical and mechanical events has not been made. The depressor tarsus corresponds closely to the levator both in potential types and contractions. If anything, it is somewhat slower in contraction. Five potential types and four contraction steps have been observed.

d. Analysis of neural and muscular electrical events.

In recordings from the main nerve branch to the levator tibia it has not been possible to distinguish the individual motor axon potentials associated with the contraction types. In the case of the depressor tibia one large axon potential is correlated with the twitch contraction. Judging from the motor axon diameters, their conduction velocities are probably the highest, their threshold lowest and their potentials largest. Antidromic potentials in the sensory

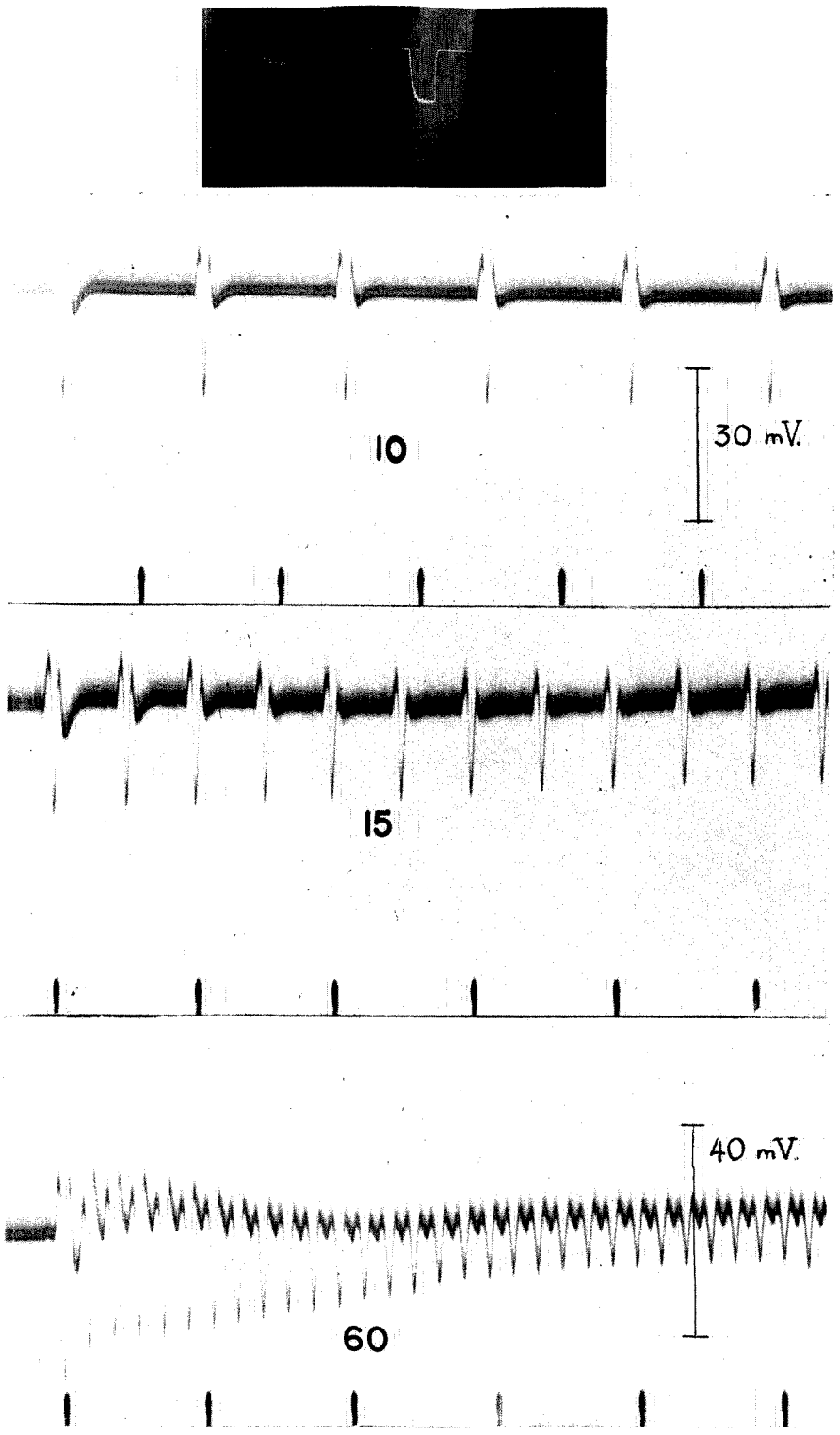

Fig. 16. Mechanical and electrical responses of the metathoracic depressor tibia to increasing intensity of stimulation. Muscle records are isometric and correspond to the three electrical records below. Numerals indicate frequency of the stimulus. Time marks - tenths of a sec. Only the beginning of each record is shown. Note lack of facilitation. Last record shows fatigue.

axons are probably smaller and slower. With the sudden appearance of each muscle potential type there is also a sudden increase in the size of the complex nerve potential, indicating that at least one motor axon is associated with each contraction type.

In an isolated nerve suspended on four electrodes (two stimulating electrodes close together and two recording electrodes about 1 cm. apart) the action potential shows the characteristic negative deflection at the electrodes so that a diphasic potential results. In the intact animal the nerve frequently gives rise to an initial positive deflection due to the complex leading-off conditions. Crushing the nerve between the electrodes results in a monophasic potential of negative sign. To determine conduction velocities and latencies within the nerve, experiments were performed with the nerve chamber described in an earlier section. As the strength of the stimulus is increased from below threshold, a series of potentials with different conduction velocities appear. At threshold for a particular axon the latency is variable and single nerve potentials are seen far to the right of their definitive position. The delayed latency observed with muscle potentials is due to nerve latency and not to junctional or muscular effects. Also the alternation of muscle potentials is due to effects in the nerve. With maximal stimulation of the isolated nerve preparation a complex picture of summated potentials is observed. Conduction velocity was measured between diphasic lead-off electrodes a known distance apart and a known distance from the stimulating electrodes. Stimulating electrodes were less than 1 mm. apart and the negative one was more peripheral. The nerve trunk of the metathoracic limb was used and results from one typical experiment will be quoted.

In the first instance the near recording electrode was 12 mm. from the stimulating electrodes and the recording electrodes were 17 mm. apart. The largest peak of the complex potential takes 4.8 millisec. to travel the 17 mm. between electrodes but subsidiary peaks take from 4 to 8 millisec. The conduction velocity therefore varies between 4.25 and 2.1 m/sec. The lower figure is probably that for the antidromic impulses in the sensory axons. After these measurements were made the electrodes were moved closer to the central end of the nerve and the experiment repeated. The distances of the near and far electrodes from the negative stimulating electrode were 3.5 and 16 mm. respectively. To travel the 12.5 mm. between the electrodes the potentials took 2.3-3.8 millisec. with the largest peak taking 2.5 millisec. The conduction velocity in this more central portion of the nerve trunk varies therefore between 5.5 and 3.3 m/sec. with the peak at 5 m/sec. Apparently as the nerve and axons get thinner the conduction velocity drops off markedly.

Estimates can be made for the latent period of nervous excitation. In cases where only one or two axon potentials are elicited, one can calculate the time taken in conduction between the stimulating electrodes and the first recording electrode. By measurement, the time taken for the nerve potentials to appear at the near electrode is known. By subtraction then, the time in excess of the calculated conduction time for an axon potential, is the latent period of excitation. Over 12.5 mm. the potential in question has a mean velocity of 5.5 m/sec. To travel from the negative stimulating electrode 3.5 mm. to the first recording electrode will take it 0.64 millisec. The measured time for the appearance of the potential is 1.2 millisec. Therefore the

latent period is approximately 0.6 millisec. These calculations are only approximate as the short lengths of nerve cannot be measured accurately and the time measurement is correct only to about 0.2 millisec. From other records similar results have been obtained. The values for the latency range from 0.3-0.7 millisec.

The latency of the muscle potential cannot be measured directly as conduction velocity in the nerve has to be taken into account. Conduction velocity in the fine intra-muscular nerve twigs has not been measured but it must be less than 3 m/sec. With one electrode on the trochantin and the other 5 mm. along the femur, nerve and muscle potentials of the levator have been recorded. The point 5 mm. along the femur is the point where the nerve first branches to the muscle and it is reasonable to assume that the muscle potential originates there. The time between nerve and muscle potentials is 2 millisec. Assuming a conduction velocity of 3 m/sec. for the nerve twigs, the nerve potential will take at least 1.6 millisec. to reach the terminations, leaving a figure of 0.4 millisec. for neuromuscular delay. This figure of 0.4 millisec. can only be considered as a first approximation.

With one electrode on the trochantin and the other an exploring electrode moving down the femur, no increase is observed in the time between stimulus artifact and nerve potential or between nerve potential and the onset of muscle potential. However a change is seen in the temporal relations of the final phases of the muscle potential. This means that the electrode on the trochantin is the active one at least for the nerve potential and the initial phase of the muscle potentials. When the position is reversed and a static electrode is placed on the femoro-tibial articulation and the exploring electrode moved towards it

from the trochantin, the onset of the potentials still shows no increased latency although phase relations are altered and the muscle potential peaks show a decided shift to the left. The nerve potential cannot be used as a reference point in this case as it is led-off not under the exploring electrode but from the point of entry of the nerve into the femur. Assuming that myo-neural delay is the same throughout the muscle and that the peak of the muscle potential represents maximum activity under the exploring electrode, then the conduction velocity along the length of the muscle can be estimated. The estimate will be extremely rough as the peak of the potential is not a good reference point. From a number of records made at different points along the muscle, the velocity was calculated as 3 m/sec.

The duration of a maximum potential in the metathoracic levator tibia varies from 9-14 millisec. The minimum potential has a duration of only 6-7 millisec. and reaches a peak in 3 millisec. From the reflex preparations (Fig. 15) where single potentials can be distinguished, the duration is approximately 12 millisec. for the 5th. potential alone. In the depressor of the third limb the muscle potential appears somewhat later than the levator potentials. In a double muscle preparation the depressor potential is evoked at higher threshold than the levator potentials and it appears as a very large triphasic spike after almost complete decline of the levator potentials. Having made an estimate for conduction time to the depressor muscle, the calculated myo-neural delay lies between 2.5 and 4 millisec. unless the nerve conduction is much slower than was estimated. This may be possible as the axons to this muscle have extremely thick sheaths and the terminal branches are very fine. The duration of the depressor potential is 14-18 millisec. and it reaches a peak in 5 millisec.

Fig. 17 shows a series of potentials from the metathoracic levator tibia. An exploratory electrode was moved in one case distally and in the other proximally. The fixed electrode was on the coxa in the first case and on the distal end of the femur in the second. The potential in all cases was maximal. An elaborate phase shift is shown but the onset of muscular and nerve potentials is not altered with progression of the electrode along the muscle. Thus conduction time in the intra-muscular nerve twigs is not recorded. With a conduction velocity of 4 m/sec. it would take the nerve potential at least 7 millisec. to traverse the length of the femur. As the records show no such delay we must assume that the leading-off conditions are such that the nerve potential is always led off from close to its point of entry into the femur. The whole question of the leading-off conditions is very complicated. The chitinous attachment and the tracheal system may play a large part and the complicated arrangement of muscle fibres in bundles which splay out serially from the central apodeme makes interpretation impossible. Muscle potentials led off under these conditions usually have an initial positive sign but this is quite capricious and depends not only on the placement of the electrodes but on a number of unknown factors which may cause reversal of the potential sign during an experiment. Even the nerve potential appears to be positive under these conditions.

Consider Fig. 17. The muscle potential must originate at the point N where the nerve first innervates the muscle. Due to the recurrent branches, the muscle potential and the contraction must spread in both directions from this point. When the exploratory electrode is on one or the other side of this point, i.e. in

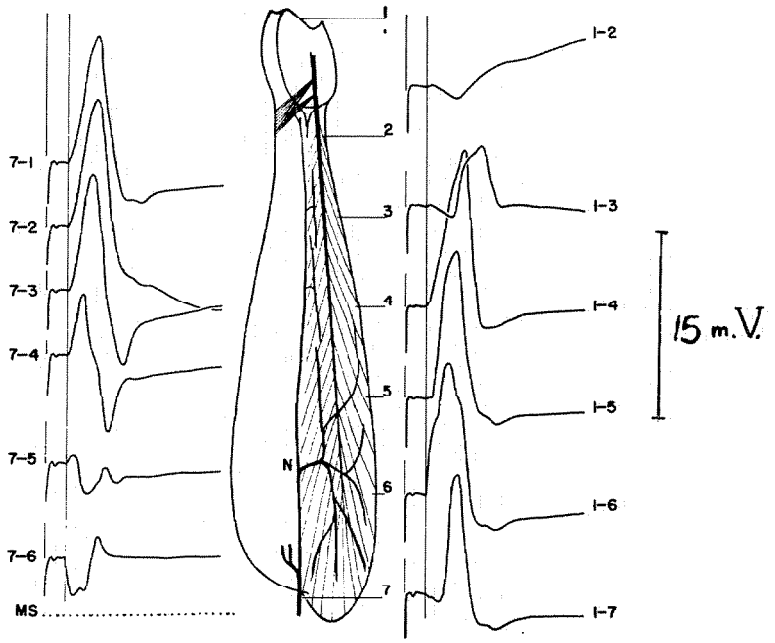

Fig. 17. Muscle potentials in the metathoracic levator tibia with various positions of the recording electrodes. Left column - Maximum potentials recorded with one electrode fixed at position 7 on femur and the other electrode at successive points 6, 5, 4, 3, 2 and 1. Centre - The femur with the nerve, levator muscle and apodeme. Numerals 1-7 represent small holes in the exoskeleton through which electrodes made contact with the muscle. Point N is where the muscle is first innervated. Right column - Potentials recorded with the static electrodes at position 1 and the exploring electrode at successive points 2-7 on the femur. Time calibration in millisecs. Note the constancy of the time of onset of the potentials and in the righthand column, the shift of their peaks to the left. Vertical lines have been drawn through the potentials as an aid to comparison.

positions 5 or 6, the initial sign of the potential changes. This shows that the active electrode is not the same in both cases. With lead 7-6 (left vertical row in Fig. 17) the impulse starts at point N which, because of the leading-off conditions, becomes positive to the rest of the limb. As nerve conduction carries the excitation towards electrode 7, electrode 6 is first encountered and it becomes positive to 7. As the excitation reaches point 7 this electrode becomes positive to electrode 6. Thus a diphasic potential is recorded. With lead 7-5, one electrode is on each side of the active region which is spreading in both directions. Electrode 7 is first affected and becomes positive. The larger activity in the rest of the muscle is delayed due to the nerve conduction time and is recorded a little later at 5. As the recording electrode is moved further down the limb so the relative sizes of the potential recorded at both the electrodes varies. Electrode 7 receives the activity first except when the other electrode is between it and the active site. This accounts for the very slight shift to the right of the onset of the potential recorded at lead 7-6. The line of reasoning used for the 7-1 series of potentials may be applied to the 1-7 series (right vertical row in Fig. 17). The lack of delay in the onset of the potential as the exploring electrode moves nearer to the tip of the femur must be due to the initial activity being led off near to the proximal end of the femur and conducted non-physiologically by some exoskeletal structure. The first part of the positive deflection is due to activity at point N. A complex relationship must exist between physiologically and non-physiologically conducted electrical events.

In the levator muscle the different potential types appear to be essentially monophasic but this is deceptive. Differences in the duration and latency put them slightly out of phase so that for example, a large negative phase of potential 5 is cancelled by the slower positive peak of potential 4 so that the resultant complex potential appears monophasic. In reflex records, when they are simple enough, the diphasicity of certain potentials can be seen (Fig. 15). Also when a potential is delayed by threshold stimulation its shape and size differs from that of the complex superimposed potentials. With this as a basis it is possible to obtain at least a rough estimate of the individual potentials by subtracting from each complex potential all those on which it is superimposed. This type of algebraic subtraction assumes that the various potentials sum as though they were connected in series. This is not strictly correct, as was pointed out earlier. An analysis made on this basis is presented in Fig. 18. It is seen that all the potentials are somewhat diphasic but the most striking difference is between potentials 4 and 5. It immediately suggests that 4 is a local potential and 5 is conducted. However, the multi-terminal innervation, the initial positive sign of the potential, the large size of the potential and the general inexplicability of the results in terms of conducted potentials are some of the facts indicating that conduction of potentials in the length of the fibre does not occur. Without complete isolation of the muscle from the exoskeleton further analysis is not possible. With the fan-shaped muscles such isolation is manifestly impossible because of the numerous separate origins of the fibre bundles. Isolated nerve branches plus a single, or even a small bundle of parallel fibres, the two ends of

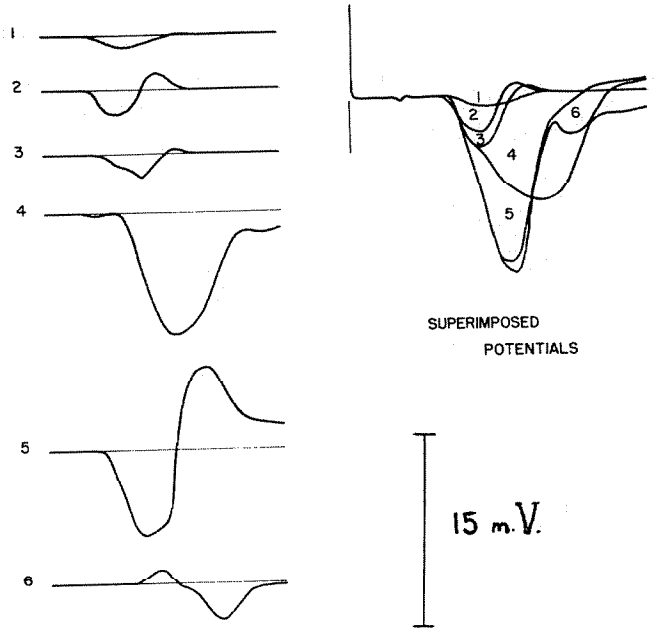

Fig. 18. The individual muscle potentials from the levator tibia of the third limb. The potentials 1-6 shown on the left correspond to the contraction types 1-6. Each one was derived by subtraction. For example, of the superimposed recorded potentials on the right, potential 4 was subtracted from potential 5, and potential 5 from potential 6, etc. These derived potentials can be considered only as a first rough approximation.

which are not connected by chitin, would answer some of the problems. Such a preparation has not yet been developed but is by no means impossible.

The long refractory period of the insect myo-neural preparations has been mentioned both by Roeder (31) and by Ripley and Ewer (57). Voskressenskaya (56) observed the phenomenon but did not appreciate its cause. Preliminary experiments have shown that in the sphinx moth, Celerio, the longitudinal flight muscles together with their innervation can be isolated. Mechanical and electrical records of the muscle action were made under various conditions. If the nerve stimulus strength is only very slightly in excess of threshold, the muscle follows the stimulation frequency up to about 35 per sec. At higher frequencies the muscle will still respond at this basic rate which is approximately the same as that recorded during free flight. The refractoriness lies in the nerve, as is shown by the fact that increasing the stimulus slightly, allows the system to follow to a higher frequency. Apparently the axons can be induced to respond to shocks falling in the relative refractory period if the intensity is sufficiently high.

The same phenomenon may be observed in isolated nerve and in neuromuscular preparations of Romalea. In the metathoracic levator tibia, potential types 3-6 exhibit skipping of shocks at frequencies over 15-20 per sec. If the intensity is increased all the axons can be induced to follow the frequency of stimulation up to about 70 per sec. Above this frequency, responses 4 and 5 cannot be driven although responses 1-3 will follow to about 150 per sec. In the isolated nerve the same type of phenomenon may be observed but it is not known which axons were responding. It appears then that the relative refractory

period may be as long as 50 millisec. and absolute refraction varies from 7 to 14 millisec.

This is confirmed to a certain extent by muscle potentials recorded from animals subjected to extreme reflex stimulation such as burning or pinching. Of any one potential type, the highest reflex frequency ever recorded is about 150-175 per sec. The large potential 5 of the levator and the single potential type of the depressor appear to have a maximum rate around 40-50 per sec. This latter figure is surprising as stimulation studies show that maximal tension is developed at about 80 shocks per sec. Either the observed reflex responses were not maximal or there is some physiological limiting mechanism which could conceivably be the relative refractory period of the axons.

A third type of confirmation has come from experiments involving pairs of shocks. Each shock of a pair was individually adjusted so that it resulted in a response to every stimulus at very low frequency. The nerve was then stimulated with both shocks separated by various intervals of time. In the depressor tibia only one action potential results, provided the shocks are separated by less than 25 millisec. With a longer time between the shocks two potentials arise and show no summation as the duration of each potential is only about 15 millisec. The nerve potentials show the same long refractory period. If the shocks of a pair are increased in intensity to well above the threshold then each will elicit a muscle potential when they are separated by only 10-15 millisec. This indicates that the relative refractory period is about 25 millisecs. and the absolute refractory period about 10 millisec. The observations are in extreme contrast to those on vertebrate axons where the dimensions of the refractory periods are much smaller.

As no drug is known which specifically blocks nerve terminations in insect muscle, the only method by which denervated muscle can be studied is by severing and allowing the nerve to degenerate. Roeder and Weiant (31) have shown that in the cockroach, degeneration is complete five days after the operation and that the muscle is electrically inexcitable. An operation was devised so that the nerve trunk to one of the metathoracic limbs in Schistocerca could be severed. A small flap of tissue above the metathoracic ganglion was loosened after swabbing the venter with alcohol. Surgical sterility was maintained as far as possible. After reflexing the flap which is about 1 sq. mm. in area, a hooked needle was inserted and the nerve lifted to the surface. Extreme care was taken to avoid damaging salivary glands, trachea or malpighian tubules. Damage to any of these organs seriously affects survival of the locust. After severing or crushing the nerve it was then pushed back into position, the flap of chitin returned to its place and the wound sealed with a thin layer of low melting-point paraffin. Experience some years ago had shown that limbs treated in this wise dry progressively from the tarsus upwards. This can be prevented by exercising them. The tarsus of the denervated limb was therefore tied to that of the sound leg so that the active leg exercises the immobile one. Some animals treated in this way were still in good condition 50 days after the operation.

Six animals were operated on in this manner and were killed at various intervals after the operation. In none of them was the region around the operated site infected or congested. The animals were made ready for testing by preparing both metathoracic limbs and their nerves in the usual way except that the tibia was not removed as it was necessary

to observe all the muscles. The first animal was prepared 14, and the last one 50 days after the operation. Particular attention was paid to the appearance of the nerve and muscles on the operated side. The electrical and mechanical responses of the muscles of control and experimental limbs were then compared.

In all cases the nerve and muscles on the operated side appeared completely normal. No significant differences existed between threshold intensity, frequency response, muscular strength, fatigue or action potentials of the two limbs. A very slight degeneration of the nerve could be seen at the point where it had been crushed or severed. There was no sign of regeneration. Wiersma (unpublished data) observed that there was no degeneration of peripheral axons in the crayfish, Cambarus, three months after severing the nerve, and responses of the muscles were normal. It is not known why there is so much difference in degeneration rates between Periplaneta and Schistocerca but the possibility of infection in the former must be considered.

IV. DISCUSSION

Insect muscle appears to be innervated in much the same way as crustacean muscle. Histological data from early anatomical studies have shown that there is multiterminal innervation of the muscle fibres (1, 5, 11, 14), and as many as 9 terminations of the nerve have been observed over one millimeter of a single muscle fibre. There is some histological evidence for di- and quadruplotomic innervation (5, 21), but this cannot be considered as conclusive. Physiological studies by Rijlant (24) and Pringle (27) left little doubt that at least double innervation occurs, and Pringle suggested that each axon innervated all the muscle fibres although the evidence was not conclusive. From this study of Romalea, both histological and physiological evidence show conclusively that there is multiple innervation, and at least six contraction types can be distinguished in the metathoracic levator tibia muscle. Individual bundles of muscle fibres receive identical numbers of axons. Wherever the nerve branches, the number of motor axons is the same, even in very fine branches. The final distribution of each axon to the individual muscle fibres has not been observed but it seems highly probable that each muscle fibre receives many branches of each axon. Whether or not the same maximum tension can be obtained by stimulation of each axon is not known. Such data will not be available until a satisfactory method is devised for separating the individual axons. Even when this can be done unequivocal conclusions as to the innervation of all the fibres by every axon may not be possible.

If Pringle is correct in concluding that in the cockroach extensor tibia, all the muscle fibres are innervated by the 2 motor axons, the same type of arrangement almost certainly occurs in Romalea. Pringle made

no histological study of the innervation but the physiological observations on Romalea are strongly supported by histological data. The strengths of the maximal contraction in the levator and depressor tibia are so nearly equivalent to that of maximally contracting vertebrate muscle that it seems improbable that only some of the muscle fibres would be active. Of course, if there is really but a single functional fast system in the depressor then each fibre is obviously innervated by the same axon. All the data for Romalea are in complete agreement with the concept of innervation of every muscle fibre by each of the several axons.

There appears to be a very close correspondence between the crustacean and insect efferent innervation of muscle. Crustacean innervation data presented in Part I of this thesis show four motor axons in the main flexor muscle. This appears to be of general occurrence at least in the higher Crustacea. van Harreveld and Wiersma (65) have shown that these axons produce a series of graded contractions in the muscle. In Romalea the position in all the tibial muscles, except the depressor tibia of the third limb, is very similar. The latter muscle is apparently specialized for leaping and seems to lack the small contraction systems found in the other muscles. The histological data suggest that it has at least a double innervation. This could easily have been missed in the physiological study, despite the special scrutiny that was devoted to it. However, not even in reflex activity has a second contraction type ever been observed and were it not for the doubt aroused by the histological data and the observations on the cockroach, it would be concluded that there was but a single innervation. Of course, the two axons observed may be branches of the same parent axon

before it leaves the nerve trunk and there is some reason for believing this to be so.

On the other hand, there remains the puzzling observation that a walking grasshopper apparently exhibits a slow, smooth contraction of the depressor which cannot be attributed to activity of the fast system. Some preliminary experiments with the animal indicate that in walking, the front pair of limbs pull the animal along, the second pair both pull and push, and the third pair do not push at all, except in jumping. Graded tension in the metathoracic levator could adjust the position of the tibia so that it would act much as does a crutch used by a lame person. Experiments are planned to test this further.

Data strongly suggest that there is a seventh axon innervating the levator, and a second or third innervating the depressor tibia. The mesothoracic levator tibia was not studied histologically but from physiological evidence it appears to have an innervation identical with that of the metathoracic levator. In only one case was a seventh muscle potential observed and it was of a doubtful nature. The possibility of inhibitory innervation is therefore suggested. In the Crustacea, the action of inhibitory axons vary greatly in the effect they have on different contraction types and in different muscles. In general, the fast systems cannot be inhibited, or if they can, the Rc ratio is very high. As most of the contraction types in Romalea muscle belong in the fast category, it follows that the action of an inhibitor could have been missed. In the case of the first slow contraction type, which one might suspect of being easily inhibited, the inhibitory axon could again have been overlooked if its threshold was high. The fact that contraction type 1 shows appreciable tension only at high

frequency might indeed suggest that it is always inhibited during electrical stimulation. Similarly, in the depressor there may be triplotomic innervation; one inhibitor, one slow and one fast axon. Only the fast is observed as the slow contraction and its action potentials are always inhibited. Inhibition has never been conclusively demonstrated in insects as axons cannot be stimulated individually. Although there are axons in Romalea muscle which are not accounted for, no conclusion can be drawn with respect to inhibition.

The general conclusion to which one is forced is that the myo-neural picture in Romalea is almost identical to that in crustacean limb muscles. In view of the close phylogenetic relationship of the two sub-phyla this is not surprising. In the non-specialized limb muscles each muscle fibre is innervated numerous times by at least six motor axons and probably by a seventh which is of unknown function. The axon terminations are close together, there being at least 1-2 endings of each axon per millimeter over the whole length of the muscle fibre. Axon terminations probably penetrate the sarcolemma and from evidence presented by Hilton (11) and others, the intra-fibre axon branches are distributed more or less evenly throughout the sarcoplasm at the innervation focus. Thus there would be a fairly continuous feltwork of six different sorts of axon terminations within the fibre. In interpreting the events in a muscle fibre during various types of contraction, the close proximity of these terminals to one another must not be lost to sight.

The interesting, although incomplete, data on Celerio which were briefly mentioned previously, indicates that in the flight muscles there is also a multiple innervation. With this muscle however, there does not

appear to be a symmetrical distribution of the nerve endings. By observing the exposed muscle surface, the smallest contraction elicited at the lowest stimulus intensity seems to involve only the distal one fourth of the fibre length. The next larger contraction involves half the fibre, the third, three quarters and the fourth, the whole fibre. This suggests that each axon innervates either progressively more of each fibre or a discrete section of the fibre. Another interesting feature which may be mentioned in passing is that with electric stimulation of the nerve, an incomplete tetanus can be elicited which is never as powerful as that observed at the same frequency with a reflex preparation of the single muscle. Only if pairs of shocks are applied to the nerve can the reflex tension be approached. Now the refractory periods of the axons are too long to permit repetitive discharge in high frequency bursts in each axon. Thus the observations might indicate that the four axons are firing sequentially during each twitch contraction in normal flight. In order to overcome the disadvantage of the long refractory period, the moth may have resorted to a series of impulses in different axons which innervate the same muscle fibres. For the moment this can only be considered as speculative.

Although the innervation of Romalea is anatomically very similar to that of many crustacean muscles, the functions of each axon and the characteristics of each contraction type differ somewhat from those of the Crustacea. Both Pringle (27) and Roeder and Weiant (31) present data which are very akin to that obtained with certain fast systems in Crustacea. The muscle responds to a single shock with a large, fast twitch in an all-or-none fashion. With a series of shocks the responses fuse and produce a higher tension. The associated potentials do not

exhibit any marked facilitation and appear to be maximal. Of the six contraction types demonstrated in Romalea, four fall into this category. These are contractions 2-5 of the levator tibia. In the depressor tibia of the third limb the single contraction type is also fast, as are most of the types shown for the depressors and levators of the first and second limbs. However, the fast contraction systems are of graded sizes in any limb muscle of Romalea. This multiplicity of fast contractions has been observed neither with Insecta nor with Crustacea although Pringle's innervation maps indicate that there is more than one fast axon to some of the muscles in the cockroach. In the flexors of certain Crustacea there are several quasi-fast systems but there appears to be an almost continuous gradation between the characteristic fast and slow types.

In neither mechanical nor in electrical recordings have any data been obtained which show the presence of facilitation. The contraction type 1 approaches very closely to the slow system in the cockroach depressor but it is probably best termed a quasi-slow contraction as there is no facilitation of action potentials although the contraction is slow developing, and has a very low fusion frequency. The 6th. contraction, from what little is known of it, appears to be a quasi-fast contraction. Although it responds with twitches at very low frequency the contraction becomes either smooth, or almost so, at a frequency of 10 per second, whereas the typical fast systems in Romalea have very pronounced clonic contraction up to at least 20 per second and the fusion frequency is at about 60 shocks per second. The possible occurrence of inhibition must again not be overlooked. Its activity might account for failure to observe muscle potential facilitation. The rate of

fatigue may also obscure facilitation although in fresh preparations stimulated at moderate frequency this seems improbable.

In considering the way in which the animal might use the various contraction types, two facts should be emphasized. Previous mention was made of the peculiarities of the femoro-tibial articulation. Although the directly recorded isometric contraction may show discrete twitches of a constant size, these tensions, when transferred to the tibia by way of the articulation, may have quite different characteristics. Not only will they be damped to a certain extent so that the contraction appears smoother, but they are capable of moving the tibia over an increasing arc. Thus individual twitches would be observed as an almost smooth contraction flexing the tibia and not causing it to exhibit a series of twitches while being almost static with respect to the femur.

From reflex preparations, data on the frequency of discharge of the various contraction types indicate that in normal movement a fairly high frequency of activity is exhibited by contraction types 2 and 3, resulting in a small contraction which can be speeded up and strengthened by the interjection of activity in axons 4 and 5. In moderate contractions, axons 4 and 5 discharge at a low frequency, whereas in the fastest contractions, types 4 and 5 may be the only active systems. The maximum frequency of their potentials does not exceed about 50 per second. Whether contraction types 1 and 6 are associated with the tonic posture maintaining mechanism is not known.

Little can be added to what has already been said about the electrical events in the muscle. Roeder's interpretation of data from the cockroach fits in well with the observations on Romalea. Both he, Wiersma and Wright (66) and Katz and Kuffler (67) have pointed out the complexity of

the leading off conditions of invertebrate muscle *in situ* and have offered an explanation for the positive polarity observed. Roeder observed only positive monophasic potentials and, in agreement with Wiersma, concludes that the negativity associated with the depolarization of the muscle membrane is limited to the side of the fibres away from the leading off electrodes. This would tend to make the chitinous attachment and the inactive portions of the muscle act as a source, and hence activity is recorded as a positive deflection. Roeder and Weiant suggested that the muscle bundles might all be innervated from their inner faces, thus producing a core of negativity during activity. The histological evidence from *Romalea* lends further weight to this postulate as the majority of nerve endings are towards the centre of the muscle bundles. The diphasicity has been accounted for by nervous conduction of the excitation over the length of these long muscles.

It is more difficult to explain the monophasicity of potential 4 and the diphasicity of potential 5 on such a basis. In view of the fact that the small potentials 1, 2, 3 and 6 also appear to be diphasic, this is even more confusing. It is possible, although extremely improbable, that potential four is a complex potential resulting from two axons with the same threshold. Thus phase differences in two such potentials might account for the observed monophasicity. On the other hand, potential 4 may represent a local potential on one side of the muscle fibres and the other potentials may be conducted, not in the length of the muscle, but around the circumference of the fibres at each locus of innervation. In this way an electrode on the side of the muscle might first register a positive potential while the depolarization was on the inside of the muscle bundles, and then a negative deflection as the depolarization

reached the outside of the bundles. Longitudinal membrane conduction would be prevented by the numerous innervation sites.

The position is so complicated by the geometry of the muscle, the speeds of conduction in the various axons and the possible variation in the delay at their terminations, that further speculation is not profitable. There is a great need for a myo-neural preparation in which these many possibilities may be studied without the numerous extraneous influences which make further interpretation of data from the whole muscle impossible. With our present concept of the nature of the innervation along each of these muscle fibres it is not possible to entertain the notion of muscle potentials conducted in the length of the fibres by an agency other than the axons. In addition the potentials are far too large and their sign is always initially positive. This in itself almost certainly precludes the possibility of propagated muscle potentials. Katz and Kuffler (67) have expressed the opinion that local potentials (negative at the site of innervation) reach a certain size and then give rise to a potential propagated in the length of the fibre. Many objections can be levelled at this work, but if one assumes only that large local potentials occurring at one nerve ending can, at a certain critical value, stimulate the intra- and/or inter-fibre axon branches, it is possible to explain their results on the basis of the concept of local potentials and nerve conduction of excitation throughout the muscle. The possibility of preparing an innervated single muscle fibre in the Crustacea appears bleak because of anatomical details of the innervation. In the grasshopper, however, there is sufficient difference in innervation so that the chances are much better. Some of the small tibial flags of the depressor pretarsus, together with their double axon supply, may answer this demand.

Pringle has expressed the view that both slow and fast axons cause maximal contraction of single muscle fibres and that the observed facilitation of slow potentials and gradual development of tension in the muscle would be due to recruitment of fibres. This assumes the involvement of more and more terminations of the nerve twigs and in view of the multiterminal innervation of each fibre, it seems improbable. There is no evidence pointing to the occurrence of either of these two phenomena, and in Romalea, the existence of a number of non-facilitating fast systems makes the postulate untenable unless there are different categories of muscle fibres. Gradual development of contraction and growth of action potentials can be adequately explained on the basis of local graded activity at all the nerve endings. The transmission process between muscle potential and contraction would cause increase in contraction with higher frequency of stimulation. Where action potential growth accompanies increase in tension, such as with the slow system in the cockroach extensor, local increase of the area and the degree of depolarization, can be envisaged. The numerous different axon terminations in the same minute area of muscle fibre might each result in depolarization of only certain sites on the membrane so that when all the terminals are active, a mosaic of independent but indistinguishable sites occurs. In Romalea there appears to be no influence of one contraction type on the others although it has not been possible to test this fully. The relation between these postulated sites and the transmission and contraction processes, must vary for each contraction type, giving rise to differences in contraction characteristics, fusion frequencies, frequency response, etc. Characteristics of each contraction type could also be imposed at the postulated transmission

process between nerve impulse and muscle potential and by the topographical inter-relationships of the axon terminations.

The possibility of growth of potentials and contraction being due to decremental conduction in the extremely fine terminal twigs of the nerves, might be considered from a speculativepoint of view. A postulate much like that of Pringle's could then be arrived at, with the important difference that it is the number of axon terminations on each muscle fibre that increases during recruitment, not the number of muscle fibres, and that the action of a muscle fibre is not all-or-none. Microelectrode recording techniques could conceivably test such a postulate.

Some of the problems of flight in higher insects are among the knottiest in invertebrate neurophysiology. The two chief theories of the action of the flight mechanism differ widely from each other but are not entirely mutually exclusive. A third theory can be postulated on the results obtained by the various workers in the field, together with certain observations made in this laboratory. This postulate is, of course, of a highly speculative nature, but in a field such as that of insect flight where the existing postulates do not encompass all the facts, speculation may be profitable. In common with the two extant theories the following postulate also does not account for all the observations but it introduces some new thoughts and attempts some unification of the data.

Pringle (27) has shown the existence of a small slow contraction in the cockroach leg muscle which is associated with very small potentials. In Romalea the very small potentials (except the first one) are associated with fast small twitches of the muscle. These potentials

are very much smaller than those associated with the maximal twitch. Roeder and Weiant (31) have observed a similar fast contraction. These observations may be supporting evidence that the very small potentials which occur in the thorax of the fly during flight are associated with twitch contractions (42, 43, 51). I propose that in the longitudinal and tergosternal muscles there are small potentials associated with a quick twitch of the muscles. With the techniques which have been used for recording the thoracic flight potentials, potentials from antagonistic muscles might well partially cancel one another as the muscles are contracting 180^{o} out of phase with one another. The very small flight potentials previously mentioned have a frequency which is the same as that of the wing beat. Now it can be objected that if the twitches associated with the small potentials are very weak they might not be adequate for flight. Furthermore, McEnroe (64) has claimed that during flight the tergosternal muscle contracts smoothly. However, it is not certain that his recording method would have shown the small tensions associated with incomplete tetanus.

I propose that the muscle has a double innervation. This certainly occurs in many insect muscles. For the fly, Pringle states that there is only a single axon, but his published illustration is far from convincing. One of the proposed two axons would be responsible for the small twitch contractions at the beat frequency. This tension alone is too small to bring about flight movements but the other axon would be responsible for the large asynchronous potentials observed and would cause a large, smooth contraction at low frequency. Thus in effect two axons firing at different frequencies, together might produce an incomplete tetanus at the flight frequency. This is very similar to

the relation between contraction types 5 and 6 in the levator tibia of Romalea. The large constant tension would supply the necessary power for the Boettiger-Furshpan 'click' mechanism. The small twitches would supply the slight extra tension necessary to release the wing from the up and down locked positions. I would also invoke the idea that the conditions of contraction are those of quick stretch and quick release as suggested by Boettiger and Furshpan.

In deep anaesthesia large potentials have been observed which are not accompanied by wing flapping. This indicates that the ganglion is active and the observation has proven an obstacle to the Boettiger-Furshpan hypothesis. I suggest that flight does not occur because the small potentials and their associated twitches are absent. The wings would then be fixed in either up or down position, or possibly some intermediate position, by action of the large contraction and 'click' mechanism.

It has been argued that the very high frequency of flight precludes reflex control. In the first place Pringle himself states that he has observed reflex nicotine discharges of over 800 per second in the large non-specialized cockroach. In the smaller insects with more specialized muscles discharge frequencies may well be much higher. This, however, accounts for only one part of the arc. Myo-neural junctional transmission need not be considered as it can be occurring in the relaxing phase of the muscle cycle. One might visualize a mechanism whereby the incoming sensory impulses bear a 1:10 relation to the efferent discharges so that the limited time involved would not present a serious problem. Practically the whole thorax is involved in movement during flight and an adequate number of sense organs exists, even after amputation of the

wings. If the muscles were functioning under high loading conditions, then amputation of half the wing would allow the muscle to contract faster. Loading would have a direct effect on the flight muscles which could be signalled by sense organs and give rise to a higher rate of discharge of the small motor axon. The axon responsible for the large potentials need not change its discharge rate, thus accounting for the observation that the large potential discharges do not increase as the wing loading is decreased.

In many records the beginning and end of flight often show a slow increase or decrease in amplitude of wing beat but no change in frequency. In other records the cessation of flight is abrupt. This has proven difficult to explain on the existing theories but with a double innervation it is obviously easily explicable if the large potentials give rise to a fairly slowly rising or relaxing contraction and the rate of beat is maintained by the small twitches. During abrupt stops the cessation of twitch activity would cause a lock of the wings via slow contraction and 'click' mechanism. Phase changes in the contractions of longitudinal and vertical flight muscles might also account in part for the gradual decline of flapping. This theory offers no explanation of why the muscles cannot be electrically excited, wherein it falls short of the Pringle-Roeder hypothesis and it shares with the latter the lack of any explanation for Pringle's observation of sustained oscillation of the denervated haltere muscle.

The myo-neural systems of insects show great variation in the orders, families, muscles of the same animal, and even in the contraction types in each muscle. This is hardly surprising in a sub-phylum which is in the process of rapidly evolving, and whose members show great

diversity of habit and habitat. The resemblance of many aspects of insect myo-neural physiology to that of Crustacea, bears out the postulated close relationship of the two sub-phyla. However, little is yet known of the physiology and the great diversity of function and anatomical arrangement gives scope for research on many of the fundamentals of nerve and muscle activity. Some of the most baffling problems are exhibited, and correlated histological and physiological studies are badly needed before even the simpler problems can be approached. The occurrence of local potentials makes the Arthropoda a particularly suitable study object for the fundamentals of muscular activity and much future effort will have to be devoted to devising better techniques for study of the isolated building blocks, the axons and single muscle fibres.

V. SUMMARY.

1. The neuromuscular physiology of the leg muscles of the grasshopper, Romalea microptera (Beauv.) has been studied. Certain experiments were also performed on the locust, Schistocerca sp. Serial sections of the metathoracic limb of Romalea were stained with Mallory's triple stain. Reconstructions from these sections together with microdissection of the limb, have resulted in detailed information on the innervation and musculature.

2. The metathoracic levator tibia muscle has a sexo-or septuplotomic innervation. The muscle fibres are divided into discrete bundles which splay out from a central apodeme. Each bundle receives an identical innervation. It has not been possible to determine the number of axons in the terminal nerve branches although it appears probable that each muscle fibre is innervated many times by each axon. Individual muscle fibres have at least one or two innervation sites per millimeter.

3. There remains some doubt as to the exact number of motor axons in the metathoracic depressor tibia. Branches carrying one, two or three axons have been observed. Individual muscle bundles may have single, double or triple innervation.

4. Preparations which allow simultaneous study of muscular contraction and muscle and nerve potentials, have been described. Responses were elicited either reflexly or by stimulation of the main nerve trunk to any of the three limbs. The nerve was exposed in the thorax for stimulation. By leaving the tracheal system intact, muscles were kept in excellent condition. Single muscles were studied either by removing the other muscles of the limb or by severing the nerve branches to the unwanted muscles. Contractions were recorded isometrically from the apodemes of the muscles. Nerve potentials were led off from the

relevant nerve branch at its point of entry into the femur.

5. With one exception, the tibial and tarsal muscles of the three limbs exhibit a number of contraction types. These have been studied in most detail in the metathoracic levator tibia, but in the other muscles they appear to be very similar. By variation of the stimulus strength, a series of discrete contractions can be elicited. The first contraction type is smooth at moderate frequency and response to single shocks has not been observed. Its tension is only a fraction of that of a maximal contraction at the same frequency. Contraction types 2 to 5 are of increasing size but otherwise differ little from one another. Each responds to single shocks with twitches of constant tension. At moderate frequency incomplete tetanus occurs and the fusion frequencies vary from 50 to 70 per second at room temperature. Maximal tensions occur between 90 and 160 per second. At frequencies higher than this the muscle responds maximally but does not follow the stimulus frequency. Contraction type 6 has the highest threshold. It responds to single shocks but in contrast to types 2 to 5, its fusion frequency appears to be at about 10 per second.

6. Only one type of contraction can be observed in the metathoracic depressor tibia although the depressors of the other limbs exhibit a number of types. This single contraction type has properties which are characteristic of contraction types 2 to 5 of the levator. It is accompanied by large, complex, triphasic muscle potentials.

7. The contraction types of the mesothoracic and metathoracic levators tibiae could be correlated with characteristic muscle potentials. The potentials increase in size from potential 1 to potential 5 but the sixth potential is much smaller than the fifth. Each has a characteristic

size and shape. With the leading off conditions employed, the potentials show various degrees of diphasicity and usually have an initial positive sign. With the muscle *in situ*, leading off conditions are very complex and even the nerve potential may exhibit initial positive sign with respect to the supposed active electrode.

8. The electrical data are compatible with the idea that excitation is conducted over the length of each muscle fibre, and over the whole muscle, by the agency of the axons.

9. The relative refractory period of the nerve varies between 25 and 50 milliseconds and the absolute refractory period between 10 and 15 milliseconds. The estimated time for myo-neural delay is 0.5 milliseconds. Nervous conduction velocity varies between 2.5 and 5.5 metres per second. Due to the long refractory period of the nerve, skipping of shocks occurs around the threshold intensity. Repetitive discharge was observed only under the most severe conditions.

10. In *Schistocerca*, the nerve to one of the metathoracic limbs was severed under surgical conditions and the wounds then sealed. At periods up to 50 days after the operation the nerve had not degenerated and tests showed that the myo-neural system of the experimental limb was in all respects the same as that of the control limb on the opposite side of the animal.

11. It is concluded that the leg muscles of the grasshopper are innervated by a small number of axons, in much the same way as are crustacean muscles. Each axon evokes a contraction of characteristic strength and speed. The vast majority of these contraction types are non-facilitating, typically 'fast' and the twitch to tetanus ratio for each is unity. The several axons innervating each muscle give rise to contractions whose maximum strengths form a stepwise series. Neither muscle action potential facilitation nor peripheral inhibition, both of which are characteristic of the decapod Crustacea, have ever been observed in *Romalea*.

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