STUDIES ON CERTAIN SENSORY AND MOTOR SYSTEMS OF DECAPOD CRUSTACEANS

Thesis by

Edwin Jean Furshpan

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1955

ACKNOWLEDGMENTS

It is a pleasure to acknowledge my debt to Professor C. A. G. Wiersma: for his continued guidance and interest; for introducing me to the study of crustacean neuromuscular physiology and alerting me to some of the subtleties and complexities to be found there; and for numerous constructive criticisms.

To Professor A. van Harreveld I wish to express my sincere thanks for his constant encouragement and willingness to share his illuminating thoughts on many phases of neurophysiology.

I have received financial support from the Biology Division in the form of two Assistantships. The McCallum Foundation has provided Summer Scholarships, as well as support during two other terms. E. I. du Pont de Nemours provided one of their Fellowships during one year. I feel a palpable debt to the people concerned.

I also wish to acknowledge the prodigious typewriting abilities of Mrs. Alethea Miller and to thank her for her good humor throughout the task.

ABSTRACT

Part I. Muscle Receptor Organs of the Crayfish.

Some interesting structures were discovered in the abdomens of certain Crustacea by J. S. Alexandrowicz (Quart. J. Micr. Sci. (1951) 92: 163) and in the present study their function was investigated. From his histological observations, Alexandrowicz deduced that these structures would serve as stretch receptors and his assumption was confirmed by the present work. It was found possible to isolate the organs and remove them from the animal. When the organs were stretched, using a specially constructed apparatus, trains of impulses were recorded from the nerve supplying them. There are two types of receptors in each half of each abdominal segment and the responses of each were found to differ from those of the other. One type (RM1) exhibited a prolonged slowly adapting discharge in response to a constant stretch; while the other type (RM2) adapted rapidly. The organs each consist of a muscular strand on which end the dendrites of the nearby sensory cell. It was shown that the muscular parts are unnecessary for the evocation of the sensory discharge by stretch; but that the former, by their contraction, can initiate impulses. The reactions of the receptors to several drugs were tested. Acetylcholine (ACh) in concentrations above a certain low value (10^{-6} g/ml.) augmented or initiated discharge in RM1. The effect was abolished by previous administration of atropine and enhanced by eserine. The discharge

accompanying stretch was not abolished by atropine and it was concluded that ACh probably did not serve as a mediator of the normal stretch-discharge.

Part II. Excitation in Decapod Crustacean Muscles.

Several muscles in a number of species of decapods were studied using a combination of two techniques. On the one hand was the procedure for isolating and stimulating single motor axons; and on the other, the microelectrode method for recording intracellularly from single muscle fibers. The existence of two classes of responses was corroborated. The first type, termed "junctional potentials," constituted the first muscular response to nerve stimulation. These potentials were found to be distributed to all parts of the muscle fiber by the motor nerve, in corroboration of previous evidence. The junctional potentials could summate when repeated and upon exceeding a certain threshold of depolarization evoked the second type of response: the spike potential. The latter was found to have the unusual properties of being graded and local. The spike was, however, followed by refractoriness. The distribution of motor axons to the individual muscle fibers was also studied. Two types of muscles were studied in this regard: those receiving two motor axons and those receiving four. In the muscles innervated by two axons, most muscle fibers were found to receive each of the axons, although several exceptions were noted. Each axon evoked a response of different character in any particular

fiber; and, in addition, the size of the response elicited by either one of the axons varied markedly from muscle fiber to muscle fiber. The situation was even more complicated in the muscles innervated by four motor axons. Fibers were found which received only one axon, some received two, others three, and a small number were innervated by all four. In this muscle, then, aside from heterogeneity of response size, a marked variation in the axon complement of individual fibers was found. The responses evoked by different axons in a muscle fiber were found to summate and could thus act in concert to evoke a spike. The crustacean neuromuscular apparatus is thus a very complex reaction system and has some of the attributes of central nervous systems.

REPRINTED FROM THE JOURNAL OF EXPERIMENTAL BIOLOGY,
VOL. 30, NO. 1, PP. 136–150, MARCH 1953
 PRINTED IN GREAT BRITAIN

PHYSIOLOGICAL AND PHARMACOLOGICAL OBSERVATIONS ON MUSCLE RECEPTOR ORGANS OF THE CRAYFISH, *CAMBARUS CLARKII* GIRARD

BY C. A. G. WIERSMA, E. FURSHPAN AND E. FLOREY*
Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

(*Received* 13 *October* 1952)

(With Plate 8)

INTRODUCTION

Alexandrowicz (1951) has recently described organs in the abdomen and thorax of the lobsters, *Homarus vulgaris* L. and *Palinurus vulgaris* L., which he considers to be stretch receptors. Their structure is reminiscent of that of the (vertebrate) muscle spindle. These organs are few in number and were found only in the dorsal regions of the body. Two pairs in each of the six abdominal segments and a small number (perhaps only four) in the thorax may constitute the animal's entire complement of these receptors.

The organs of the abdomen are attended by an interesting, if complex, innervation. One of the several axons supplying each unit has its cell body in the periphery, in close apposition to a specialized area which Alexandrowicz calls the 'intercalated tendinous region'. The cell sends short dendritic processes to this area and is considered to have a sensory function; its axon would conduct impulses centripetally in response to stretch. Also observed were fibres typical of crustacean motor axons. They branch extensively along the muscular regions of the unit, 'giving off abundant ramifications ending amidst the myofibrils'. Other fibres, which seemed to branch and innervate the tendinous region as well as the muscular areas, were described and referred to as 'accessory' nerves.

It seemed of special interest to study the physiology of these crustacean receptors in view of the physiological deductions made by Alexandrowicz and of the recent intensive investigation of the vertebrate muscle spindle, both anatomically (Barker, 1948) and physiologically (Lecksell, 1945; Katz, 1949, 1950*a*, *b*; Kuffler, Hunt & Quilliam, 1951; Hunt & Kuffler, 1951*a*, *b*; Hunt, 1952*a*).

Several attributes of these receptors contribute to making them more amenable to experimentation. Unlike the vertebrate spindle, which lies within the body of a muscle, these are separate and external organs, free from other musculature and having only nervous connexions. They are thus rather easily isolated and, if necessary, removed from the animal. Carefully dissected preparations, if perfused

* Hixon Fellow.

occasionally with fresh solution, maintain their ability to respond to stretch for long periods, often for over 12 hr.

The present paper aims to present a first approach to the study of some of the reactions of these units. Several aspects of the present work will be treated in more detail in subsequent research, but since these organs represent one of the more easily obtained single-unit sensory preparations, and since their exposed position allows their being bathed directly in solutions of known drug or ion concentration, it has seemed appropriate at the present time to follow our short preliminary note (Wiersma, Florey & Furshpan, 1952) by a more detailed description of technique and results.

METHODS

Most experiments were performed with the fresh-water crayfish, *Cambarus clarkii* Girard, and some, as noted in the text, with the lobster, *Panulirus interruptus* (Randall). Several methods of preparation have been employed. One dissection has been found best for most purposes, however, and although similar to one given by Alexandrowicz, it will be described in view of some anatomical differences between lobster and crayfish.

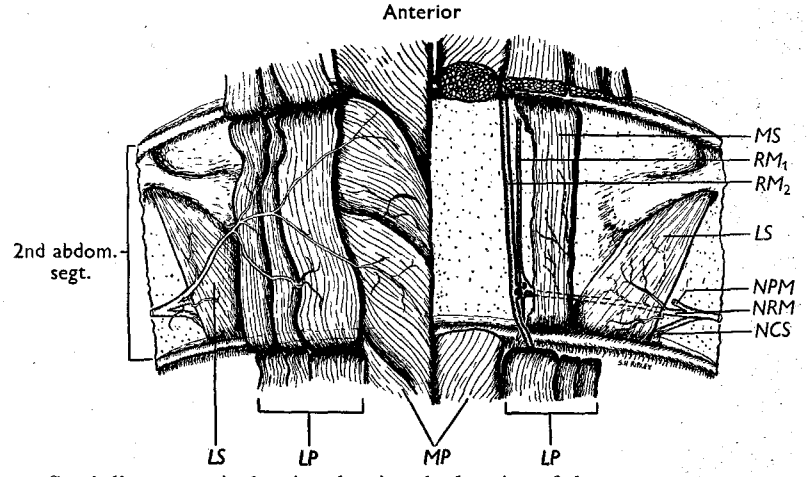
The tergal parts of all the abdominal segments are dissected free from the rest of the abdomen in the form of a single strip containing all the extensor musculature. It is found convenient to hold the strip, ventral surface up, with a bulldog clamp which can be attached with a spring clip to the side of the perfusing vessel. The clamp is attached to the proximal end of the first abdominal tergite. Varying amounts of the dorsal attachments of the flexor muscles remain, and these are removed from the half-segment to be prepared. After severing the nerve branch supplying it (Text-fig. 1, *NPM*), the more ventral layer of extensor musculature (profundis lateralis and medialis) is cleared, care being exercised to cut nerve branches which connect the thicker of the two receptor organs with the profundis musculature. Now the superficial extensor muscles are exposed and the receptor units can be found by carefully pulling aside the medial border of the medial superficial muscle, which partially covers the units (Text-fig. 1, right side). Removal of the tergal strip necessarily severs the nerve trunk which supplies the organs, but a sufficient length of nerve remains to allow for the placement of electrodes. If the organs are to be excised this section of nerve may be freed along its entire length by carefully removing the remaining extensor musculature to which it sends numerous small branches. If the units are to be left within the tergal strip, however, only the medial and not the lateral superficial muscle is removed, as the latter then serves to protect the units from being stretched by pull on the nerve. This same trunk also sends at least one large branch (Text-fig. 1, *NCS*) dorsad to tactile receptors in the tergum, and this branch is always cut since impulses from these receptors otherwise confuse the recording.

To test the response to stretch, the end of the cut nerve trunk was lifted above the surface of the perfusion solution on a micromanipulated platinum electrode. The other electrode was immersed in the surrounding fluid or attached to the clamp

which holds the strip. Most of the recordings were taken from organs of the second segment. A thread was attached to the posterior part of the strip so that pull upon it caused movement of the third segment on its articulation with the second similar to that in natural flexion.

After suitable amplification the impulses were led into an oscilloscope, or in some experiments concerned only with low-frequency response, into a power amplifier which drove a signal magnet writing on a two-drum kymograph. With this device long continuous records have been easily and inexpensively obtained.

Preparations were dissected in, and perfused with, van Harreveld's crayfish solution (van Harreveld, 1936). Some of the preparations for testing motor response



Text-fig. 1. Semi-diagrammatic drawing showing the location of the receptor organs, nerve trunk and branches, and the extensor musculature in the crayfish. It represents a ventral view of a section of the tergum which has been removed from the abdomen in a single strip. The letters denote the following structures. Extensor musculature: *MS*, superficialis medialis; *LS*, superficialis lateralis; *MP*, profundis medialis; *LP*, profundis lateralis. Nerve branches: *NRM*, nerve to the receptors and the superficial extensor muscles; *NPM*, nerve to the profundis muscles; and *NCS*, nerve to the various receptors of the exoskeleton. RM_1 and RM_2 are the muscle receptor organs.

were stimulated in a solution containing one and one-half times the normal amount of potassium and no magnesium (Waterman, 1941). To study the effect of drugs on the receptors, drops of the appropriate solutions were applied to either stretched or unstretched preparations, and the concavity of the tergal segment served conveniently to retain the added solution for a short period of time. If it was desired to have the drugs act for a longer time, the entire vessel within which the strip was held was filled with the solution. Concentrated standard solutions of drugs were prepared in van Harreveld crayfish solution containing no bicarbonate, and were diluted with buffered solution prior to being used.

Anatomy

A short comparative study of the anatomy of the crayfish and lobster organs was made. The anatomical data for the lobster are, for the most part, from Alexandrowicz; but several preparations of the California rock lobster, *Panulirus interruptus*

(Randall), were made for further comparison. In preparations dissected as described above, the two receptors are easily seen under the binocular microscope without the aid of staining. It was necessary to stain, however, in order to see the nerves and ganglion cells. For this purpose rongalite-reduced methylene blue (Pantin, 1948) was found quite satisfactory.

In the crayfish as in the lobsters, the two organs of each half-segment are of unequal length and thickness. The difference in thickness is much more pronounced in the crayfish than in the lobster, but in both the thicker organ is also the longer. As is the case in the lobster, the larger of the two organs exhibits finer cross-striation of the muscular regions. As already indicated in the section on methods, there is a surprising difference between crayfish and lobster in the position within the abdominal segment. In all of the lobsters these organs are situated in the space between the lateral and medial superficial extensor muscles. This places them lateral to the superficialis medialis, whereas the organs of Cambarus lie medial to this muscle (cf. Text-fig. 1). Another difference between the two animals exists in the amount of connective tissue surrounding the organs. Whereas in *Panulirus* the two organs of a half-segment are almost entirely enclosed within an encapsulating sheath, those of the crayfish are separate and rarely bound together by any external connective tissue except in the region of the nerve entrance.

In both animals it was possible to show the presence of ganglion cells sending dendrites to a differentiated region. Fibres typical of motor axons, whose branches ramified along the length of the muscular parts, were also seen; and in one case fibres were observed which seemed to fit Alexandrowicz's description of accessory nerves. Thus, despite the differences, the similarities seem sufficiently manifest to justify carrying over Alexandrowicz's terminology to the crayfish. The longer and thicker organ, having the finer cross-striations, will be called RM_2, and the other, RM_1.

Response of the receptors

Flexion of the tail strip is followed by trains of action spikes in the trunk innervating the *RM*'s (muscle receptors). If the precaution of severing the branch to the receptors in the exoskeleton has been taken, spikes of not more than two heights are observed, each falling into a regular rhythm (Pl. 1, fig. 1). The two rhythms are easily distinguishable from one another, since one adapts very slowly and the other by comparison, rather rapidly. A marked difference in stretch-threshold for eliciting the two different rhythms is also apparent. The slowly adapting rhythm follows much slighter stretch and, in fact, is occasionally recorded in the absence of any externally applied stretch. It is therefore easily obtainable by itself (Pl. 1, fig. 3). The regularity of this rhythm, as well as the constancy of the spike height, can leave no doubt that it arises from a single receptor unit. This slowly adapting unit can maintain continuous discharge for periods exceeding an hour, and in one experiment, where the preparation was constantly perfused at a slow rate, response to a sustained stretch did not cease until $3\frac{1}{2}$ hr. after its initiation. As intimated, the other rhythm arises from a unit which has a higher threshold and is fairly rapidly adapting. It is usually necessary to flex the isolated tergal strip to an extent which

approaches the maximum possible in order to induce discharge in this receptor unit. Even on maximal flexion the response usually lasts for about 30 sec. and seldom longer than 1 min. By the standards of tactile receptors, a discharge which is maintained for a minute is long-lasting. In the crayfish, however, where this unit is contrasted with another which is capable of sustained response for several hours, it has been convenient to refer to it as the 'fast-adapting' receptor unit.

There are, then, two distinct types of rhythm which can be recorded from the nerve trunk to the *RM*'s. Histologically two types of organs, RM_1 and RM_2, have been shown to be present, and the question arises which receptor gives rise to which type of rhythm. If a moderate amount of stretch is applied to a preparation so that only the slow-adapting unit is active and then the terminal attachments of RM_2 (the longer-thicker organ) are severed, no appreciable change in the frequency of the discharge ensues. But if the attachments of RM_1 are then cut, all discharge ceases. If in other preparations, RM_2 is left intact, and only the attachments of RM_1 are cut, some response, though always diminished in frequency, may follow upon gentle stretching. This is due to connective tissue binding the two organs together in the region of the ganglion cells, and this response can be shown to originate in RM_1 despite its severed attachments. Further, it can be shown more directly, by the converse experiments, that the fast-adapting rhythm originates in the organ RM_2. It has recently been found possible completely to isolate and remove one or the other of the *RM*'s from the tergal strip, and to study it in isolation. These experiments leave no doubt concerning which type of rhythm arises from which receptor. In most preparations the nerve-action potentials recorded from RM_1 were smaller than those of RM_2 (Pl. 8, fig. 1 A). In the rock lobster a similar pattern of two spike rhythms was observed. Because of the connective tissue enclosing the two receptor muscles in this animal, no cutting experiments were performed; but, as in the crayfish, the more slowly adapting discharge with the lower threshold consisted of spikes of smaller size in almost all preparations (Pl. 8, fig. 1 B).

Only one of the nerve fibres supplying each receptor unit has its cell body in the periphery and is thus probably the sensory axon. Further evidence in support of this interpretation was obtained physiologically. The most direct and conclusive method would involve splitting the receptor nerve trunk and recording from single axons. Successful isolation of single axons has not yet been accomplished on account of the extremely tough connective tissue surrounding the nerve fibres in the periphery, and a less direct method had to be employed. The muscular elements of the receptor unit receive branches from all the attending nerve fibres with the exception of that one whose cell-body lies peripherally and whose terminals are restricted to the narrowly circumscribed tendinous region. It was found that crushing the muscular parts of an organ with a fine forceps did not alter the response to stretch. But if the approximate area innervated by the dendritic processes of the ganglion cell was probed with a sharp needle, a high-frequency discharge resulted and was usually followed by a loss of the receptor's ability to respond. Both of these facts are in full accord with the assumption that the fibre with the peripheral cell-body is indeed the sensory axon.

It was then found feasible to clamp the tendinous region with two pairs of screw-locking forceps, one on either side, so that very little muscle tissue was interposed. One pair of forceps was secured and the other moved by a rack and pinion device, thus stretching the receptor region almost exclusively. Responses were readily obtained in this manner, and it was found that the characteristic differences in the responses of the two receptors were not altered with this method of stretching. The comparatively rapid adaptation was still displayed by RM_2, and it is thus a property of the receptive mechanism or is an attribute of its nerve fibre (Gray & Matthews, 1951), rather than a function of its attachment or of the viscous-elastic properties of its in-series muscle elements.

These differences in the response to stretch of RM_1 and RM_2 are paralleled by disparities in the response of the receptors to various drugs and to alteration of the ionic content of the perfusing solution. Differences in the effects of drugs will be noted in a subsequent section, but some differences in the responses to changes in calcium and potassium ion will be noted here.

The increase in excitability caused by a reduction in the external calcium concentration is well known and has been specifically studied for receptors by Talaat (1933). Elimination of this ion from the external environment of these muscle receptors also produces dramatic increases in their excitability. Shortly after their immersion in a calcium-free solution, the receptors begin to fire spontaneously. If allowed to remain in this medium, the frequency of the response continuously increases until a point is reached at which impulses seem to drop out of an otherwise regular train. The dropping out continues and eventually the spontaneous activity, as well as the ability to respond to stretch, disappears. It has been consistently noted, however, that the response of RM_2 to the decreased calcium ion is delayed with respect to that of RM_1. Often impulses have started to drop out of discharge from RM_1 before RM_2 starts to fire spontaneously. Differences have also been found between the responses of the two receptors to increases in the external potassium concentration. Results with this ion were not as consistent as those obtained with calcium, but a general pattern was observable. The high-potassium solutions were made by adding crystalline potassium chloride to normal crayfish solution. Potassium concentrations of three and four times normal caused a short (*c.* 30 sec.) decrease in excitability so that a moderate discharge resulting from stretch would be inhibited. This phase was followed by one of increasing excitability, and with higher concentrations ($4\times$) spontaneous activity would ensue in RM_1. The increase in excitability often continued, and a dropping out of the spontaneous impulses and a cessation of discharge similar to that described above for calcium lack would eventually occur. Such spontaneous activity, and the associated behaviour, was never seen for RM_2 with these potassium concentrations.

The dropping out of impulses followed by complete cessation of spontaneous discharge found in calcium-free solutions resembles, at least superficially, another phenomenon noted in the behaviour of RM_1. If stretch on this receptor is continuously increased, a point near the extreme limit of flexion will be reached at which impulses start to drop out. Then almost immediately, the high-frequency

discharge stops completely. Following a slight decrease in tension, the discharge is again resumed. Recovery will also occur if the receptor merely remains in this over-stretched state for some time. In one such experiment the receptor was over-stretched and maintained in this condition until, at the end of approximately 4 min. discharge was resumed. Then tension on the receptor was increased further until the discharge again ceased, and again recovery occurred after 4 min. This was repeated for three additional trials with recovery times of 4, $4\frac{1}{2}$ and 5 min. The tensions needed to produce over-stretch are rather high, and it is possible that these recovery effects find their explanation in the slow rupture and slipping of the in-series elements of the organs. Some of the other attributes of the over-stretch phenomenon will be mentioned, although it is difficult to evaluate their significance without further study. The frequency of discharge immediately prior to cessation in over-stretch is dependent upon the rate of stretching; the higher the rate, the higher the frequency. This frequency is also dependent upon the state of fatigue of the preparation; the less fatigued, the higher the frequency. Over-stretch occurs at lower tensions, and is preceded by lower frequencies if the external potassium concentration is higher than normal.

Motor innervation

Because of the ubiquity of multiple innervation and peripheral inhibition in crustacean motor systems, it is usually necessary to obtain single axons before an effective study of these systems can be undertaken. Numerous attempts to induce contraction in the receptor musculature by stimulating the whole nerve trunk have been made. These have been unsuccessful, with a few exceptions, despite the apparent good condition of the animals and the care with which the dissections have been executed. This lack of success may, perhaps, be attributable to the above explanation (an interaction of motor and inhibitory stimulation), but attempts to isolate single axons have also been unsuccessful, as the connective tissue band in which the axons lie is extremely tough. Nevertheless, the few preparations which have given contraction on stimulation of the nerve trunk, demonstrate the existence of a functional motor system. It has also been shown that, as in the vertebrate spindle, this motor system is capable of altering the response to a given stretch. Pl. 8, fig. 2 shows several superimposed sweeps of the oscillograph. The recording electrode was placed on the nerve not far from the stimulating electrodes, and thus the stimulus artefact and deflexion of the base-line are rather large. The impulses which were set up at the stimulating electrodes and travelled directly to the recording electrode are included in the stimulus artefact and are not seen in the picture. The musculature of both organs gave visible contractions and impulses in both sensory axons are seen in the record.

There are several other muscle systems whose contractions interact with the receptor organs and affect their sensory discharge. It is known that contraction of the flexor muscles will stretch the receptors, and it is probably this system which is capable of imposing the widest range of tensions on the organs. Contraction of the extensor muscles would also be expected to affect the receptor's discharge, and the

following experiments were performed to see if these expected effects were present. A tergal strip was removed as usual, the remnants of flexor musculature cleared, but no further dissection was performed. Then the nerve trunk of the right half of the second segment was picked up on a recording electrode and that of the left side of the same segment on two stimulating electrodes. A base-line discharge was set up by moderate flexion of the strip and then the stimulating electrodes were activated at a frequency of about 30/sec. A visible contraction of the extensors of the left side was accompanied by a decrease or cessation of the discharge from the receptors of the right side. It is presumed that contraction of the extensors of the same side of the same segment would inhibit discharge of the receptors even more readily. Experiments performed in a similar manner demonstrated that contraction of the extensor muscles of the right side of the third segment had an opposite effect and augmented discharge from receptors of the right side of the second segment. It can be seen that very complex interactions of at least four different muscle groups are possible, all of which affect the tension on the receptor terminals, and these are considered further in the discussion.

Pharmacological reactions of the receptors

Drops of the appropriate solutions were added to the half-segment of the tergum containing the dissected receptors. At the end of 3 sec., in most experiments, the response was recorded. After the record was taken the tergal strip was thoroughly washed and then the perfusion vessel allowed to drain in preparation for addition of the next test solution. The mechanical agitation caused by the application of drops of the drugs was frequently sufficient to induce a small and transitory discharge in the more sensitive RM_1. Most of the effects with which we have been concerned, however, have been so large and long-lasting that confusion could seldom arise between mechanically caused and drug-induced responses. Where doubt existed drops of physiological solution, applied in the same manner, were used as controls.

A large number of drugs have been tested on this preparation. Of these acetylcholine gave the most interesting results, and only the effects of this drug and of a few other drugs which seemed most closely bound up with the ACh-induced response will be reported here. All drug concentrations are given in grams per millilitre of solution.

Acetylcholine. When applied in concentrations of 10^{-6} or greater, ACh consistently induces spontaneous impulses in RM_1, the slowly adapting receptor. RM_2 has about a hundred-fold lower sensitivity to ACh. In a number of fresh preparations solutions of 10^{-9} (and, in a few, 10^{-10}) ACh initially enhanced the discharge from slightly stretched preparations (that is, with a background discharge of about 10/sec.) as shown in Pl. 8, fig. 3. Subsequent applications of the same or even higher concentrations (10^{-8} and 10^{-7}) often gave no increment. Because of this lack of correlation between concentration and response the results obtained with these low concentrations are rather difficult to interpret. The frequency of discharge following application of the higher concentrations (10^{-6} to 10^{-3}) was

always related to concentration, and a typical record is shown in Pl. 8, fig. 4A. The numbers inserted refer to the concentration of ACh added. The variation in height of the nerve-action potentials from one record to another is not significant but merely reflects slight differences in the level of the surface of the solution in the tergal concavity and thus in the point at which the active lead from the nerve is located. These responses were obtained from a preparation of RM_1 with intact terminal attachments but to which no external stretch had been applied.

Hunt (1952*b*) reports that ACh exhibits a large excitatory effect on the vertebrate spindle, but he concludes that it is the contraction of the intrafusal muscle fibres which causes the heightened response rather than any direct effect of the ACh on the sensory terminals themselves. Two types of experiment indicate that this is not the case, however, in the crayfish organs. In the first, the terminal attachments of the receptor were severed so that it floated loose in the ACh solution being tested, attached only by its nerve to the tergal strip. This usually resulted in only a slight decrease in the ACh-sensitivity of the preparation. For example, in one experiment an eserinized receptor (RM_1) gave a slight response to 10^{-8} ACh prior to cutting its ends free from its attachments. After this operation ACh in a concentration of 5×10^{-8} was needed to give a similar response. It is felt that this slight decrease in sensitivity to ACh reflects the decrease in the level of excitability following removal of the subliminal stretch to which the organ is subject when *in situ*. This is corroborated by a number of preparations which have discharged spontaneously at a low level without the application of external stretch. The majority of preparations of RM_1, in fact, are very close to firing, even though the tergal strip is fully extended, as only the slightest amount of flexion is necessary to initiate discharge. A second type of experiment also indicates that ACh does not produce its excitatory effect on this organ by first initiating contraction of the receptor musculature. RM_1 was completely isolated with the nerve trunk and clamped with forceps (see p. 141) on either side of the intercalated tendinous region. Fixed in this manner there is only a small amount of muscle tissue remaining in series with the receptor region, and this must certainly be damaged by the clamping procedure. But even when so clamped, the sensitivity of RM_1 to ACh was essentially unchanged.

Eserine. Despite negative effects obtained in a few preliminary experiments (Wiersma *et al.* 1952), eserine (physostigmine salicylate Merck) has since been found to enhance the ACh response of all preparations tested, to a rather marked extent, when they are bathed in this drug for some time (3–5 min.). It has recently been found that high concentrations of eserine can also show a depressant action on ACh excitation. If drops of 10^{-4} eserine are followed immediately by ACh, a much smaller excitatory effect is obtained than if a washing is interposed between the applications of the two drugs. We have not studied this effect further, since it is not directly concerned with the problem in hand, but it might be that in this direction lies an explanation of the preliminary negative results.

Pl. 8, fig. 4E shows some responses of the same preparation of RM_1 as is shown in Pl. 8, fig. 4A, but between the times of the two sets of recordings the receptor had

been bathed in eserine 10^{-5} for 3 min. No external stretch was applied, and the numbers inserted again refer to the concentration of the single drops of ACh which were added 3 sec. prior to the taking of each record. It can be seen that there is an approximately ten-fold increase in the sensitivity of the eserinized receptor to ACh. Similar results have now been obtained in a large number of experiments. After being bathed in the lower concentrations of eserine (10^{-5}, 10^{-6}), the receptor shows no noticeable change in its sensitivity to stretch stimuli. Higher concentrations (10^{-4}), however, usually induce a low-frequency discharge in the absence of externally applied stretch. Once obtained, this spontaneous discharge is difficult to suppress and may persist after a number of washings.

Atropine. Many drugs, when applied to the receptors in high concentrations, induce effects which may not be related to their more typical and specific actions. In fact, one is hard pressed to find a compound which in high concentrations does not have some effect on the response of this naked receptor; and, especially in the invertebrates whose pharmacology is less well defined than that of the vertebrates, it is difficult to decide where the boundaries between 'typical' and 'unspecific' effects should be drawn. This difficulty is well illustrated by the effects of atropine. When this drug is applied in a concentration of 10^{-4}, it causes considerable augmentation of response in discharging receptors (Pl. 8, fig. 5*c*, *d*), and can initiate spontaneous activity in resting ones. Atropine 10^{-5} also has a slight excitatory effect. But more striking is the effect of the atropine on the ACh-induced excitation. Pl. 8, fig. 5*a* shows the response of a preparation of RM_1 to a slight stretch. Pl. 8, fig. 5*b* shows the response of this same receptor three seconds after the addition of one drop of ACh 10^{-4}. After washing for a short time the discharge (Pl. 8, fig. 5*c*) returns to its original level. Then atropine 10^{-4} is added and the excitation which it induces is shown in record 4*d*. Subsequent addition of another drop of ACh 10^{-4} causes only the slight increment of discharge (over that induced by atropine) seen in Pl. 8, fig. 4E. After washing ACh 10^{-4} is again capable of inducing the same large response (Pl. 8, fig. 5*f*) as prior to the administration of atropine. These effects of atropine were consistently observed in a large number of preparations, and in no case did it cause a depression of the normal stretch response.

DISCUSSION

These organs have thus proved to be receptors responding to the stimulus of stretch. This fact and several other facts were correctly predicted by Alexandrowicz. He also foresaw that the fibres ramifying along the receptor musculature subserved a motor function, and that the receptor designated as RM_2 would have the higher threshold. Not only was the predicted difference in threshold found, but further, the two receptors were seen to have markedly different adaptation rates. RM_1 adapts very slowly, has a very low threshold, and in some instances discharges even when the tergal strip is fully extended. It can maintain both very low (about 2/sec.) and fairly high (over 200/sec.) frequencies of discharge and can do so for long periods of time. RM_1 may thus be termed a tonic receptor, for it is well suited to transmit continuous information about the degree of flexion of each segment of the

abdomen. RM_2, on the other hand, fits the definition of a phasic receptor. Its high threshold and fairly rapid adaptation restricts its response, so that in the whole animal it probably fires only during rather extreme tail flexion as is found in the swimming reflex. Alexandrowicz also felt that it would be involved in this reflex, and further conjectured that RM_2 might initiate impulses which inhibit the powerful tail flexors. It seems to us more likely that impulses from this receptor operate to induce reflex contraction of the extensors so that the tail is readied for its next flexion.

A knowledge of all the functional connexions of the motor nerve fibres innervating the receptors will certainly facilitate the formulation of the role of the receptors in reflexes, in view of the complicated interactions which could conceivably exist in such a system. Contraction of the *RM*'s has been shown to facilitate discharge; but if these organs have been phylogenetically derived from the extensor muscles and share with them one or more motor axons, it would be difficult to state, *a priori*, how the combination of in-parallel extensor-muscle contraction and in-series receptor-muscle contraction might affect the discharge. There is, in addition the possibility of interaction between the receptors of one segment with the muscles of adjacent segments. Both of the receptors have one attachment on an articular membrane which connects tergites of adjoining segments (see Text-fig. 1), and RM_2 has its other attachment at the anterior tendon of the medial superficial muscle of the immediately posterior segment. Thus contraction of the extensor muscles of adjacent segments would be expected to increase discharge from the receptors, and this was demonstrated experimentally. Briefly reviewing the possible interactions of the abdominal receptors with other musculature of the tail, it can be seen that contraction of the flexor muscles will tend to increase discharge from these receptors. Contraction of the in-series muscle elements of the receptors themselves can also increase discharge frequency, whereas contraction of the extensor muscles of either side of the same segment will diminish the response. And lastly, contraction of the extensor musculature of adjoining segments will augment discharge. Deduction of the pattern of the discharge for any natural movement of the tail cannot be made until it is known what combination of these various motor systems may be activated simultaneously.

It is interesting to note that the difference in response of RM_1 and RM_2 to various conditions is not solely a function of the viscous-elastic properties of their in-series elements or the places of their terminal attachment parts. There must be in addition an inherent difference in their sensory mechanisms, as shown by their reactions to drugs and by the reactions to stretch of their isolated intercalated areas held in forceps. At present we are not yet able to judge the relative importance of these factors in the overall responses obtained from the organs *in situ*.

A comparison between these organs and the spindle of the vertebrates suggests itself. Alexandrowicz has already undertaken such a discussion, but since the appearance of his paper the work of Kuffler *et al.* (1951), and Hunt & Kuffler (1951 *a*, *b*) on the function of the small nerve fibres in the ventral roots of cats has been published. These investigations suggest the nature of the augmentation of

afferent discharge upon small-nerve stimulation first observed by Leksell (1945). Their evidence indicates that the small-nerve fibres are motor axons for the intrafusal muscle fibres, and it is by contraction of this spindle musculature that the increase in discharge is effected. A similar mechanism is now seen to exist in the crustacean muscle receptors. Although it has not been possible to split the main nerve trunk, it has been demonstrated that when stimulation of the whole nerve results in visible contraction of the receptor muscle elements, one or several impulses can be recorded from the sensory axons following each stimulus with a delay of about 10 msec. Leksell discusses the possibility of a direct facilitation of the sensory mechanism by a depolarization induced by the efferent impulses. Although it now seems that this is not the case in the spindle, the possibility of such an additional facilitating mechanism in the *RM*'s cannot yet be excluded. This, as suggested by Alexandrowicz, might be the function of the axons that he has named the 'accessory' fibres, but no evidence concerning this point has yet been found.

With respect to the response of these organs to ACh, several previous reports of a sensitivity of afferent endings to this drug are in the literature. Coon & Rothman (1939*a*, *b*) found that ACh, in an optimal concentration of 1:40,000 (as well as nicotine, 1:100,000 and α-lobeline, 1:1,000,000), can initiate axon reflexes, causing transient pilomotor and sweat responses. Concentrations higher than optimal of ACh or nicotine only inhibit further response to the optimal concentration, and the pilomotor response, at least, is not blocked by atropine. ACh can, therefore, be said to exhibit nicotine-like effects. Brown & Gray (1948) have demonstrated a similar nicotinic action of ACh in initiating spontaneous activity in nerves of the mesentery of the cat and the skin of the dog. By the elimination of other known possibilities they deduced that the recorded response must have been set up in the sensory endings by a direct action of ACh at these sites. Here, as in the pilomotor axon-reflex responses, atropine had no blocking action, and inhibition was obtainable by prior administration of higher doses of the ACh (or nicotine). This inhibition of the ACh response was not accompanied by any apparent loss of sensitivity to the stimulus of pressure and they concluded that ACh is not involved in the normal sensory function of the receptors concerned. Hellauer (1950) found that 1 % ACh solutions, dropped on the cornea of the eye, caused sensations of pain; and Umrath (1951) reports that in sensitive persons, ACh 10^{-5} can be felt as pain.

With the crayfish muscle receptor, RM_1, however, it has been possible to demonstrate directly that ACh, in low concentrations, can initiate discharge in some part of a receptor mechanism. It has been shown that ACh 10^{-6} is usually a sufficient concentration to initiate spontaneous activity when applied to the isolated receptor region of RM_1. If this preparation has been previously sensitized with eserine, ACh in concentrations as low as 10^{-8} is able to produce a definite response. Not enough is known of the pharmacology of the Crustacea to assess the validity of a distinction between nicotinic and muscarinic actions of ACh in the crayfish. It was observed, however, that the ACh response could never be blocked by increasing the concentration of this drug; atropine was seen to be an effective inhibitor of the ACh excitation; and a marked potentiation was obtained by pre-treatment with

eserine. If it cannot be concluded with certainty that a muscarinic action of ACh is evident here, it is at least noteworthy that the characteristics of the response differ in these respects from those previously reported for vertebrate receptors (see above).

Although atropine was able to inhibit the ACh excitation, it demonstrated no such action against the normal response of the receptor to stretch, but, in fact, its application was usually followed by an augmentation of the discharge. In order to maintain the hypothesis that ACh acts here as a normal transmitter substance, it is necessary to invoke some mechanism like the following. The terminal surfaces of the ganglion cell dendrites would be enclosed in compartments within which the ACh would be released during stretch. Neither the added ACh nor atropine is able to penetrate the pockets, and these drugs have their entire action on the cell or dendrite surfaces external to the compartments. There is, as yet, no histological evidence for the separation of the terminal dendrite surfaces from the rest of the neuron. If one is dissatisfied with the construction of such an hypothesis, one is left with at least two possible alternatives. The first is that ACh is not in any way involved in the normal mechanism of these crayfish muscle organs and their response to it is merely fortuitous. The second takes into account the sensitivity of the receptor to such low concentrations of ACh and the potentiation of this effect by eserine. ACh would have a role in the normal receptor mechanism as a regulator of the afferent discharge, rather than being the direct cause of its initiation. This latter hypothesis has the advantage of making compatible all the observed effects of the various drugs; but, as in the case of the others, no direct evidence can be brought to its support at present.

SUMMARY

1. The muscle organs recently described by Alexandrowicz in the tails of *Homarus vulgaris* and *Palinurus vulgaris*, have also been found to be present in the crayfish (*Cambarus clarkii*) and the rock lobster (*Panulirus interruptus*), and a study of them in the latter animals has been undertaken.

2. The position of these units within the abdomen of the crayfish differs from their position in the lobsters. In almost all other respects, however, there is substantial correspondence in their morphological features.

3. The organs are easily isolated and it is found that stretching them is accompanied by a discharge in one of the axons supplying each unit. There are two receptors in each half-segment of the abdomen and their responses show considerable differences. One has a very low threshold and can be sustained in continuous discharge for several hours. The threshold of the other is high and this receptor usually adapts completely to the most extreme stretch in less than 1 min.

4. A phenomenon which is designated 'over-stretch' has been observed. All-but-maximal stretch causes a reversible cessation of the previous high-frequency discharge. The discharge can be restored by slight release of tension, but even if the tension producing 'over-stretch' is maintained for a sufficient length of time, discharge will resume spontaneously. The possibility that this spontaneous return is due to a slow rupture of the receptor tissues is not excluded.

5. The muscular regions of the organs are not necessary for their sensory function.

6. It has been possible to show that stimulation of the nerve trunk can induce contraction in these muscular regions and that such contraction results in discharge in the sensory axons.

7. The unit with the low stretch-threshold responds to low concentrations of ACh and this effect is potentiated by eserine. In concentrations above 10^{-6} before eserinization and above 10^{-8} after eserinization, ACh consistently initiates rhythmic responses in relaxed units and augments the discharge from those under tension.

8. ACh affects the sensory mechanism directly; the muscular regions of the organs are not necessary for its action.

9. Atropine inhibits the ACh excitation, but only increases the response of the receptors to stretch. Eserine, except in high concentrations, has no consistent effect on the normal stretch discharge.

10. The possibility that ACh has a role in the normal receptor mechanism is discussed, but the question is left open.

REFERENCES

ALEXANDROWICZ, J. S. (1951). Muscle receptor organs in the abdomen of *Homarus vulgaris* and *Palinurus vulgaris*. *Quart. J. Micr. Sci.* **92**, 163–99.

BARKER, D. (1948). Innervation of the muscle spindle. *Quart. J. Micr. Sci.* **89**, 143–86.

BROWN, G. L. & GRAY, J. A. B. (1948). Some aspects of nicotine-like substances and their relation to sensory nerve endings. *J. Physiol.* **107**, 306–17.

COON, J. M. & ROTHMAN, S. (1939*a*). Nature of the pilomotor response to acetylcholine. *Proc. Soc. Exp. Biol., N.Y.*, **42**, 229–31.

COON, J. M. & ROTHMAN, S. (1939*b*). Nature of a sweat response to drugs with nicotine-like action. *Proc. Soc. Exp. Biol., N.Y.*, **42**, 231–3.

GRAY, J. A. B. & MATTHEWS, P. B. C. (1951). A comparison of the adaptation of the pacinian corpuscle with the accommodation of its own axon. *J. Physiol.* **114**, 454–64.

HELLAUER, H. F. (1950). Sensibilität und Acetylcholinegehalt der Hornhaut bei verschiedenen Tieren und Menschen. *Z. vergl. Physiol.* **32**, 303–10.

HUNT, C. C. (1952*a*). The effect of stretch receptors from muscles on the discharge of motoneurons. *J. Physiol.* **117**, 359–79.

HUNT, C. C. (1952*b*). Drug effects on mammalian muscle spindles. *Fed. Proc.* **11**, 75.

HUNT, C. C. & KUFFLER, S. W. (1951*a*). Further studies on efferent small nerve fibres to mammalian muscle spindles. Multiple spindle innervation and activity during contraction. *J. Physiol.* **113**, 283–97.

HUNT, C. C. & KUFFLER, S. W. (1951*b*). Stretch receptor discharges during muscle contraction. *J. Physiol.* **113**, 298–315.

KATZ, B. (1949). The efferent regulation of the spindle in the frog. *J. Exp. Biol.* **26**, 201–17.

KATZ, B. (1950*a*). Action potentials from a sensory nerve ending. *J. Physiol.* **111**, 248–60.

KATZ, B. (1950*b*). Depolarization of sensory terminals and initiation of impulses in the muscle spindle. *J. Physiol.* **111**, 261–82.

KUFFLER, S. W., HUNT, C. C. & QUILLIAM, J. P. (1951). Function of small-nerve fibres in mammalian ventral roots; efferent muscle spindle innervation. *J. Neurophysiol.* **14**, 29–54.

LEKSELL, L. (1945). The action potential and excitatory effects of the small ventral root fibres to skeletal muscle. *Acta physiol. scand.* **10**, suppl. 31, 1–84.

VAN HARREVELD, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. Exp. Biol. N.Y.* **34**, 428–32.

PANTIN, C. F. A. (1948). *Notes on Microscopical Technique for Zoologists*. Cambridge University Press, p. 54.

TALAAT, M. (1933). The effect of ions on the cutaneous sensory endings of the frog. *J. Physiol.* **79**, 500–7.

UMRATH, K. (1951). Über die Erregung freier sensibler Nervenendigungen durch die Erregungssubstanz sensibler Nerven. *Z. vergl. Physiol.* **33**, 457–61.

WATERMAN, T. H. (1941). A comparative study of the effect of ions on whole nerve and isolated single nerve fiber preparations of crustacean neuromuscular systems. *J. Cell. Comp. Physiol.* **18**, 109–26.

WIERSMA, C. A. G., FLOREY, E. & FURSHPAN, E. (1952). Effect of acetylcholine on a stretch receptor of crayfish. *Fed. Proc.* **11**, 172.

EXPLANATION OF PLATE 8

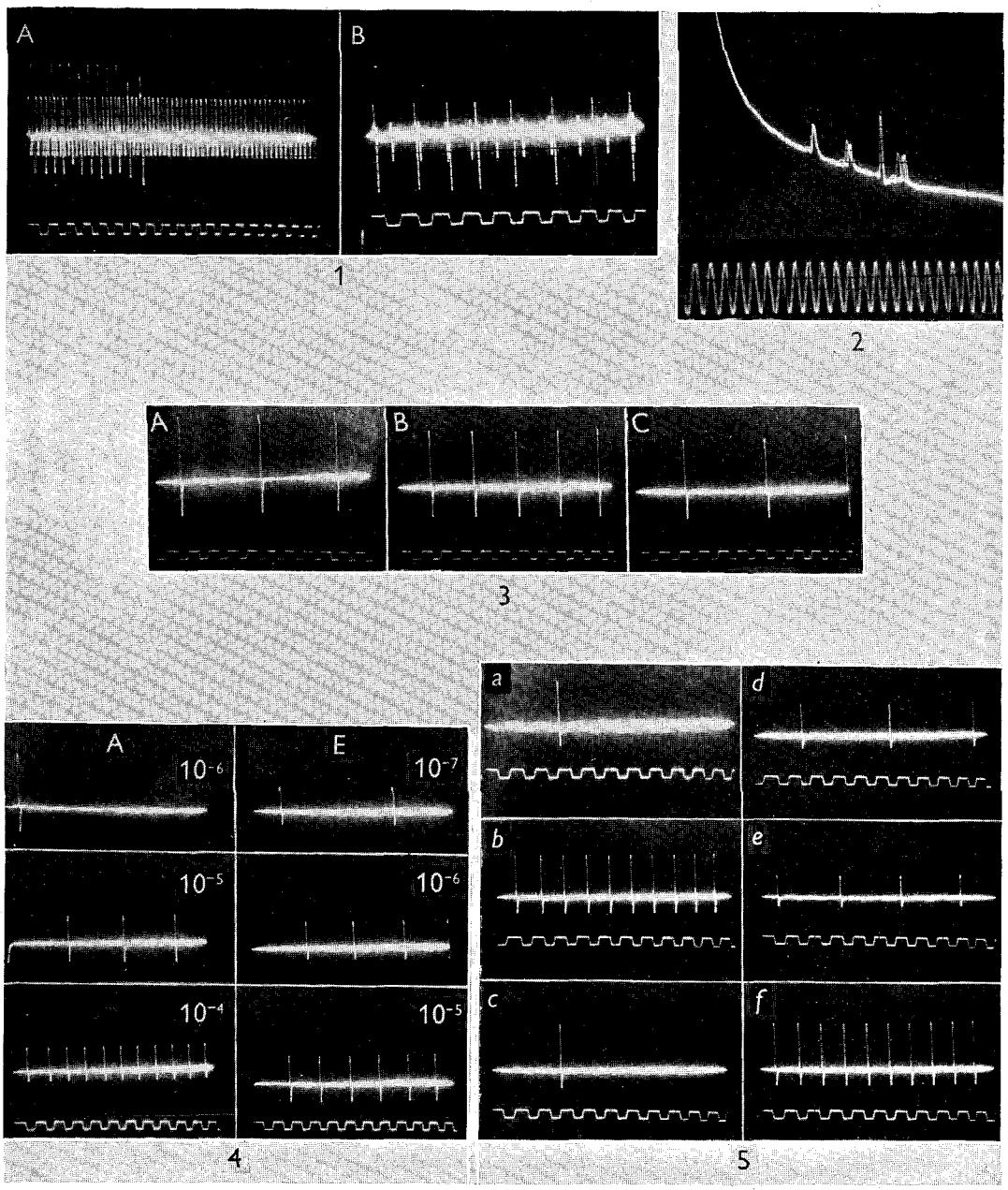
Fig. 1. Action potentials recorded from the nerve to the muscle receptors of A, the crayfish (*Cambarus clarkii*) and B, the lobster (*Panulirus interruptus*). A moderate amount of stretch has been applied in each case. In both A and B the larger spikes are from RM_2, the more rapidly adapting organ. Time signal: 60 cyc./sec.

Fig. 2. Response of sensory fibres to motor stimulation of the nerve trunk. One unit responds to each stimulus with a repetitive discharge, whereas the other responds only once. The direct response of the whole nerve trunk to each stimulus is hidden in the stimulus artefact. Time: 1000 cyc./sec.

Fig. 3. The effect of ACh 10^{-9}. A, the response of a slightly stretched preparation of RM_1; B, the increase in this response following addition of one drop of ACh 10^{-9}; and C, the return to the original discharge frequency following washing. This response to very low concentrations was not always reproducible nor related to concentration; but see fig. 4. Time: 60 cyc./sec.

Fig. 4. Response of RM_1 to various concentrations of ACh. A, before bathing in eserine 10^{-5} for 3 min., and E, after. The numbers refer to the concentration in grams/millilitre of the drop of applied ACh solution. The tergal strip was fully extended and there was no stretch-discharge prior to addition of drugs. Time signal: 60 cyc./sec.

Fig. 5. The effects of atropine. A preparation of RM_1, slightly stretched and giving the response shown in *a*. This stretch is maintained throughout the experiment. *b* shows the result of adding one drop of ACh 10^{-4}. After washing, the discharge returns to its original level, *c*. Then a drop of atropine 10^{-4} is added and a slight excitation follows, *d*. A second reapplication of a drop of ACh 10^{-4} now increases the response only slightly, as shown in *e*. Washing re-establishes the ACh sensitivity as shown in *f*. Time: 60 cyc./sec.



WIERSMA, FURSHPAN AND FLOREY—MUSCLE RECEPTOR ORGANS OF THE CRAYFISH

ADDENDUM TO PART I

S. W. Kuffler (J. Neurophysiol. (1954), 17: 558) and, Kuffler and C. Eyzaguirre (Fed. Proc. (1955), 14: 89) have recently reported some additional studies on the crustacean abdominal stretch receptors. In the first paper, Kuffler used preparations from lobster and crayfish (Homarus americanus and Procambarus alleni) and confirmed the physiological results presented above. He also studied the contractile responses in greater detail and showed how they correlated with the resultant afferent discharge. He applied stimulation to the whole nerve trunk and with these stimulating conditions the contractions of RM1 and RM2 were characteristically different. If RM2 contracted at all it gave a twitch response, whereas only slow shortening was seen in RM1. At frequencies of stimulation at about 10 per sec. discrete twitches were recorded in RM2 by means of a "strain gauge," while at higher frequencies (75 per sec.) smooth plateaus of tension appeared. Depending upon the amount of initial stretch, the twitches of RM2 could each be accompanied by one or several afferent impulses in the sensory axon. Contraction in RM1, however, was accompanied by a smooth increase in the afferent discharge frequency, and the sensory impulses did not follow the frequency (or some multiple of the frequency) of the stimulation. If the initial tension

was sufficiently large, a single efferent impulse could result in a train of sensory nerve impulses. He does not consider the possibility, especially in RM1, that some of the augmentation of sensory discharge following efferent stimulation could be due to an excitatory nerve fiber ending directly on the sensory cell. Although the presence of such a nerve fiber has not been demonstrated, Kuffler and Eyzaguirre (see below) have proven the existence of a direct inhibitory axon; and there are still histologically demonstrable axons whose function is unaccounted for. Kuffler has also made some observations on electrical events occurring in the receptor muscle fibers and these will be considered in Part II.

Kuffler and Eyzaguirre (see above) have published a note concerning electrical changes in the sensory cell bodies of the stretch receptors. They used microelectrodes for intracellular recording and found steady depolarizations accompanying stretch. If the latter was sufficiently strong and the steady depolarization exceeded a certain threshold, a train of impulses was evoked. The frequency of the sensory impulses was higher with larger depolarizations. The inhibitory axon mentioned above was found to function by counteracting the steady depolarization. In particular it tended to return the membrane potential to its normal level and caused depolarization if the cell was hyperpolarized. The steady depolarization or "generator potential" had previously

been observed in frog muscle spindles by B. Katz (J. Physiol. (1950), 111: 261). The action of the inhibitory axon is similar to that reported by Fatt and Katz (J. Physiol. (1953), 121: 374) for inhibition in crustacean muscle.

Part II.

ELECTRICAL EXCITATION IN CRUSTACEAN MUSCLE

A. Introduction.

The experimental section of this part concerns the electrical responses which are evoked in crustacean muscles by stimulation of their motor nerves. The significance of these responses lies in the role usually assigned to them in the sequence of events leading from motor nerve impulse to muscle contraction. This sequence is: (a) conduction of the impulse in the motor axon to the junction between axon and muscle; (b) transmission of excitation across this neuromuscular junction; (c) a state of muscular excitation which in some manner is spread along the muscle fiber; (d) coupling of the muscle excitation to contraction; and finally (e) the muscle contraction. Although some of the boundaries implied above may be artificial, each of the processes is conveniently studied separately, often using different techniques. The electrical responses of muscle enter the scheme as the only known concomitants of the state of excitation mentioned in (c). As well as an interest in the excitation process itself, part of the motivation for these studies was the hope that they would serve as a basis for experiments on excitation-contraction coupling, (d), for several attributes of crustacean muscle seem to make them promising objects for such an investi-

gation.

In order to facilitate subsequent comparisons, a discussion of vertebrate neuromuscular systems will ensue. That which immediately follows does not apply to the "slow" muscle fibers of the frog, nor to the muscles of spindle organs. With these exceptions it is known that a vertebrate muscle is organized into many motor units. The motor unit may be defined as a single motor neuron plus all of the muscle fibers which it innervates. Aside from mechanical summation of contraction, the response of the motor-unit is generally all-or-nothing. The motor-unit concept implies that a particular muscle will receive many motor axons; that the contractile response resulting from the stimulation of any one axon will be small, constant, and similar to that of any other; and that variation in the number of activated motor neurones will be a very important way of grading contraction. In short, the motor unit provides the quantum of response. Estimates of the number of motor axons innervating a single muscle (and thus of the number of motor units) vary from about 95 in the rabbit sartorius (1) to 4000 in sheep eye muscle (2). Intermediate values ranging from 275 to 600 have been given for various cat limb muscles (3). All of these values are too high, however. Those for limb muscles include the "small nerve fibers" (4) which are now known to innervate spindle musculature, and that for the eye muscles includes proprioceptive afferents. Estimates are also available of the num-

ber of muscle fibers in a single motor unit. These calculations involved the number of motor axons innervating the muscle and are similarly in error, being too low. Values for rabbit sartorius are given as 100-125 (1), for leg muscles of the cat as 120-165 (5), and for sheep eye muscle as about 5 (2).

It has been maintained (6,7) that vertebrate "twitch"* muscle fibers receive only one nerve-ending each. It is, in fact, implied by the motor unit concept that a muscle fiber will not be innervated by more than one motor axon. There is, however, good experimental evidence that single twitch muscle fibers can receive two or more endings and that these can be from branches of the same axon or from different axons (polyneuronal innervation). As early as 1924, Cattell and Stiles (8) showed that stimulation of either of the ventral roots supplying the gastrocnemius of the frog gave almost as much tension as stimulation of both simultaneously. Katz and Kuffler (9) have given more direct evidence for the presence of two and more end plates in almost every muscle fiber of the frog sartorius. In one type of experiment they recorded the compound muscle action potential at either end of the muscle before and after severing one of its two main nerve

---

*The word "twitch" is used here to signify those muscle fibers which have conducted action potentials and contract rapidly in response to one of these action potentials; and are thus distinguished from the "slow" muscle fibers of the frog and perhaps the muscle fibers of the spindle organs.

branches (pelvic or tibial). Before either nerve branch is cut, the size and latency of the action potentials recorded from both ends are very similar. After cutting the pelvic branch, however, the latency of the potential recorded from the pelvic end is increased by an amount equal to the time necessary for conduction of the muscle potential from the focus of tibial nerve endings to the focus of pelvic endings. Further, although the height of the potential now recorded from the pelvic end is decreased, this is at least partially due to temporal dispersion because of the increased length of the conduction pathway in the muscle. These observations were interpreted as follows. Most of the fibers in this muscle receive innervation near both ends, from the tibial and pelvic branches of the nerve respectively. Action potentials initiated at the two end plates, as well as propagating to the ends of the fiber, also travel toward the center where they extinguish one another halfway between the endplates. Thus, only the action potential from the nearby endplate is recorded at either extremity of the muscle. When, however, one of the nerve branches is cut, the electrode at that end then records the action potential initiated at the far endplate as evidenced by the temporal dispersion and increased latency. It is interesting that in this same paper, Katz and Kuffler cite a histological study by Sandmann published in 1885 (10) which had been overlooked in the more recent literature of their time. In it Sandmann reported two, three and

more endplates on most fibers of the frog sartorius. In a very recent study Hunt and Kuffler (11) reported further experiments using the techniques of measuring contraction-tension and recording muscle potentials with intracellular electrodes. Seven different muscles, four of them in the cat and three in the frog, were studied. These authors were able to confirm and extend the observations of Cattell and Stiles (8), that the sum of the muscle tensions obtained when each of two ventral roots is stimulated separately is greater than that obtained by simultaneous stimulation of both roots. With intracellular recording they were able to show in one of the cat muscles and several frog muscles that stimulation of different portions of the motor nerve supply can elicit action potentials in the same muscle fibers; and these potentials usually had different latencies, presumably due to the time for conduction in the muscle fiber. They showed that, in some cases, much of this multiple innervation is poly-neuronal. The cricothyroideus of the cat is particularly interesting, for it receives two separate nerves; the superior laryngeal branch of the vagus and a nerve from the pharyngeal plexus. The large majority of muscle fibers showed action potentials, as measured with an intracellular micro-electrode, when either nerve was stimulated. Further, they found that in many of the fibers the two end plates were sufficiently close to each other to show summation of their respective local potentials.

It will also be interesting to compare the way in which the neurally induced excitation is spread over the muscle fibers of different animals, and this attribute of vertebrate twitch muscle will be discussed next. Typically, a single nerve impulse arriving at one of the few endplates on a muscle fiber initiates a local depolarization across the muscle fiber membrane which is called the endplate potential (usually abbreviated as e.p.p.). At most endplates this depolarization is sufficiently large to activate the muscle fiber's conduction mechanism and an action potential propagates longitudinally until it either arrives at the end of the fiber or collides with an action potential initiated at another endplate. Endplates may also be present in normal muscle, however, which require facilitation to initiate the propagated wave (12,13). But in either case the conducted muscle action potential qualitatively resembles that of nerve. Both of them overshoot beyond zero potential, are all-or-nothing, leave a wake of refractoriness, and are dependent upon the presence of sodium ions in the external medium. The height and time course of both are similar. Thus, in summary of the several preceding paragraphs, vertebrate twitch muscle fibers receive one to several endplates which terminate branches of one to several axons. All of the muscle fibers innervated by one neuron constitute a motor unit. The contraction of this relatively small unit has been thought of as the quantum of which the normal contractile response is additively composed;and although

there may be some overlapping of motor units (polyneuronal innervation), and some fibers of a unit may require facilitation before a conducted impulse is initiated, the concept is for the most part valid and useful. Excitation spreads along the muscle fiber as a self-propagating wave which resembles the nerve impulse. The conduction mechanism resides in the muscle fiber's own excitable membrane.

As intimated above, however, not all vertebrate muscle has these properties. The most notable exception is the category known as "slow" or "tonus" muscle fibers which have been found occupying parts of certain muscles of the frog. They have most recently been studied by Kuffler and Vaughan Williams (14). These fibers receive many endings which seem to be mostly from different axons. A good estimate of the number of endings is not yet available, but it is probably more than several per mm. The action potentials of this type of muscle fiber are also unusual in several respects. They do not propagate actively along the length of the muscle fiber; the density of nerve-endings providing for the spread of excitation. They do not overshoot the resting potential in magnitude, having on the average an amplitude of about 10 mv. There is no refractoriness following them and successive ones may summate to give a larger depolarization. The size of the summated depolarization is, however, limited and the potential response to a train of nerve impulses always shows a plateau. The time course of the slow muscle poten-

tials has a peculiar aspect; for although the initial part of their decay is approximately exponential, as one might expect for a local, passively declining potential, the depolarization is followed by a smaller and prolonged phase of hyperpolarization. The contraction of the slow muscle fibers also differs from that of the twitch fibers; for the former display smooth graded contractions even at low frequencies of stimulation and the response to a single maximal nerve volley is very small. The time course of shortening is slow and is measured in seconds, while relaxation may take minutes. The exact values vary considerably with the conditions of recording and stimulation (15).

The muscle fibers of the spindle organs (intrafusal fibers) have not been studied in as much detail as have those of the twitch and slow systems because of difficulty in technique, but there are some things known about them. Discrete endplates similar to those of twitch fibers have been demonstrated with histological methods by Barker (16) and shown to be few in number. A spindle organ most often has twice, or slightly more than twice, as many motor endplates as intrafusal fibers, about half of them being distributed on either side of the sensory area. Since it seems unlikely that conduction of muscle action potentials proceeds across the highly modified sensory region, it may be said in these cases that there is only one endplate per contractile element. This might seem to argue for the presence of conducted action potentials;

however, it is not conclusive, since the length of the muscle elements is small. In the rabbit this value is about 1 mm. which is approximately the same as the length constant of muscle (as measured in the frog (17)). Consider the case of an endplate located in the center of a 1 mm. length of muscle fiber. A local potential set up at the endplate would have to spread only 0.5 mm. in either direction and, since it decays exponentially with distance and falls to 37% (1/e) in 1 mm., it would only decrement to 60% of its original value in 0.5 mm. Thus no decision on this basis is really possible and the question of local as against propagated action potentials must await more direct investigation. Several other features of the intrafusal motor innervation were noted by Barker. It was quite common that a motor axon would branch before terminating and contribute to more than one endplate either on the same fiber or more often on adjacent fibers. Further, single endplates frequently received branches from several axons which were separate as far as he was able to follow them.

With the vertebrate, somatic neuromuscular system just described serving as a frame of reference, an account of the development of the present concepts of this same system in the crustaceans will be presented. Prior to 1936 several of the characteristic phenomena of crustacean muscle had been observed, but agreement as to their cause was not general. For example, in 1907, Keith Lucas (18) described two types of

contraction obtained from the muscle which closes the claw of the lobster. He referred to these as slow and twitch contractions; and found that the strength-duration curve for indirect (by way of the nerve) stimulation showed a discontinuity at the same point at which one type of contraction was replaced by the other. In 1917 (19) he extended these studies to the crayfish and further showed that a curve relating height of contraction to the interval between two nerve stimuli was often bimodal. From these results he concluded that the two contraction types were elicited by stimulation of different "substances" (types of nerve elements). This interpretation was not universally accepted at the time, however, and alternative explanations for the two contraction types were offered. Wiersma, in 1933 (20), advanced the hypothesis that the frequency of the motor nerve impulses was the important parameter determining which contraction type ensued; and that the impulses eliciting both would travel in the same nerve elements. He postulated that one nerve impulse would evoke no contractile response, but that two in rapid succession would give a twitch. A longer train of impulses at lower frequency would, on the other hand, give rise to the slow response. In 1934, Wiersma and van Harreveld (21) brought additional support to this idea from experiments on the muscle opening the claw of the hermit crab. This muscle was shown by histological methods to receive only two axons, one of which was assigned the function of mediating peripheral inhibition. (Inhibition in

isolated legs was first described by Biedermann (22) in 1887). These authors were able to demonstrate both twitch and slow responses in this muscle, even though only one axon remained to subserve a motor function.

C. F. A. Pantin (23,24,25) also presented evidence that crustacean muscles, with one exception (the crusher claw of Homarus), would be innervated by only one excitable element. He, too, emphasized the importance of frequency of nerve impulses in determining the character of the muscular response and explained this dependence in terms of all-or-nothing recruitment of additional muscle fibers following facilitation at their neuromuscular junctions. In support of this he presented a continuous curve relating contraction to stimulation frequency throughout the responsive range. Katz (26) corroborated these results. His response-frequency curve was sigmoid and very steep on either side of the inflection point so that most of the response range was obtained with a narrow band of stimulation frequencies. He also emphasized the importance of the recruitment of muscle fibers following neuromuscular facilitation and showed that response to nerve stimulation was much more frequency dependent than that to direct muscle stimulation. Both Katz and Pantin worked with relatively slow moving marine crabs (Carcinus maenas). This choice of muscle preparation was, however, unfortunate in one respect, and this will be discussed further at a more appropriate place below.

It is now known that more than one type of motor axon can innervate a single crustacean muscle and that the electrical and mechanical responses evoked by each can differ with respect to height, time course, facilitation, and fatigue. The first conclusive demonstration of this was provided by van Harreveld and Wiersma (27) in 1936. They isolated single functioning motor axons from crayfish claw nerves and stimulated them independently. (Previous to this Wiersma (21) had shown that it was possible to split limb nerves into small bundles which would still conduct impulses.) In particular these authors studied axons evoking contraction in the muscle which closes the claw (abbreviated as "the closer"). In normal preparations two such axons were always found and stimulation of the remainder of the nerve never resulted in any contraction of this muscle. A single impulse in the thicker of the two axons evoked a rapid twitch, while a train of impulses was needed in the thinner fiber to obtain even a small contraction. About 30 seconds was required to attain maximal tension during stimulation of the thin axon at 40 shocks per second. This time for the thick nerve fiber was only about 1 sec. and the terms "slow" and "fast" were adopted to describe the thin and thick axons, respectively. These same terms are also used with reference to the muscle action potential and contraction evoked by the thin and thick axons. The muscle potentials showed even greater differences, the slow potentials being very small, but growing with repetitive stimulation. Despite this growth

(facilitation) they remained smaller than the single fast muscle action potential. In this muscle, which was later shown to be atypical in this respect, the fast action potential exhibited no augmentation with repetition.

In a number of succeeding papers these authors used the method of isolating single axons, as well as observing the stained nerve fibers branching on the surface of the muscle, to determine the pattern of innervation of the major limb muscles in a number of different crustaceans. This type of study, limited, however, to the use of histological techniques, goes back as far as Biedermann (22), who observed that the nerve fibers ramifying on the surface of the opener of the claw of the crayfish were apparently all branches of only two axons. At every branch point the two nerve fibers could be seen to divide at the same place. Mangold (23) corroborated these findings and introduced the term "diplotomic" branching for this type of simultaneous divisions of two axons. Hoffmann (29) added some further observations, one of the most interesting being that one of the axons innervated two different muscles: a fiber innervating the extensor of the carpopodite ("stretcher" muscle) continued into the next podomere to end in the abductor of the dactylopodite ("opener" muscle). He showed diplotomic branching for the opener, stretcher and "bender" (flexor of the carpopodite) muscles. Although a number of the observations of these older workers proved to be correct, the histological methods by themselves were insufficient; and it was only when the latter were combined with

physiological techniques that more extensive information on the limb muscle innervation patterns was obtained.

As stated above van Harreveld and Wiersma (27) demonstrated physiologically that the closer of the crayfish claw received two motor axons. Staining with methylene blue, however, revealed three axons branching on the muscle surface in a "triplotomic" manner (30,31,32). It was suspected that the third was an inhibitory fiber and this was confirmed physiologically by Marmont and Wiersma (33). The original contention by Biedermann, that the crayfish claw opener received only two axons, one excitatory and one inhibitory, was confirmed (31,33). The stretcher muscle was found to be similar, receiving one each of inhibitory and excitatory axons; and the observation of Hoffmann (29) that one of the axons was also common to the opener muscle was also confirmed. The common axon was found to be excitatory and served as the sole motor nerve supply for both muscles (31). Further studies revealed that in some marine species (brachyurans and anomurans) these two muscles receive three nerve fibers (30,34) and the additional axon was subsequently shown to be inhibitory (35). It has now become clear, in fact, that almost all variations in the innervation patterns of decapod limb muscles may be ascribed to differences in the inhibitory nerve supply (36). Inhibition will not be considered further here, but for details of the comparative anatomy and physiology of this system the following references should be consulted: 33 and 35 through 40. The numbers of motor axons to the seven most peripheral limb

muscles were subsequently worked out for numerous decapod species and are as follows: opener - one (31); stretcher - one (same axon) (31); closer - two (27); bender (flexor of propodite) - two (31); main flexor (of carpopodite) - four (41); accessory flexor (of carpopodite) - one (41); and extensor (of carpopodite) - two (31). There may very well be exceptions to this scheme. For instance, in Blepharipoda occidentalis, four axons can be seen branching on the stretcher muscle which normally receives only one motor axon. It would be necessary for three of the axons to be inhibitory to keep the scheme inviolate, but this has not been tested by stimulation of single axons. Nevertheless, the above values are very useful in predicting the number of motor axons to a given limb muscle of a decapod.

A question then arises concerning the innervation of the individual muscle fibers. Can muscle fibers receive branches from each of the two or several axons (polyneuronal innervation) or are there several non-overlapping motor units? The first case requires that a muscle fiber respond in different ways to the stimulation of different nerve fibers. The first experimental results concerning this question were obtained by van Harreveld (34) using histological techniques. He initially studied the opener of the crayfish claw, which was known to receive one inhibitory and one motor axon. From the physiological evidence that the contraction of the opener can be completely inhibited (33), it was to be expected that all fibers of this muscle would receive branches from both axons;

and, indeed, he was able in many cases to observe both pairs of axon branches from a terminal diplotomic division ending on a single muscle fiber. Although the case was not as simple for a triply innervated muscle (two motor and one inhibitory axons), histological evidence was obtained that in such muscles all three axons can end on one muscle fiber (34). Physiological evidence concurred. Wiersma and van Harreveld (42) showed that stimulation of one of the motor axons to the crayfish claw closer augmented a contraction evoked by stimulation of the other shortly afterwards. No mutual influence with respect to fatigue or facilitation of the action potentials could be demonstrated. These results indicated a pathway susceptible to mutual influence somewhere between the muscle action potential and contraction; and thus it seemed likely that at least some of the contractile substance was activated by both axons. van Harreveld (43) presented more direct evidence from microscopical observations of the contraction of individual muscle fibers. Movement of a fiber is not a sufficient criterion for contraction, since active fibers passsively move their neighbors. He noticed, however, that slight curves in the relaxed fibers were straightened out, unaccompanied by wrinkling of the surface, during contraction. He was thus able to observe the two types of contraction in single muscle fibers. In fact, every fiber observed to contract during stimulation of one of the motor axons also responded to stimulation of the other.

These studies of polyneuronal innervation were extended by van Harreveld (34) and van Harreveld and Wiersma (41) to quadrupally and especially quintupally innervated muscles. The manner of branching of the motor axons in the two cases was analagous with the diplotomic type and the terms "quadruplotomic" and "quintuplotomic" were used to describe them. The analogy was taken as inconclusive evidence that the four or five axons would also all innervate individual muscle fibers. Some physiological experiments bearing on this point were performed with the quintupally innervated muscles (flexor of the carpopodite in Panulirus interruptus). Four of the axons proved to be excitatory and one, the thinnest, inhibitory. Interaction between the four motor axons was studied and a small heterofacilitation of contraction was found. The test contraction was about equally augmented by previous stimulation of any of the other three motor axons. It was also observed that the ratios of the sizes of the four muscle action potentials was constant for different parts of the muscle. These results will be considered at greater length in the discussion section as they pertain to the present findings.

Little has been said, as yet, concerning the nature of the contractions evoked by the one to four motor axons innervating a particular muscle. It was noted above that Lucas distinguished twitch and slow contractions in the crayfish claw closer (19). van Harreveld and Wiersma (27) confirmed this in their isolated axon experiments which were performed on the homologous muscle of a crayfish of a different species.

If one examines other crustacean limb muscles, however, one often finds the term "twitch" inapplicable, as more than one nerve impulse is necessary to elicit even small contractions (31). This tendency away from twitch-like shortening becomes extreme in certain crab muscles as studied by Wiersma and van Harreveld (44). They found that in the flexor of the dactylopodite of the walking legs (homologous with the claw closer) of Cancer anthonyi, stimulation at frequencies of less than 100 shocks per sec. evoked smaller and slower contractions when applied to the thicker of the two motor axons (fast axon). Above this frequency the reverse situation was obtained (i.e. response to the thin axon was slower). Nevertheless the terms "fast" and "slow" were retained for the thick and thin axons, respectively, for two reasons. Firstly, the muscle action potentials evoked by the thick axon were larger than those set up by the thin at all stimulation frequencies; and secondly, even though the frequency of stimulation of the fast axon had to be high to evoke a contraction, the number of impulses needed was very small. In fact two shocks separated by less than 30 msec. resulted in contraction when applied to the thick axon, but never when applied to the thin one. A shorter latency of contraction for the fast system would thus be expected and this was actually observed. These findings allowed an understanding of the results of Pantin (24) who also worked with crab muscles and found smooth frequency-response curves for stimulation applied to the whole nerve. He interpreted this incorrectly to signify the absence of two

qualitatively different contraction systems. The absence of a discontinuity in these curves merely reflected the lack of a sharp transition from one contraction type to the other. It will be recalled that in their early experiments Wiersma and van Harreveld (21) also obtained results discordant with the idea of two different types of motor axons. In experiments with the opener muscle of the hermit crab claw, which receives only one motor axon, they obtained both twitch and slow contractions by varying the strength and frequency of stimulation. In 1941, Wiersma investigated this muscle again (45) and confirmed the original results: only one motor axon could be found and twitch and slow contractions could result from its stimulation. The twitch probably finds its explanation in a phenomenon called the spike potential, which will be described below. Nevertheless, this muscle may be atypical in this regard and was an unfortunate choice of object for these early studies.

A phenomenon of some considerable interest was observed by Wiersma and van Harreveld (46). In certain muscles (e.g. Blepharipoda occidentalis, claw closer), low frequencies of stimulation (10-15 shocks/sec.) applied to the fast axon could elicit large muscle action potentials unaccompanied by any visible contraction; while a stimulus of the same frequency delivered to the slow axon evoked much smaller muscle potentials which were, nevertheless, accompanied by contraction. This phenomenon was referred to by the authors as the "paradox." Taken with previous evidence that both axons seem

to innervate the same muscle fibers, the paradox is of theoretical significance in attempting to understand the coupling between muscle action potential and contraction. This additional emphasis placed upon polyneuronal innervation of single muscle fibers motivated some of the present studies.

Another basic attribute of crustacean muscle is the large number of endings which any one axon has on individual muscle fibers. Ripley (47) used the term "multiterminal innervation" to describe this situation in insect muscle and it will be used here to avoid the confusing term "multiple innervation." This type of innervation has been known for a long time and was first described for insects by Foettinger (48) in 1880. Mangold confirmed and extended his observations, also histologically, in 1905 (28). It is interesting that Mangold found diplotomic and quadruplotomic branching mentioned above for the crustacea. Marcu (49) described multiterminal innervation for fibers of fly muscle (about 20 endings per fiber).

Wiersma and van Harreveld (46) anticipated the finding of multiterminal innervation in crustacean muscle from their physiological experiments. They reasoned that since fast and slow action potentials could reside in the same muscle fiber and in some preparations the slow potential would be followed by a contraction, while the larger fast potential was not (the paradox), then the potentials must occupy different sites on the fiber and must not be all-or-nothing. This reasoned against conducted action potentials and for many separate sites of excitation. In 1939, van Harreveld (34)

was able to see the very fine terminal regions of the motor axons, using a silver stain, on the surface of crayfish muscle. He was not able to observe any discrete structure, such as the endplate of vertebrates, but saw the axons tapering continuously to invisibility. In one very good preparation of a doubly innervated muscle (opener), van Harreveld was able to see twenty-eight such endings on a single muscle fiber (3.5 mm. long). At each of the twenty-eight places both of the axons terminated together. All of the errors of counting and histological preparation tended to diminish the number of visible endings, so that this number may be below the average. In similar studies on triply innervated muscles of the crayfish (extensor, bender), van Harreveld observed that the three components of a terminal branch did not all end close to one another (34). The thinnest fiber usually ended first and probably did not stain in its terminal regions. Of the two thicker elements one usually continued beyond the other, branching by itself and thus showing more endings. Because of the numerous terminations on each muscle fiber and the fact that even small nerve branches may run for considerable distances on the fiber surfaces, a dense tangle of intramuscular nerve elements is seen. van Harreveld (43) described this as a feltwork.

Physiological evidence was also presented to support the contention of multiterminal innervation. It was reasoned that if excitation could be distributed to all parts of the muscle fibers by the feltwork of axons, conducted muscle action poten-

tials of the vertebrate type would be superfluous. van Harreveld (43) stimulated muscle fibers directly with very small electrodes. This was done in preparations which had ceased to respond to axon stimulation to avoid the possibility of conduction in the intramuscular nerve branches. The only contraction observed was localized in the region of the electrodes and did not propagate along the remainder of the muscle fiber. Further, the potentials usually recorded by Wiersma and van Harreveld had many of the properties of graded, local potentials. They could, in most cases, summate; were not followed by refractoriness; and, as usually recorded, were monophasic (44,50). Exception was taken to these results by several authors. Holmes (51) disputed van Harreveld's histological data, maintaining that the feltwork of nerve fibers was in reality only connective tissue and that each muscle fiber would receive only one or a few nerve endings. And in 1946, Katz and Kuffler (52) demonstrated conducted action potentials and contended that the local-type potentials could only be recorded focally like the endplate potentials of curarized vertebrate muscle. Twitch contractions were observed to accompany spikes; while with the local-type potentials, slower smoother contractions were seen. Thus they viewed crustacean muscle as essentially similar to that of the vertebrates with two quantitative differences. In the crustaceans (1), the safety factor of transmission would be lower so that facilitation and recruitment would be more important and (2), the tendency for muscle to contract in

response to endplate potentials would be intensified. They first demonstrated propagated muscle potentials following direct stimulation of the muscle fibers and measured conduction velocities of about 25 cm. per sec. They were also able to show diphasic potentials arising out of nerve-induced "local" potentials if the latter were sufficiently large. It seems likely that in their preparations these nerve-induced spikes were also propagated (see fig. 3 of that paper). Concerning their finding of sharply localized endplate potentials, it is significant that the experiments were performed using "strip" preparations. These are obtained by cutting main nerve branches to most parts of a muscle, leaving only a small strip of fibers still innervated. This preparation represented an attempt to approach the physiological ideal of working with single units, but it is probably because of it that the presence of focal endplate potentials has not been confirmed by more recent investigations. For example, in 1953 Fatt and Katz (53) reported the results of experiments using the intracellular recording technique (glass microelectrodes) to study the distribution of the local-type potentials along the length of muscle fibers in two species of crabs. They found that wherever a microelectrode entered a muscle fiber an "endplate" potential could be recorded following nerve stimulation. Further, they were able to enter a particular muscle fiber at a number of points along 5-6 mm. of its length, and measure the amount of variation in the sizes of the potentials so recorded. In

experiments on fourteen fibers the ratio of the highest to the lowest value (for each fiber) averaged 1.4±0.045. The duration of the rising phase of the potentials was practically constant. These results, taken with the fact that the length constant of crustacean muscle is about 1 mm. (distance in which local changes in potential passively decrement to 1/e of their value at the source), constitute a very convincing demonstration of multiterminal innervation. Confirmation of these data is presented in the section treating the present results (IIC). In this same paper, Fatt and Katz report several instances of being able to record a response in one muscle fiber to stimulation of each of two axons.

There has also been discussion concerning the conducted muscle potentials reported by Katz and Kuffler (52). Wiersma (54) suggested that an additional regenerative process or spike potential did not exist and offered an alternative explanation for these conducted waves. He argued that the experimentally evoked, local muscle potentials would in turn stimulate the hypolemmal branches of the motor axons in the adjacent regions. These in turn would elicit a local muscle potential in the new region and this cyclic process would continue, resulting in propagation. He felt that in normal muscle this situation would not arise since nerve impulses would tend to arrive at the endings simultaneously. Recent experiments with intracellular microelectrodes, however, have demonstrated clearly that an additional process signalled by a

spike potential can occur in crustacean muscle (55),(also see present results). Fatt and Katz (55) used two internal microelectrodes, each forming a pair with an external electrode in the bathing solution. One pair was used for passing current across the muscle fiber membrane (stimulating) and the other for recording. No attempt was made to observe responses to nerve excitation and it seems unlikely that they stimulated terminal axon branches unintentionally. When sufficiently large depolarizing currents were passed across the membrane by the stimulating electrodes, a spike potential was often seen at the recording microelectrode arising from the resultant electrotonic potential. By entering a muscle fiber at several points successively, it was shown that the spike potential could be propagated and at a velocity averaging about 30 cm. per sec. Local action potentials, apparently restricted to the region of the stimulating microelectrode, were also often observed. Evidence will be presented in the Results section, that in neurally activated crustacean muscle, the role of the spike is not primarily that of a distributor of excitation. Another interesting finding was also presented by these authors (55). Replacement of the sodium ions of the external medium by choline only resulted in the augmentation and prolongation of the spike potential. These effects were even more pronounced when tetraethylammonium and tetrabutylammonium were used as the sodium substitutes. Spike potentials which have been studied elsewhere (squid giant axon (56), frog muscle (57), and frog myelinated nerve (58)) are uniformly

diminished by the replacement of sodium by choline. Lorente de Nó (59), however, has shown that tetraethylammonium, as well as some other quaternary ammonium compounds, can substitute for sodium in certain small fibers in frog nerve.

The older experiments on crustacean muscles can be summarized by describing the two opposing views which they engendered. On the one hand was the concept of a novel excitatory system. The muscle fiber surface was considered as a mosaic of discrete spots at which local electrical events were evoked by numerous nerve endings, and the activity at some of these spots could differ from that at others. The other view stressed the similarities of crustacean neuromuscular mechanisms to those of the vertebrates. Only one or a few endplates were visualized at which propagated muscle action potentials could be initiated, although it was also felt that considerable contraction could accompany the local endplate potentials. Considerable reconciliation of these two ideas has been effected by the recent work of Fatt and Katz (53), as well as by the present experiments.

The remainder of this section is, in a sense, a digression, for few additional references to crustaceans will be made. It is an attempt to present what is known of the neuromuscular mechanisms of other animals, in order to consider the extent to which some of the above-mentioned attributes might be general ones. In this respect the insects are most interesting. These arthropods also have a small number of motor nerves fibers innervating any particular muscle (47,60,61,62), and

different contraction types have been observed. For example, Pringle (60) distinguished twitch and slow contractions in the flexor tibia of the cockroach. By varying the strength of stimulation to the nerve both before and after subjecting it to a certain amount of drying, he was able to evoke the two contraction types separately. He observed visually that the same muscle fibers could be involved in either type of contraction. He thus assumed double motor innervation of all the muscle fibers and speculated that the difference between twitch and slow contraction would lie in the number of muscle fibers activated by the two nerve fibers. Thus he attributed the facilitation observable in the slow system to a progressive recruitment of additional muscle fibers, each fiber giving an all-or-nothing response. Wilson (63) has recently worked with the same muscle and offered an alternative explanation of the two contraction types. He stimulated the muscle through its nerve and recorded the resultant electrical activity in numerous of its fibers with intracellular microelectrodes. He distinguished two types of electrical responses: (1) one had a duration of 3-4 msec., was followed by a refractory period and often overshot beyond zero potential (size range 22-85 mv.), while the other (2) lasted about 6-8 msec., showed summation rather than refractoriness and was smaller (8-20 mv.). He concludes that the two types of potential underlie the two types of contraction and that each occurs in different muscle fibers, much as in the frog (14). His arguments in support of the latter conclusion are not very convincing. For example,

he points out that both responses occur in fresh, unfatigued muscle and therefore the small slower potential would not merely be an endplate-like response which failed to elicit a spike. Katz and Kuffler (52), however, showed that endplate-like potentials, too small to evoke spikes, are obtainable from the muscles of intact crabs. And it will be shown below that both types of potential (endplate and spike) can be elicited from the same muscle fibers of various crustaceans. Wilson also reports that he never observed the transition from one type of potential to the other while recording from a single fiber, but it seems very probable that some type of prepotential precedes the spike response.

The most comprehensive study of an insect muscle is reported in several very recent and very interesting papers by Hoyle (62,64,65). He worked mostly with the extensor tibia of the migratory locust and showed that it received three motor axons. One of the axons, which runs in a separate nerve, seemed to innervate all of the fibers of the muscle and evoked in them an action potential consisting of a spike arising from an endplate-like potential. This axon was referred to as "F," indicating that it gave rise to a typical fast response. A second axon, referred to as S_1 (signifying a slow response), seemed to have two different types of endings and could produce qualitatively different effects in two classes of muscle fibers. S_1 innervated only about 30% of the fibers of the muscles. In about two-thirds of these, its stimulation evoked small, slow potentials lasting over one second and capable

of summating to plateaus of depolarization of 50 mv. The other one-third of these fibers showed more rapid responses to stimulation of fiber S_1, which were only slightly smaller and slower than those evoked by F. The slow responses were designated as S_{1a} and were accompanied by slow contractions, while the more rapid action potentials were referred to as S_{1b} and gave rise to twitches. The third axon (S_2), which was smaller than either F or S_1, produced an electrical response in only some of the fibers and was not seen to be accompanied by contraction. The form of the electrical response was a brief depolarization followed by a longer lasting hyperpolarization. Although both phases were small (less than 1 mv.), the hyperpolarizations could summate during repetitive stimulation and thus raise the resting potential of the fiber. The S_2 response was most clearly seen in fibers with low resting potentials and could not raise the membrane potential above the level of about 70 mv. Hoyle has not been able to demonstrate that S_2 causes any inhibition of either contraction or action potentials evoked by the other two axons. In fact stimulation of S_2 sometimes seemed to augment the contraction elicited by F and Hoyle ascribes to S_2 the function of raising the membrane potential briefly before a maximum effort is required by the animal. This might be useful inasmuch as muscle fiber resting potentials were found by him to be low in locusts feeding on certain diets (66). Since the F response occurred in all fibers tested, it can apparently be present in combination with the other three types of potential,

and records are given from two fibers showing F and S_{1b}, and F and S_2. Responses S_2 and S_{1a} recorded from a single fiber are also shown and presumably this fiber received axon F as well. No fiber was found which showed both S_{1b} and S_2 effects, and other combinations are not mentioned.

Insect muscles may receive more than three axons. Ripley (47) found as many as six contraction steps accompanying gradual increase of the intensity of stimulation applied to the nerve supplying the flexor of the tibia, in a different species of grasshopper (Romalea microptera). Four of the contraction types were twitches, while two were slower. Hoyle also found up to four steps of twitch contraction in the homologous muscle of Locusta (64), and although he did not observe the slow response, he reports from a histological study (62) that this muscle receives "a relatively large number of motor axons." He concluded that not more than one of the twitch-evoking axons innervates any one of the small bundles into which these muscles are organized. A microelectrode inserted into individual muscle fibers never recorded more than one fast action potential and this would always be associated with a particular contraction step. In this case the concept of the motor unit seems more applicable than in any other arthropod system yet studied.

del Castillo, Hoyle, and Machne (67) reported that the size of the endplate response does not vary appreciably along the length of the fibers of the flexor tibia of Locusta. Hoyle corroborated this in his later paper for the extensor tibia of

the same species (64). Thus, multiterminal innervation is apparently present in insects as well as in crustaceans. The original histological demonstrations of multiterminal innervation were, in fact, on insects (28,48).

In brief summary of the studies on insects, it may be said that both polyneuronal and multiterminal innervation have been demonstrated. As well as endplate-like potentials which evoke spikes, there are slower potentials which can cause steady changes in the resting membrane potential level and which do not seem to elicit spike potentials.

Very few of the details of muscle innervation given above for vertebrate and arthropod systems are available in any other of the phyla. The numbers of motor axons to a muscle, the numbers of these which innervate the individual muscle fibers, or the numbers of endings on a typical muscle fiber, are essentially unknown for other animals. But there are indications that further study of these systems would be interesting. Two types of contraction have been recognized in various molluscs for some time. Winton (68) showed that the anterior byssus retractor of Mytilus edulis responds differently to alternating or direct current stimulation. Following the cessation of a.c. stimulation, the muscle relaxes rapidly, but after d.c. stimulation does so over one hundred times as slowly. In this slowly relaxing state the muscle may support considerable tensions for long periods. Winton showed that d.c. stimulation applied for 14 sec. of every minute resulted in a sustained or steadily increasing con-

traction. A.c. stimulation given in the same time relations resulted in only one discrete contraction per minute. A.c. stimulation after d.c. abolishes the delayed relaxation. Fletcher (69) confirmed these findings and tried to stimulate the muscle through its nerve supply, but was unsuccessful. He recorded the muscle action potentials with a mechanical device writing on smoked paper and found that the response to a single shock was propagated at less than 20 cm. per sec. and was of the order of 1 sec. in duration. The electrical response to repetitive or d.c. stimuli was a plateau with irregular potentials superimposed upon it. Pumphrey (70) obtained evidence for the mediation of twitch and tonic contractions by separate nerve groups, but in a different species. He recorded impulses from intact motor nerves, primarily from those to the anterior adductor muscle, in the clam, Mya arenaria. Contractions were elicited reflexly through tactile stimulation. Accompanying contraction in response to different strengths of stimulation were particular sequences of motor nerve impulses. Correlated with the rapid initial closure of the valves following strong tactile stimulation were large nerve impulses which eventually dropped out, although the animal remained closed for some time. The remaining small nerve impulses were also the sole concomitants of very weak slow contractions following slight stimulation. It appears from his records that impulses in the small nerve fibers evoke very little contractile response by themselves, but can cause maintainance of tension which has been initiated by the

large nerve impulses. Pumphrey refers to it as a delay of relaxation and from this deduces that the large and small axons must innervate the same muscle fibers. A phenomenon observed by Blashko, Cattell and Kahn (71) should, however, prompt caution in the acceptance of this reasoning. Working with crustacean muscle they found that if, during stimulation of the nerve to the closer muscle of the claw at a frequency eliciting only slight contraction, they passively closed the claw against the resistance of the recording lever, the claw remained more or less closed after removal of the passive, externally applied tension.

Ramsay (72) developed a nerve-muscle preparation of the retractor of the buccal mass in the snail, Helix pomatia. He recorded summating muscle potentials, a single one of which could be followed by a slow contraction in some preparations. Gradually increasing the strength of stimulation of the nerve, several contraction steps were observable. From histological studies he discerned two types of nerve fibers, thin deeply staining (1-3 μ) and thick (6-10μ) faintly staining ones. He confirmed an observation by Röchling (73), that a thick and a thin axon could run together and branch diplotomically within the muscle. This was not, however, the only manner of branching that could be seen. These observations are very enticing, but not complete enough to validate a comparison with the crustaceans.

Some doubly functioning molluscan muscles are anatomically differentiated into two parts. For example, in the scallop,

Pecten, the largest portion of the adductor muscle is yellowish, clear and striated; while the remainder is white, opaque and unstriated. The striated portion is responsible for the twitch-like swimming movements of Pecten, while the other part shows slower tonic contractions (74). (The muscle with which Pumphrey worked (see above), however, does not correspond to this description, being homogeneous and unstriated throughout.) Lowy (75) has recently recorded contractions and action potentials from the two parts of the adductor of Pecten. Confirmation was supplied that twitch-like closures of the shell were due to contractions of the striated portion; while during maintained closures, the smooth muscle portion was active. The action potentials of the smooth part have the slower time course of the two. This muscle, then, is roughly analagous to those of the frog which have separate twitch and slow areas, but different from the crustaceans where twitch and slow contractions proceed in the same muscle fibers. It is interesting to note that Lowy was always able to record action potentials in the slow portion during its prolonged contractions; and this was also true for the wholly smooth adductors of Mytilus (76), which could remain contracted for up to 300 hours. These observations do not support the idea of a molecular catch-mechanism which would allow prolonged contractions without expenditure of energy. Such an idea has been put forth by several authors (e.g. 77,78).

J. Z. Young (79) performed experiments with nerve-muscle preparations from the squid, Loligo. The mantle of this animal

is composed almost entirely of striated muscle fibers running circumferentially and radially in a matrix of connective tissue. Nine to eleven pairs of nerves from the stellate ganglion form its complete innervation. Each nerve contains one giant axon (250-700 μ) and numerous smaller axons (less than 50 μ). Young was able to stimulate these stellar nerves and record contraction from the appropriate area of the mantle. The response to a single shock to one of the nerves was a practically all-or-nothing twitch of one area. If all of the small nerve fibers were cut away leaving only the giant axon, the response was essentially the same. If, however, the giant axon was pricked with a needle, but the small fibers left intact, smaller contractions of the same mantle area were obtained; and these were graded according to the stimulus strength. The two types of contraction correlate with the two types of mantle movements performed by the live squid. One is a violent contraction accompanying jet-propelled swimming and the other is a gentle, rhythmic movement associated with respiration. It is not known if both giant and small axons innervate the same muscle fibers, or what the mechanism of gradation is in the small-axon system. Neither has the action potential nor its manner of distribution been studied. Prosser and Young (80) found that the height of the contraction evoked by the giant fibers is initially independent of frequency. The entire duration of a single twitch is about 200 msec. Thus it has the fastest time course so far found for molluscan muscle and in this respect, and in the absence of facilita-

tion, it resembles the closer muscle of the crayfish and somatic muscle of vertebrates (with the exception of frog slow muscle fibers).

Prosser and Melton (81) have recently found evidence of qualitatively different motor nerves in the smooth muscles of the sipunculoid worm Phascolosoma. The muscles studied, the proboscis retractors, were known from previous work (82) to display two types of action potentials, a spike and a slow wave. The spike was found to fatigue easily on repetition, to evoke a twitch contraction and to be followed by refractoriness. The slow wave facilitated and summated and was associated with tonic contractions. With stimulating electrodes applied directly to the muscle (excitation nevertheless proceeds through the intramuscular nerves) the spike was for the most part all-or-nothing, but the slow wave was graded according to the intensity of stimulation. Both spike and slow wave were conducted away from the stimulating cathode at velocities of about 1-2 m. per sec. and 0.3-0.4 m. per sec., respectively. Evidence was presented that this conduction also proceeds by way of intramuscular nerves. Using very small stimulating electrodes, Prosser and Melton, were able to elicit either spikes or slow waves, or both. The type of response depended upon the position of the stimulating electrodes with respect to nerve fibers and small bundles of nerve fibers which they could discern on the muscle surface. From histological studies they were also able to distinguish two types of nerve fibers. One group had diameters in the vicinity of 2 μ, while the others

were below 1 μ. A detailed diameter spectrum for comparison with spike and slow-wave conduction velocities would have been interesting but was not provided. Evidence was presented that each of the two types of muscle response arises from stimulation of one of the nerve fiber groups, although this could not be proved directly. The spike potential usually preceded the slow wave and the latter is thus not a prepotential (endplate-like potential) for the former. It also suggests that the function of evoking the slow response may be assigned to the small axon group on the basis of the relative conduction velocities. No data were given which would help to decide whether the innervation of individual muscle fibers was by one or both axon groups (i.e. whether both types of response could be elicited in a single muscle fiber).

Prosser, Curtis and Travis (82) not only studied Phascolosoma proboscis retractors, but also recorded action potentials from the latern retractors of Thyone and the byssus retractors of Mytilus. The Thyone muscles showed two types of potentials, fast and slow. The former fatigues readily, while the latter facilitates on repetition. Only one type of potential was observed in the Mytilus muscle. This is a wave which shows summation and no refractoriness; and is conducted at about 0.6 m. per sec. One suspects that conduction here is also mediated by intramuscular nerve branches, but this is not known.

Very little study has been devoted to annelid motor systems. Giant axons located in the central nervous system are very common. In some cases they apparently send branches

directly to the body musculature and thus serve as terminal motor axons. This situation has been reported for the polychetes Myxicola infundibulum (83) and Branchiomma vesiculosum (84). Stimulation of the giant axons gives an all-or-nothing contraction which rapidly fatigues and shows no facilitation unless the contraction has become considerably reduced by repetition. The contraction involves the entire worm and the mechanisms for more local graded contractions were not discussed. In other annelids, the giants are not the primary motor axons (85) and papers concerning the latter were not found.

C. F. A. Pantin and his collaborators have studied the motor mechanisms of actinian coelenterates (sea-anemones) in some detail. A net of synapsing neurones provides for the central nervous system as well as the peripheral effector axons. Much of the work has been devoted to the properties of this nerve net and is more germane to a discussion of nervous systems, but there are several phenomena which are pertinent to the present considerations. The earlier work of Pantin was mostly with Calliactis parasitica as experimental animal, while the more recent studies of Batham and Pantin have been on Metridium senile. One response studied in Calliactis was the contraction of the sphincter muscle (at the top of the column) to stimulation applied to the side of the column (86). A single electric shock evokes no visible contraction unless the intensity is extremely high. Two shocks, however, above a certain low threshold do elicit a response.

The size of the latter is dependent upon the interval between the two stimuli, provided that this is greater than about 50 msec. (absolute refractory period), but not much greater than 1 sec. (facilitation interval). The sphincter muscle will respond to a single stimulus of intensity not much greater than the two-shock threshold, if it is applied directly to the muscle itself (87). These phenomena were interpreted by Pantin as follows. The first of two stimuli applied to the side of the column would result in a nerve impulse arriving at each "endplate" of the sphincter muscle, but would be unable to activate the muscle. This would, however, facilitate the neuromuscular transmission process so that a second nerve impulse arriving soon after might effect activation. The increase of contraction with shorter intervals would be due to the success of the nerve impulse at a greater number of junctions. This implies an all-or-nothing contraction process in the muscle fibers. There are systems in the crustaceans which show augmentation of contraction with decrease of the two-shock interval, but for which an increase of graded processes in all of the muscle fibers is responsible, rather than recruitment. These do not, however, show the large difference between the responses to one and two stimuli seen in the anemones; and it is upon this that Pantin puts the greatest burden of his argument. As an alternative explanation it might be possible, for example, that the last synapse of the nerve net would require facilitation. Direct proof pertaining to this question will be difficult to obtain. The muscle fibers

are usually less than 1 μ in diameter although they may be over 1 mm. in length. Their diameter practically excludes the possibility of intracellular recording. It is not, in fact, clear that an electrical concomitant of conduction and muscular activation can be readily measured. Pantin (88) states that no electrical activity has yet been detected, but he does not disclose how assiduous was the search. An interesting histological detail appears in the same paper. This is in the form of an "endplate" photographed from a preparation of Dr. Batham's. The width of the endplate structure (about 10 μ) suggests that it supplies a number of muscle fibers, although these do not appear in the picture.

Metridium differs from Calliactis to the extent that it shows considerable spontaneous contraction of its various muscles under apparently constant external conditions. These movements are extremely slow and are barely detectable visually, but are quite apparent in kymograph records made with slowly moving drums. There is a particular sequence of contraction of several muscles, which lasts several minutes. The whole sequence may be repeated after somewhat varying intervals (several sequences per hour) giving some suggestion of rhythmicity (89). A very similar sequence can be evoked by a train of stimuli at a frequency below that which normally elicits the more rapid contractions associated with reflex activity (90). Batham and Pantin typically used five or ten stimuli at frequencies of from one in 2 sec. to one in 15 sec. In contrast to the semitetanic appearance of the contraction

following stimuli at higher frequencies (1 or 2 per sec.), these slow responses are entirely smooth and may outlast the stimuli by several minutes. There is also a prolonged latent period preceding the contraction. It is not known if the fast and slow type responses both occur in the same muscle fibers, although both can occur in the same muscle in which no obvious histological differences among the fibers are found.

It is apparent from the above discussion that outside of the arthropods very little is known of the more intimate details of neuromuscular mechanisms in invertebrates. There was one feature, however, which seemed to be quite general among the systems considered. Rapid and slow contraction were evoked by different groups of nerve fibers, and in some cases the muscle was also differentiated into fast and slow parts. In this regard, then, the mammals seem to be unusual in their total reliance on a twitch-type system in their somatic musculature. (It is not known if this is also true in birds and reptiles, however.) Part of the difficulties in studying some of these invertebrate systems lies in the nature of the materials, themselves. Most of the muscles consist of very small cells which are typically imbedded in tough connective tissue. Nor are lengths of nerve available in most animals which are amenable to manipulation. These facts emphasize the usefulness of the crustacean systems with their long, easily split nerves and large, free muscle fibers. But as might be expected, other difficulties are present and these will become apparent in the following pages.

B. Methods.

Preparations and Methods of Stimulation:

Nerve-muscle preparations from a number of decapod crustaceans have been used for these studies. In each case, some portion of the nerve containing the appropriate motor axons had to be isolated for stimulation, and in many experiments single axons were prepared. Further, some surface of the muscle needed to be exposed to allow electrical recording. The way in which the dissection was carried out to meet these requirements is detailed below for each preparation used. In addition any techniques peculiar to the use of that preparation will be described.

(1) The muscle of the quick-adapting abdominal stretch receptor, RM2:

The crayfish, Cambarus clarkii (Girard) was the only species from which this muscle was prepared. The dissection differs in only minor respects from that described in Part I, pp. 2 and 3. The tergal strip of the abdomen was isolated and the extensor musculature of one side of one segment removed. If this is properly done only a T-shaped structure formed by the stretch-receptor muscles and their nerve remains in that half-tergite. RM1 was cut free of its terminal attachments and its connection with the nerve trunk. Since the muscular parts of the remaining organ (RM2) were now the most important, a more diligent attempt was made to keep

them undamaged. In the previous experiments the ends of the muscle strands had been clamped in mounted forceps to effect removal from the tergite. For the present purposes, however, the tissue on which RM2 had its attachments was dissected out and clamped instead. This was especially difficult at the posterior attachment which is obscured by the lateral, deep extensor muscle, and damage to this end of the receptor muscle often resulted. Damaged regions are readily recognized by their opacity and were never seen to extend anteriorly beyond the sensory region. In all experiments reported below, only the portion of the muscle between the sensory region and the anterior attachment was used, so that this posterior damage probably had little effect on the results. A length of nerve suitable for stimulating is obtained in this dissection. Attempts to split this nerve with sharp needles were unsuccessful and responses were evoked by stimulation of the whole of it.

Some of the experiments required moving the recording electrode small known distances. The fine adjustment knob for the horizontal traverse of the micromanipulator was calibrated for this purpose. The smallest units were 50 μ and distances could be estimated accurately to within 10 μ.

(2) The flexor of the dactylopodite (closer muscle) of the thoracic limbs:

Several species have provided this preparation. Two grapsoid crabs, Pachygrapsus crassipes and Hemigrapsus nudus were most frequently employed. In these animals only the

four most posterior pairs of non-chelate limbs were ever used. After removing the limb at the autotomy joint in the ischiopodite, a particular strip of exoskeleton was clipped away with a pair of bone forceps (or finger-nail clippers) from the propodite. This strip of shell covers the surface of the "closer"* muscle opposite to the one which contacts the "opener" muscle. The strip is narrow and is the only region of exoskeleton on that side of the propodite which is free from muscle fiber attachments. Care must therefore be taken in removing the strip to avoid probing the underlying and adjacent fibers. This was facilitated by the fact that all the grapsoid crabs used had the still uncalcified "shell" of the next molt underlying the calcareous exoskeleton. This tough structure helped to protect the muscle fibers and could then be removed with greater care under the dissecting microscope using fine scissors. A successful dissection was signified by the translucent appearance of the muscle fibers. When damaged they became white and opaque. The animals were almost always used within several hours, and sometimes within minutes, after removal from their natural environment.

The "closer" muscle was also used from non-chelate limbs of two species of the crab, Cancer: C. antennarius and C. productus. The dissection technique was analagous with that used on the grapsoid crabs. In the specimen of Cancer,

---

* The words closer and opener are put in quotation marks when referring to muscles of non-chelate limbs.

however, a tough next-molt "shell" was not present, but the limbs were large enough to permit dissection without damaging muscle fibers. Specimens of Cancer were not as readily captured and were kept in the laboratory for longer periods of time before being used. Aquaria, supplied with running sea water served to keep them in good condition.

In the crayfish, Cambarus clarkii and Astacus trowbridgii, only the first thoracic legs, the chelae, were large enough for convenient preparation of the closer muscle. These limbs differ from the walking legs of the crabs in at least one respect. In the claw the only region of the propodite (excluding the pollex) which is free from closer muscle fiber attachments is that surrounding the opener. In this preparation, therefore, the approach was from the opposite side of the muscle. After clipping away the shell overlying the opener, that muscle was removed by severing its distal attachment and stripping it back. The surface of the closer muscle still remained covered by two large nerves and a thin membrane. After removal of the membrane, only the surface of the closer between the nerves was exposed for probing with the microelectrode; and to this extent the preparation was of limited value.

The technique for isolating the appropriate motor axons was essentially identical for all of the above preparations and has been described by van Harreveld and Wiersma (27) and Wiersma (35).

(3) The main flexor of the carpopodite:

This muscle will be referred to subsequently as the "main flexor." Panulirus interruptus, the California rock lobster, was the only animal from which successful preparations were made. The technique employed, differed in several respects from that described by van Harreveld and Wiersma (41). The animal was encouraged to autotomize a limb at the natural breaking joint in the ischiopodite. Bleeding often occurred, nevertheless, and cauterization of the stump was routine procedure (41). The muscle lies in the meropodite and for these experiments its entire free surface was exposed. This is the face of the muscle contiguous to the extensor of the carpopodite and the one on which lie the various nerves coursing through the meropodite. To effect this exposure the inner, slightly concave part of the exoskeleton of the meropodite and ischiopodite was clipped away. The area of shell to be removed is conveniently pigmented black and is approximately the region which receives extensor muscle fiber attachments. Next, the extensor tendon was severed distally and that muscle was removed. The limb was transected at the carpo-propodite joint and the distal piece discarded. The remainder then consisted of carpopodite, meropodite and part of the ischiopodite. A modified femur clamp was used to secure this piece at the mero-carpopodite joint and served to support the preparation as well as prevent flexion during muscle contraction.

The nerve containing the main flexor axons was split in a small region on either side of the ischio-meropodite joint. In some preparations there were two nerves and in others three, at this level. The nerve containing the desired axons could be recognized as the thinner (or thinnest). Any other nerves but this one were removed. Before the remaining nerve could be split it was necessary to remove a mass of sticky connective tissue surrounding it. The success of the subsequent dissection depended considerably upon the extent to which this sometimes tedious cleaning was performed. Care was also taken to keep the proximal attachment of the nerve intact. The nerve contains seven efferent fibers which are sufficiently larger than the numerous sensory axons to be readily recognized. Of the seven, only four were used in the present experiments. Of the other three, one is the main flexor inhibitor; one, the stretcher-"opener" motor axon; and the third, the stretcher inhibitor (41). It was necessary to remove these latter three and it was often possible to recognize them without having to stimulate each of the seven axons in turn. The flexor inhibitor was always the thinnest of the seven. The other two were often closer to one another than any other two axons; and they usually ran on the side of the nerve opposite to that from which the first branch to the main flexor arose.

Many dissections failed to yield four separate, functioning motor axons, one or more having been damaged in splitting the nerve. In those cases that were successful, the axons were

transected as proximally as possible and each hung over a separate platinum hook electrode. Two electrodes were carried by a Zeiss micromanipulator and two by a double rack and pinion device. A layer of paraffin oil was poured on top of the bathing solution and the four electrodes, with axons attached, were lifted through the aqueous-oil interface. A grounded Ag-AgCl electrode was immersed in the aqueous phase and by means of a selector switch, stimulating voltages could be applied between it and any of the hook electrodes. With this method the potential is effectively applied along the axon between the hook and the oil-aqueous interface. Provided that the hook electrodes were made positive with respect to ground, this method of stimulation was very satisfactory. Excitation occurred with low voltages and cross-stimulation of other axons was absent at maximum available intensites.

The live lobsters were obtained from the Kerckhoff Marine Station at Corona del Mar. They were transported wrapped in wet burlap atop a cake of ice. All animals survived the journey and could usually remain alive for an additional three weeks in their new environment. This consisted of an aquarium containing 18 liters of sea water, constantly aerated, kept at 10° C., and renewed about once a week.

Potential Recording:

Muscle potentials were recorded almost exclusively using intracellular microelectrodes (86). These were made of glass

and drawn from melting-point capillary of about 1 mm. outside diameter. The completed electrode tapered to a tip considerably less than 1 μ in diameter. Fig. 1 shows an electronmicrograph of one such electrode. It was the only one observed in the electron microscope and may not be typical. In most cases, however, the tips did not seem to be resolvable using the high power objective of a light microscope and were thus probably less than 0.5 μ in diameter. The apparatus used to draw the microelectrodes was quite simple to both assemble and use. Its essential parts were a vertically pulling spring (20 cm. long, extending 3 mm./gm.) and a heating element consisting of a loop of nichrome wire (1.5 turns of #26 wire, 3 mm. in diameter). It is not known within what limits these characteristics may be varied, but they are probably wide. Current was supplied to the heating loop by a high-wattage step-down transformer (110 v./12 v.). The primary of the latter was connected to the output of a variable transformer. The step-down transformer allowed more delicate control of the heating current, since its output voltage only showed changes about 10% as large as those of the variable transformer. In actual operation a clamp carried by a micromanipulator held the lower end of a 4 cm. length of the capillary tubing. The latter passed through the nichrome loop and was attached by another clamp at its upper end to the spring, which was extended by about 10 cm. The micromanipulator allowed the center of length of the capillary to be placed in the center of the heating loop.

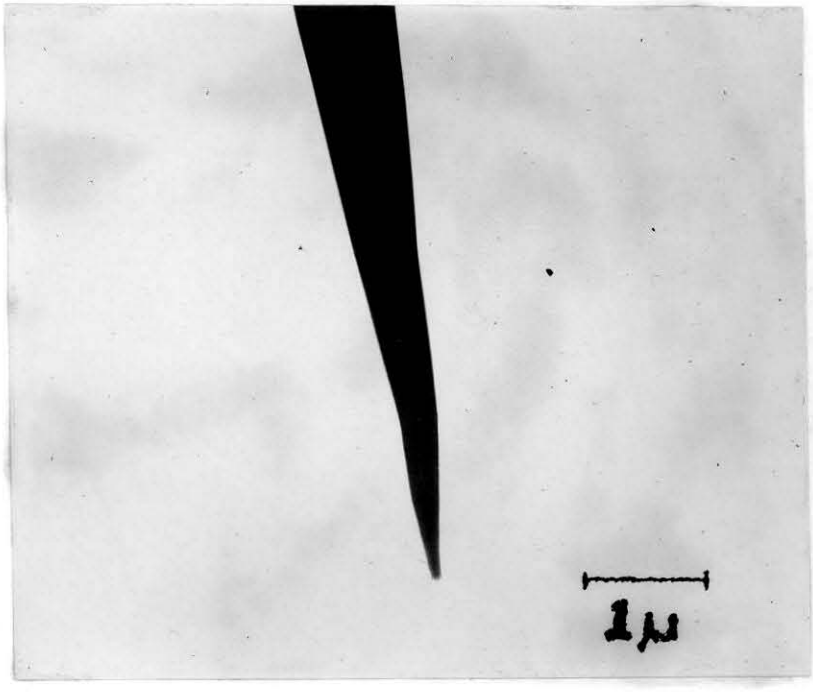


Fig. 1. Electronmicrograph of a glass microelectrode. This electrode was drawn with the apparatus described in the text. Many of the electrodes used were probably not this small.

These precautions provided two microelectrodes of equal length from each piece of capillary; and insured that the glass did not contact the hot wire.

The microelectrodes were filled with 3 M. KCl by putting them in a boiling bath of that solution for 15-20 min. The details of the technique were similar to those described by Ling and Gerard (87). It was found that if air bubbles remained in the electrode tips after boiling, they would usually dissolve completely in the surrounding fluid during the following several days. Electrodes were usually made, therefore, that much in advance of their being required and stored over water in a "dessicator." It was possible to prepare completely about 50 electrodes as a batch in about an hour. This greatly facilitated the experiments, since a dozen or two electrodes were sometimes broken on a single preparation.

The position of the microelectrode was also controlled by a Zeiss micromanipulator to which it was affixed by means of a stout, capillary-bore, glass tube. The microelectrode shank was wedged into a polyethylene collar in the end of the tube. This arrangement held the electrode firmly enough and allowed rapid replacement of broken ones. A fine silver wire, coated with AgCl, was inserted directly into the shank of the microelectrode through a hole in the wall of the stout tube. The silver wire led, by means of a short length of double shielded wire, to a compact cathode-follower input stage which was mounted on the micromanipulator. These measures helped to reduce the input capacitance and thus

the input time constant. A low input capacitance is necessary in view of the large input resistance offered by the microelectrode. A few measurements of this resistance yielded values of about 20-40 megohms. A single measurement of the input capacitance gave 3 pF. No more exact assessment of the time constant was attempted since neither the absolute magnitude nor the exact time course of the action potentials was of importance for the conclusions.

The cathode follower was built according to the design of Bishop (88). It had a balanced input to two #954 acorn tubes. Its output was divided and led (1) directly to the input of the resistance-coupled amplifier of one channel of the double-beam oscilloscope and (2) to the input of a condenser-coupled pre-amplifier. The output of the latter fed into the other channel of the oscilloscope (Dumont #322). The oscilloscope amplifiers provided a maximum sensitivity of 25 mv. per inch deflection of the trace, while the pre-amplifier (Techtronix, #22) increased this either 100-or 1000-fold. In sampling the electrical response of the fibers of a muscle, the microelectrode was poised over the desired region by observing its position through the binocular microscope. Following this, attention was directed to the oscilloscope beam registering the d.c. amplifier response while the microelectrode was slowly lowered into the muscle. The successful penetration of a fiber was then signalled by the sudden deflection of this beam in the proper direction to indicate measurement of a resting potential. The motor axons

could then be stimulated and the response observed or photographed.

The convenience of using both beams of the oscilloscope, one registering the response at a greater amplification than the other, arose from the great variation in the size of these responses. Muscle potentials of less than 0.1 mv. and greater than 50 mv. had to be observed in the same preparation.

Physiological solutions:

van Harreveld's crayfish solution (89) was used to bathe preparations of both Astacus and Cambarus. Sea water was used for Cancer and the grapsoid crabs. A solution for Panulirus was prepared from the data of Schlatter (90) and had the following composition:

NaCl	30.5 g.
KCl	0.9
$CaCl_2$	2.3
$MgCl_2 \cdot 6H_2O$	2.0
$NaHCO_3$	to pH 7.2
H_2O	to 1 liter

B. Results.

The present experiments concern, for the most part, four attributes of the crustacean neuromuscular mechanism. Two of them have been considered in greater detail than the others, but they are all interdependent. The terms that will be used to describe them are as follows: (1) junctional potentials, (2) spike potentials, (3) multiterminal innervation, (4) polyneuronal innervation. Although all of these terms have been used before in describing other systems, they have not, with the exception of (2), been applied to crustaceans. Operational definitions of them will be given in the descriptions of the observed phenomena, and a justification of their use will be attempted in the Discussion.

Muscle potentials:

During experiments on the sensory cell of the stretch receptors, using intracellular microelectrodes, entries into the strand of muscular tissue of the quickly adapting organ, RM2 (see p. 3), were also made. Following stimulation of the whole nerve, which contains motor axons for this muscle, two types of potential response were visible. Fig. 2 is a photograph of a single sweep of the oscilloscope beam, during which two stimuli were applied to the nerve. The two small diphasic transients preceding the responses mark the times of application of the stimuli. The small ripple in the record is at 60 cycles per sec. It can be seen that the response to the second stimulus differed from the first mainly in the presence

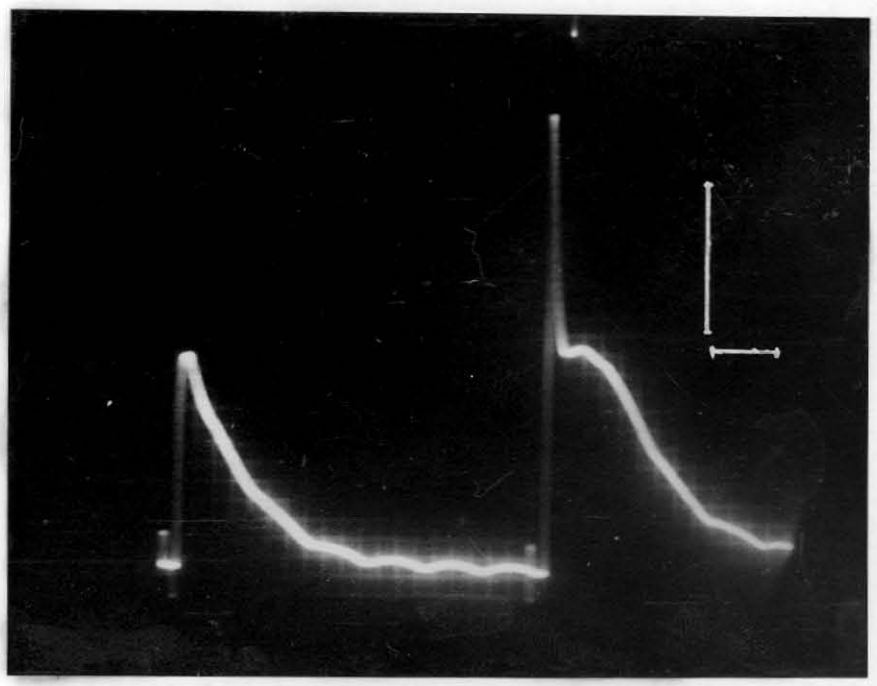


Fig. 2. Junctional potential and spike potential. From an RM2 muscle of Cambarus. The first response is a junctional potential. The additional sharp peak in the second response is a spike potential. Calibrations: time, 20 msec. (horizontal line); voltage, 20 mv. (vertical line).

of a sharp spike of potential. The additional hump following it was also a common feature, but will not be considered further. The relations between these two types of potential response and some of their other properties will be examined at greater length below. For the present this record serves to define "junctional potential" (first response) and "spike potential" (the sharp peak of the second response).

The junctional potential is quite similar in appearance to local, post-synaptic responses elsewhere observed (e.g. at the vertebrate motor endplate, and in spinal cord motoneurons). Such potentials have, in general, been shown to be local and graded, and upon exceeding a certain size to elicit an all-or-nothing propagated spike. It then becomes interesting to determine in how far these properties are applicable to the potentials under consideration; for these attributes indicate something of the mechanism of the spread and distribution of excitation.

In all experiments the junctional potentials did indeed prove to be graded, in that successive ones could summate to a greater size. Examples are given in Fig. 3. In Fig. 3a, recorded from a preparation of the main flexor of Panulirus, the junctional response to a single stimulus to one of the motor axons is shown (at two amplifications). b-d show summation of junctional potentials recorded from a different fiber of the same preparation. Note the lower sweep speed than in a. The photographs are multiple exposures each showing seven sweeps. In all sweeps the first nerve stimulus (of 2)

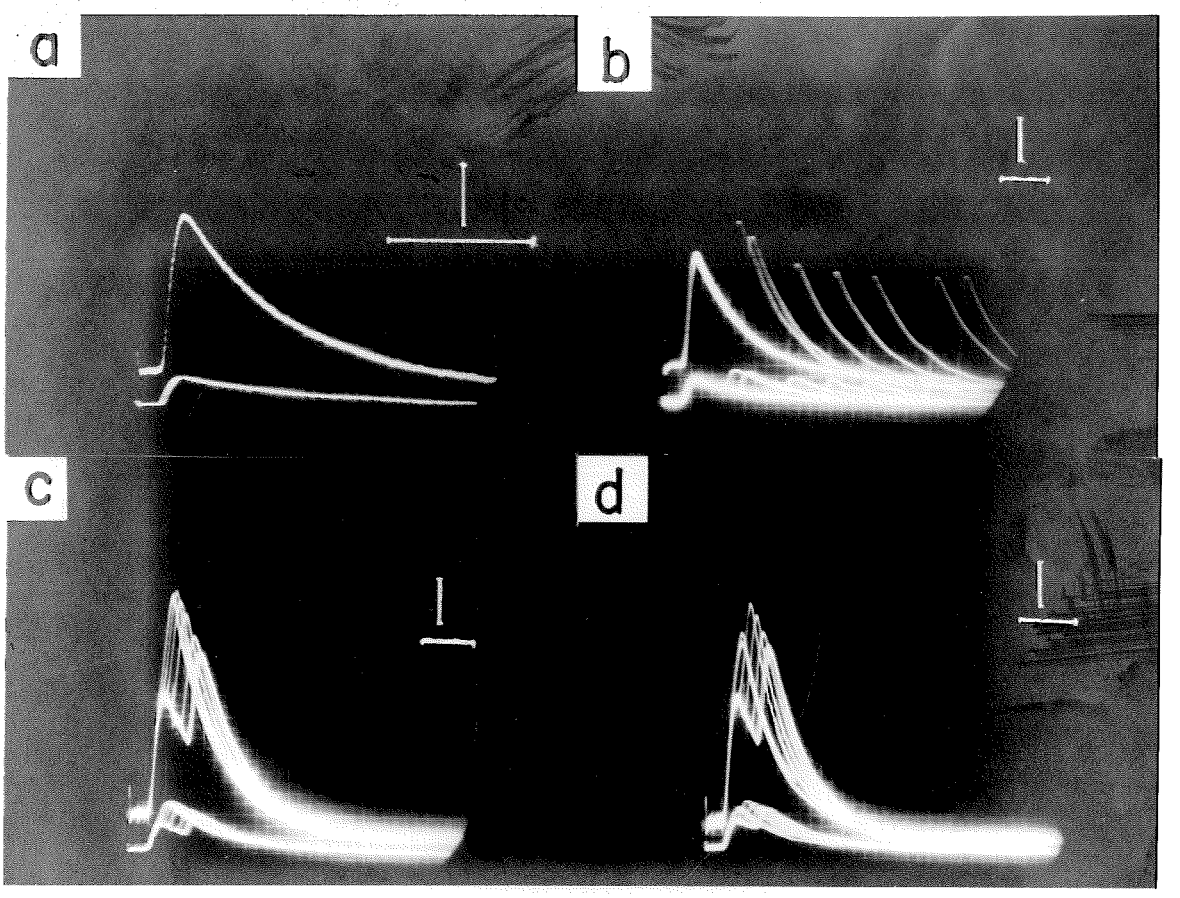


Fig. 3. Junctional potentials. Panulirus, main flexor (of carpopodite). Upper beam at 5x amplification of lower. b-d, multiple exposures, two nerve stimuli per sweep. On each successive sweep the second stimulus is closer to the first. Calibrations: time, 20 msec.; voltage, 2 mv. for upper beam.

occurred at the beginning of the trace and for each successive exposure the time interval between the shocks was decreased. b shows stimulus intervals from 110 msec. to 17 msec., while in c these times were 15 to 3.5 msec. When the two-shock interval was decreased below 3-3.5 msec., the second response was reduced in height as seen in d. These shorter intervals approach the refractory period of the nerve and the reduction in response amplitude probably finds its explanation in the refractoriness of some of the terminal axon branches to that muscle fiber.

Spike potentials were evoked by nerve stimulation only if the junctional response was of sufficient size. With the exception of the claw closer and RM2 muscle of Cambarus, in only a small fraction of the fibers of a muscle did the junctional potential exceed this threshold following a single nerve stimulus. It was, however, usually possible to evoke a spike by repetitive stimulation. Fig. 4 illustrates one aspect of this point. a and b show potentials recorded from a fiber of the claw closer of Cambarus, a to a single stimulus to the fast axon and b to two. The two-shock interval in b was 16 msec. The second junctional response, added to the declining phase of the first, exceeded the threshold and evoked a spike. c and d show a similar effect using the muscle strand of RM2. In this case two microelectrodes had been inserted and the potential recorded by each is displayed on a separate beam. The responses were to a single nerve stimulus, c, and to two separated by 2.3 msec., d. Not

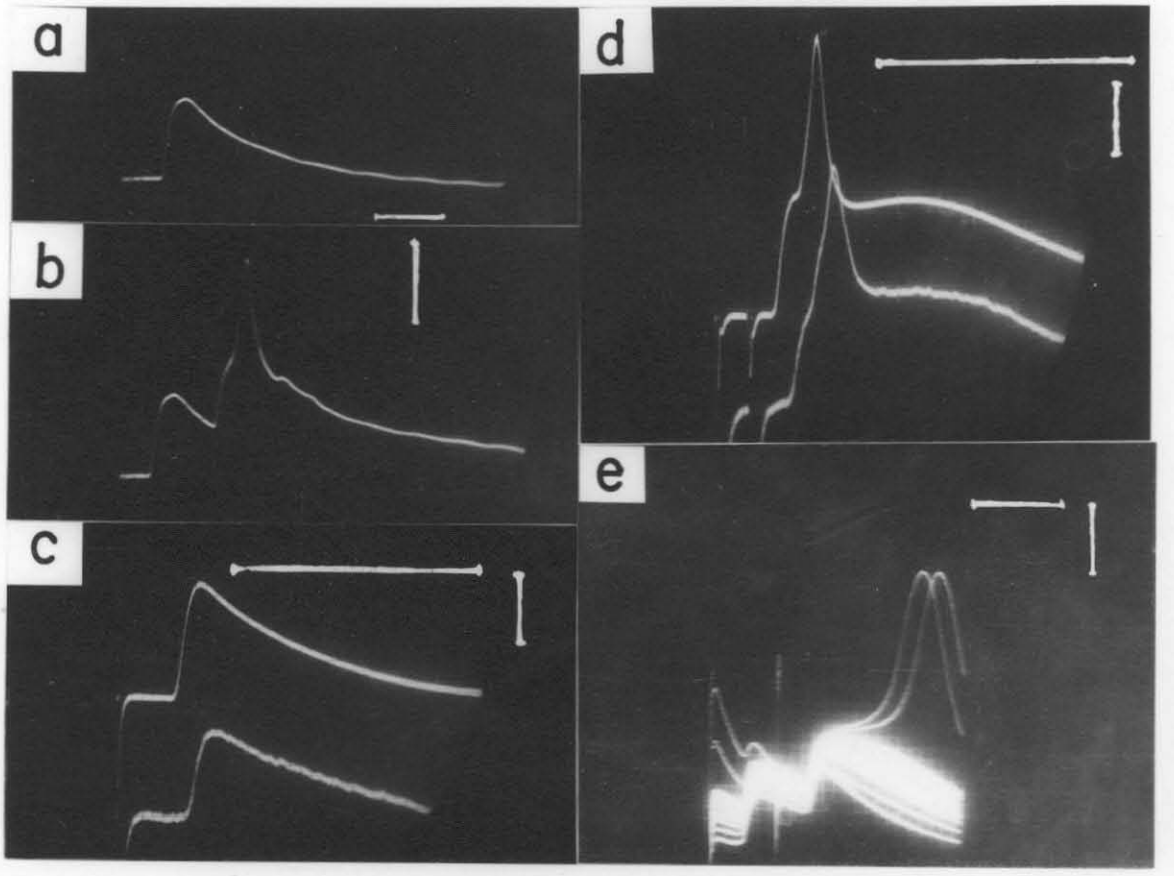


Fig. 4. The initiation of spikes. a and b, Cambarus claw closer. c and d, RM2. e, Panulirus main flexor. a and c, one nerve stimulus; b and d, two. e, multiple exposure, two stimuli repeated 25 times per sec. Calibrations: 20 mv.

all fibers, however, will show spike potentials following two nerve stimuli. In the majority, in fact, the junctional response to one nerve impulse was quite small and the summation of many of them was necessary to exceed the spike threshold. Fig. 4e is a multiple exposure showing the response to two nerve stimuli, separated by an interval of 11 msec. and repeated 25 times per sec. In this case, aside from the summation of each pair of junctional potentials, successive pairs also summed. The depolarization eventually reached threshold and two spikes were evoked. The ends of the spikes are seen at the beginnings of the two successive sweeps. The magnitude of the threshold depolarization varied considerably and probably depended to some extent on the condition of the preparation. The lowest value found was 22 mv. (Hemigrapsus, "closer") and high values in the vicinity of 40-45 mv. were quite common.

The crustacean muscle junctional response thus seems to resemble qualitatively other post-synaptic events which have been recorded from diverse systems. Does the crustacean spike potential then, also resemble the propagated action potential of nerve and vertebrate muscle? From qualitative observations made during the course of these experiments, it is apparent that there are several points of difference. The crustacean muscle spike can be a more subtle phenomenon than the explosive, all-or-nothing action potentials elsewhere observed. It is often graded, its size depending upon that of the junctional response. Although it is followed by refractoriness,

this is to some extent incomplete. And it can be present in one part of a muscle fiber without spreading to adjacent regions. Each of these points will be considered in more detail in the following.

Wide variation in the amplitude of the spike potential was observed, and is illustrated in Fig. 5. The size of the action potential shown in a is near the upper limit of those encountered. It was recorded from an RM2 muscle (Cambarus). The total amplitude was 85 mv. The arrow approximately indicates the inflection point at which the spike component arose from the junctional response. Estimated in this way, the latter was about 57 mv. and the former about 28 mv. The resting potential in this case was 74 mv., so that the overshoot was 11 mv. The potential of Fig. 5b was recorded from a fiber of a "closer" muscle of Pachygrapsus. This was also among the larger potentials observed and was 71 mv. in total amplitude. The resting potential was 64 mv. so that a 7 mv. overshoot was present. There was often, however, no overshoot and the presence of a spike component could be recognized only by the consequent change in time course. The potentials shown in Fig. 5c-d were recorded from a fiber of the main flexor of Panulirus. In c, two shocks were delivered to one of the motor axons and this was repeated three times, with a different two-shock interval on each trial. The responses to the first shock of each pair were superimposed at the beginning of the record and the three second-shock responses follow. The altered time course of the second-

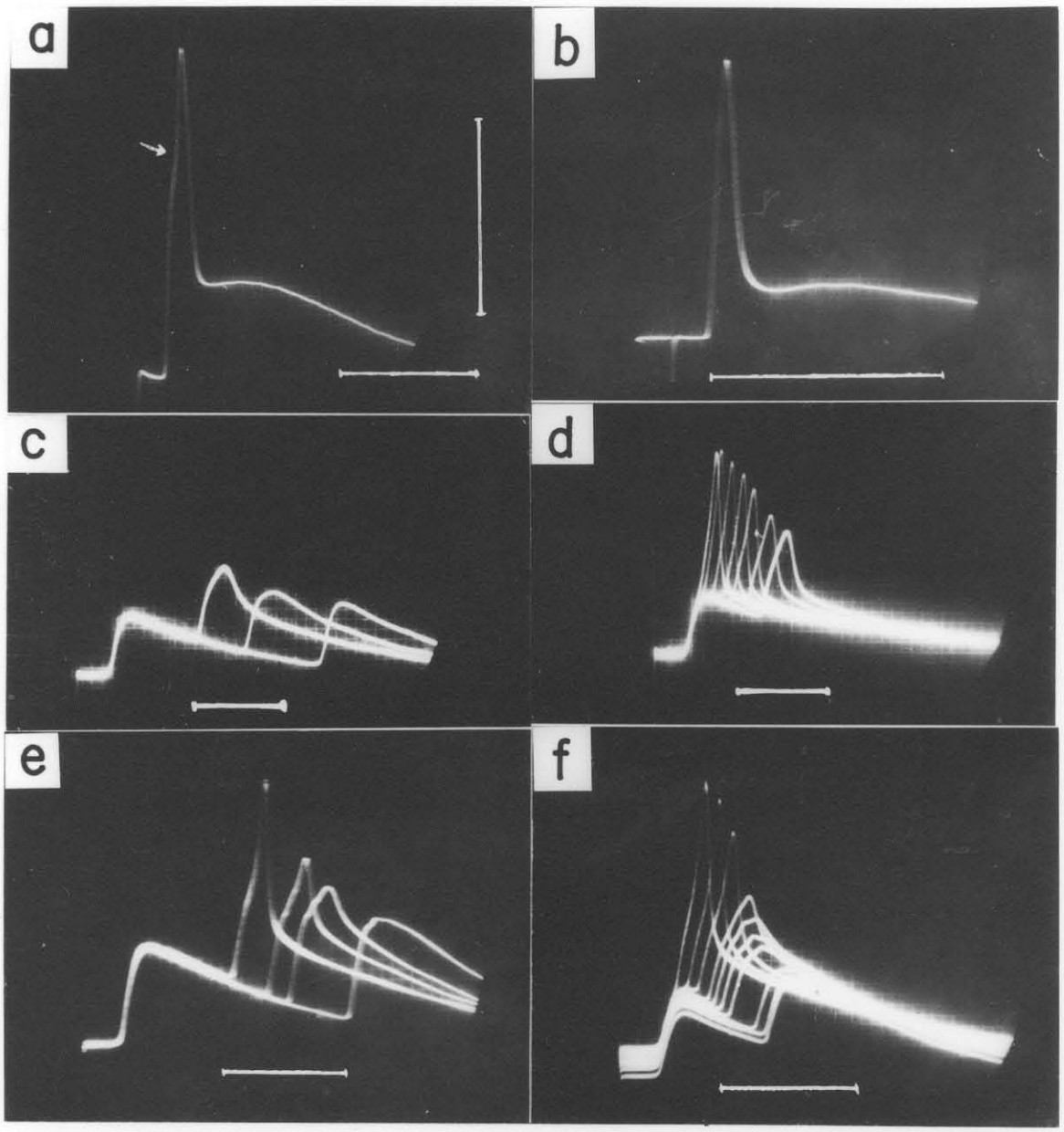


Fig. 5. The size of spikes. a, RM 2. b, walking leg "closer", Pachygrapsus. c-f, main flexor, Panulirus; multiple exposures, two stimuli per sweep. On each successive sweep the second stimulus is closer to the first. Calibrations: 50mv., 20msec.

shock response in the trial with the shortest two-shock interval, indicates the presence of a spike component. This is more apparent in d which shows responses to seven two-shock trials, using shorter interval than in c. c and d were recorded from the same muscle fiber and illustrate that the size of the spike response can vary within wide limits. It is also apparent that the size of the total junctional response is one of the factors determining the size of the spike. e and f show similar effects recorded from two muscle fibers of another preparation of this muscle.

The Panulirus main flexor exhibited gradation of the spike most readily of all the preparations used. Gradation was also observed in the RM2 muscle strand of Cambarus and in the "closer" muscle of the walking legs of the grapsoid crabs. In some cases, however, the spike did appear to arise from the junctional potential in an all-or-nothing manner (see Fig. 4e, for example). Both types of behavior were seen in the same preparation, but the factors determining which occurred are unknown.

Experiments were performed to test for the presence of refractoriness following the spike. Variability was also found in this case. When the spike response to a test shock was preceded by a conditioning spike at varying intervals, the test spike was, at different times, decreased, augmented, or unchanged in height. Fig. 6 shows some of the responses obtained in this way and perhaps gives some clue to the reasons underlying the variability. Fig. 6a and b were

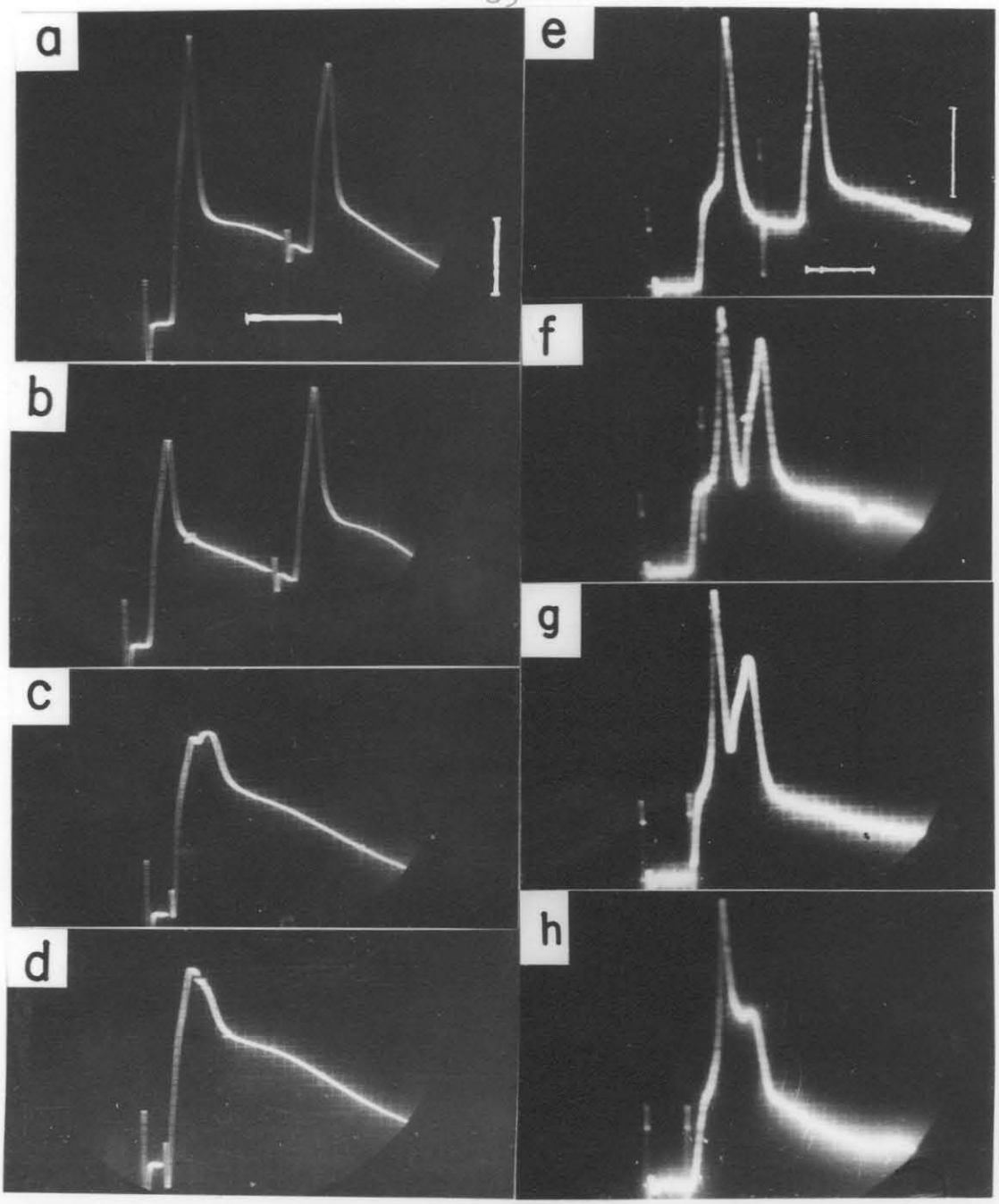


Fig. 6. Refractoriness following the spike. a-d, from an RM2 muscle fiber; e-h, from a fiber of a "closer" muscle of Hemigrapsus. Calibrations: 10 msec., 20 mv. Calibrations for a-d given in a; for e-h, in e.

recorded from the same preparation of an RM2 muscle, b at some time later in the experiment than a. The interval between conditioning and test shocks (c-t interval) was 15 msec. in both cases. Whereas the test response was diminished in a, it was augmented in b. As in this case, the test response was only found to be augmented when the conditioning spike was small. The augmentation apparently results from a larger total junctional response due to summation of the test response with the declining phase of the conditioning spike. c and d illustrate better the refractoriness seen in a. The c-t intervals were for c, 2.4 msec. and for d, 2.2 msec. Fig. 6, e-h show responses from one fiber of a "closer" muscle of Hemigrapsus. The c-t intervals are as follows: e, 17 msec.; f, 9.5 msec.; g, 7.5 msec.; h, 6 msec. g shows both the summation and refractoriness particularly well. A comparison of the junctional-spike inflection points of the conditioning and test responses in g shows that part of the test junctional potential had added to the declining phase of the conditioning spike. Despite this higher level of depolarization achieved by the test junctional potential (summation), the test spike was considerably reduced (refractoriness). It is interesting that even part of the test response can add to the declining phase of the conditioning spike and this point will be considered in the Discussion.

The next attribute of the spike that will be discussed is the manner of its distribution over the muscle fiber. Katz and Kuffler (52) and Fatt and Katz (55) have demonstrated

that under artificial conditions the spike can propagate along the length of a crustacean muscle fiber at a rate of about 25-30 cm. per sec. They assumed that the conduction was effected by the muscle fiber membrane, much as in the vertebrates. Experiments pertaining to the question were performed exclusively on preparations of the muscle strand of RM2 (of Cambarus). It was chosen because it seemed to be a single muscle fiber and because in some preparations spike potentials are elicited by single nerve stimuli. Experiments have been performed which corroborate the above assumption that the RM2 muscle consists of only a single fiber, and they will be described first.

The procedure involved passing current directly across the muscle fiber membrane with the aid of a microelectrode and observing the result either visually as a contraction or, using a second microelectrode, as a change in resting potential. The microelectrode through which current was passed will be referred to as the "stimulating" electrode, while the other will be called the "recording" electrode. A potentiometer and a 67.5 volt battery provided a variable voltage for passing current through the stimulating electrode. The latter was made positive (the anode of an electrolytic cell) except where otherwise stated. It would be expected, considering the muscle fiber as a core conductor, that with these conditions only fibers which had been penetrated by the stimulating electrode would be depolarized during the passage of current and would thus contract. Since the microelectrode

enters only one fiber at a time, if there were more than one fiber in the RM2 muscle strand, only a part of the latter should show this contraction. This was, however, often not the case. The local contraction seen in the vicinity of the microelectrode could be made to involve the entire width of the muscle. This was observed for several entries in each of six preparations. Controls were performed to show that contraction ensued only when the stimulating electrode had actually entered a fiber, using the measurement of the resting potential as the criterion for entry. These results were not considered unequivocal because of the subjective nature of the observation of the contraction. Therefore the second type of experiment was performed in which another microelectrode was used to record changes in potential. The recording electrode should measure changes in potential of different magnitude and direction during the passage of current through the stimulating electrode depending upon whether both electrodes are in the same or adjacent fibers. If in numerous entries, both electrodes always proved to be in the same fiber, it could be concluded that only one fiber was present. Four preparations were made to test this point, and with one class of exceptions to be discussed below, in all of the total of about thirty trials the recording electrode registered a depolarization during the application of a positive potential to the stimulating electrode. This is the result to be expected when both electrodes are in the same fiber. For many of the trials removal and re-entry of only one of the elec-

trodes was performed, whereas in others the positions of both electrodes were changed. The distance between the two electrodes varied from about 0.5 mm. to 1.5 mm. As mentioned above, there were some apparent exceptions, in that application of the maximum available voltage (67.5 v.) to the stimulating electrode, sometimes produced no measurable potential change at the recording electrode. This was despite the fact that both electrodes were inside a fiber as shown by their recording of resting potentials. In these cases, however, local contraction around the stimulating electrode was also absent (with the maximum voltage); and it seems apparent that only a negligible amount of current was actually crossing the fiber membrane. The absence of local contraction typically occurred with the use of very fine electrodes having a high resistance. In view of this difficulty, local contraction was used as the criterion for successful entry of the stimulating electrode, rather than measurement of the resting potential, in most of the above thirty trials. When this was done, the recording electrode registered depolarization or hyperpolarization respectively, as the stimulating electrode was made positive or negative, and with no exceptions. (See below, however, for the case in which the two microelectrodes were each on different sides of the sensory region.) The above results were taken as strong evidence that the RM2 muscle strand is only one fiber in cross section, and it will be referred to subsequently as the RM2 muscle fiber.

An incidental observation was made, however, which modi-

fies slightly the term "muscle fiber." Alexandrowicz (91) has found, with histological techniques, that a small portion of the length of a receptor muscle, that part which receives the sensory endings, has the muscular substance almost entirely supplanted by connective tissue. This fact was reflected in the present experiments by a marked decrease in electrotonic current spread across the sensory region. That is, when the two microelectrodes were placed on either side of the region, only very small changes in potential appeared at the recording electrode, even though local contraction was present at the stimulating one. Thus the RM2 muscle is not functionally a continuous single fiber along its longitudinal axis, even though it is only one fiber in cross section. This fact does not affect the following results, however, since only the portion of the fiber on one side of the sensory area was used.

Returning now to the consideration of the manner of spread of muscular excitation, it will be recalled that Fatt and Katz (53) have used the microelectrode technique to corroborate the existence of multiterminal innervation in crustacean muscle. They made a number of successive entries along the length of a muscle fiber and were able to measure junctional potentials having similar time courses at all of these points. Recently, in a study on various properties of the crustacean stretch receptors, Kuffler (92) has observed that wherever he penetrated the receptor muscles (RM1 and RM2) with a microelectrode, a junctional "end-plate" potential

could be recorded. These observations have been confirmed and those of Kuffler extended, using the RM2 muscle fiber of Cambarus.*

The typical procedure was as follows. A recording microelectrode was inserted in the RM2 fiber just anterior to the sensory region, the response to a nerve stimulus recorded, and the electrode withdrawn. This was repeated at successive positions, each 0.25 mm. anterior to the preceding, until the end of the muscle fiber was reached (usually 10-12 positions). Fig. 7 shows every other record from one such experiment (each position separated by 0.50 mm.). The tendency seen here, for the junctional potential to decrease towards the end of the fiber, was common. The gap in the baseline of each trace represents the stimulus artifact and the time interval from the beginning of the gap to the foot of the muscle response will be referred to as the latency. It can be seen that the latency increases progressively (from a to f) as the distance of the recording electrode from the sensory region becomes larger. It has been assumed that this latency increase is due to the time for conduction of the impulse in the nerve which runs along the muscle fiber. In support of this assumption is the similarity of these junctional potentials to non-conducted responses found elsewhere and the demonstration by Fatt and Katz (53) that the crustacean junctional response rapidly decrements beyond a point at

---

*The present experiments were performed before the appearance of the above two articles.

which the nerve has been disrupted. Further, it has been observed with the present preparations as well as in the detailed studies of Alexandrowicz (91), that the motor axon first makes contact with the muscle fiber in the vicinity of the sensory region and then runs along the fiber to its end, which is in the proper direction to account for the latency prolongation.

If the junctional potential is distributed along the muscle fiber by the nerve, it then becomes interesting to know how the spread of the spike is effected. To test this point preparations were used which happened to exhibit spikes in response to single nerve impulses; and then the type of experiment illustrated in the previous figure (Fig. 7) was performed. In the records of Fig. 8 the distance between successive recording positions was 0.50 mm. (The shock artifact is again marked by the gap. The initial, small, diphasic wave served to trigger the oscilloscope sweep.) There are two particular points to be observed. Firstly, the height of the spike component varied for different electrode positions and tended to be largest where the junctional response was largest. (The vertical distance from the foot of the action potential complex to the inflection point on its rising phase serves as a measure of the size of the junctional response.) And secondly, the spike and the junctional potential tended to progress along the length of the fiber at the same rate. These two points are further illustrated in Figs. 9 through 11.

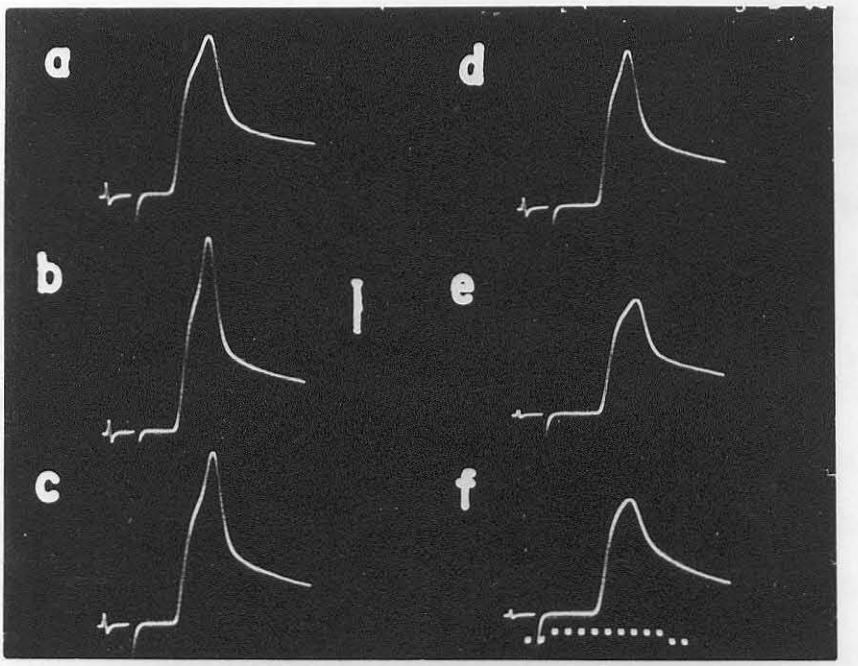


Fig. 8.

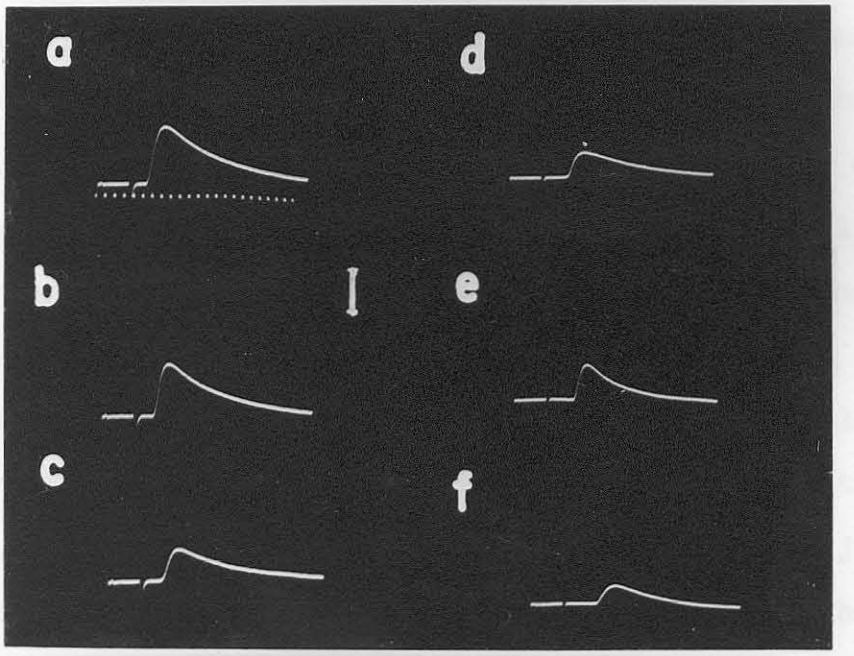


Fig. 7.

Fig. 7. Junctional responses from an RM2 muscle fiber. Each response was recorded 0.5 mm. further from the sensory region than the previous one. Note the increase in latency. Calibrations: time in msec.; voltage, 20 mv.

Fig. 8. Spike responses from another preparation of an RM2 muscle fiber. Other details are the same as for fig. 7.

Fig. 9 a and b show potentials recorded simultaneously from two points on the same RM2 muscle fiber using two microelectrodes separated by 1.10 mm. This was done to show that the variations in spike height are not due entirely to changes with time, for the spike can be quite labile. Another point is illustrated as well. Whereas the place on the fiber from which the lower-beam potentials were recorded showed practically no spike following a single nerve impulse, it is capable of exhibiting one in response to two nerve impulses, as seen in b. Thus a spike will sometimes not invade a neighboring region even though the latter is capable of producing a spike following larger depolarizations. The second point made in the preceding paragraph, that the junctional and spike potentials progress along the fiber at similar rates, is better illustrated in Figs. 10 and 11. Fig. 10 is a montage prepared from two records taken from two sites, separated by 1.75 mm., on the same RM2 fiber. The two action potentials are so superimposed that the stimulating shock artifacts for each coincide. It can be seen that the two time intervals, one between the bases of the junctional potentials and the other between the peaks of the spikes are approximately the same (ca. 1.4 and 1.5 msec. respectively). The graphs of Fig. 11 show similar data for a number of points in each of four such experiments. The abscissa in each is the distance in mm. from the first electrode position, where successive positions are further away from the place of initial contact of the motor axon with the muscle fiber. The

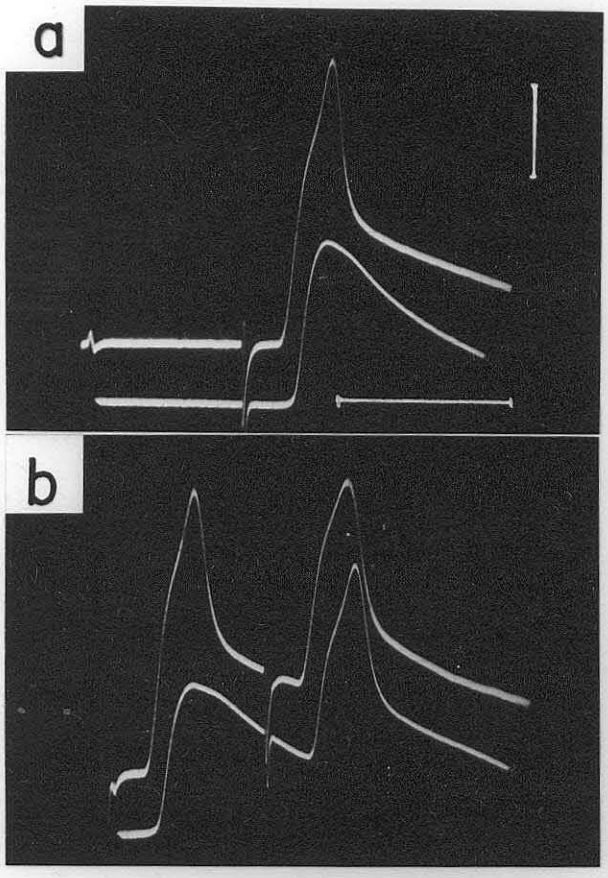


Fig. 9. Local spikes. Simultaneous recording from two points on the same RM2 muscle fiber. The two recording points are separated by 1.10 mm. a shows the response to one nerve shock; b, to two. Calibrations: 10 msec. and 20 mv.

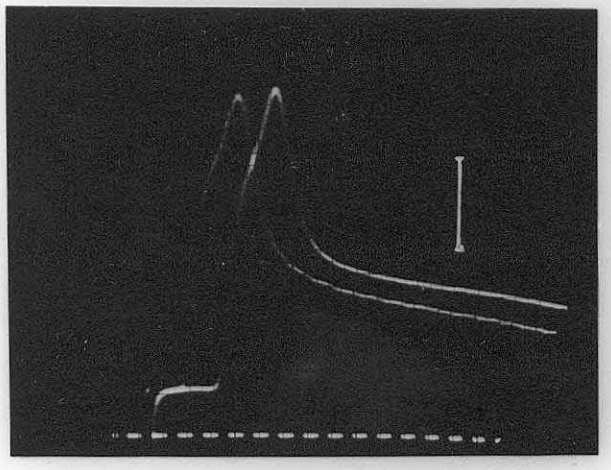


Fig. 10. The mechanism of conduction of spikes. A montage of two action potentials recorded 1.75 mm. apart from the same RM2 muscle fiber. The shock artifacts have been superimposed. Note that the interval between the bases of the junctional potentials is similar to that between spike peaks. Calibrations: time in msecs., voltage as in fig. 9.

ordinates give the delay in msec. from the shock artifact to (J) the foot of the junctional potential or (S) the peak of the spike. The straight lines were fitted to the points visually and too much emphasis should not be placed upon them, as the increase in delay time with distance is often not linear. The lines merely show that, in general, the two delays tend to increase at about the same rate. This statement does not apply very satisfactorily to the experiment shown in C. The deviation is in the proper direction to be explained by the progressively diminishing size of the junctional potential along the length of the fiber. The result of this circumstance would be that the spike threshold would be reached at successively later parts of the junctional response.

The results of Fig. 11 also provide a measure of the rate at which the excitation is conducted along the muscle fiber (presumably by the motor axon). This rate is given by the reciprocal of the slope of the J-lines and the numerical values for the four experiments are as follows:

Table 1.

	Rate at which the delay of the junctional potential increases with distance. msec./mm.	Conduction velocity of excitation along the length of the muscle fiber. m./sec.
A.	0.80	1.3
B.	0.70	1.4
C.	0.65	1.5
D.	0.50	2.0

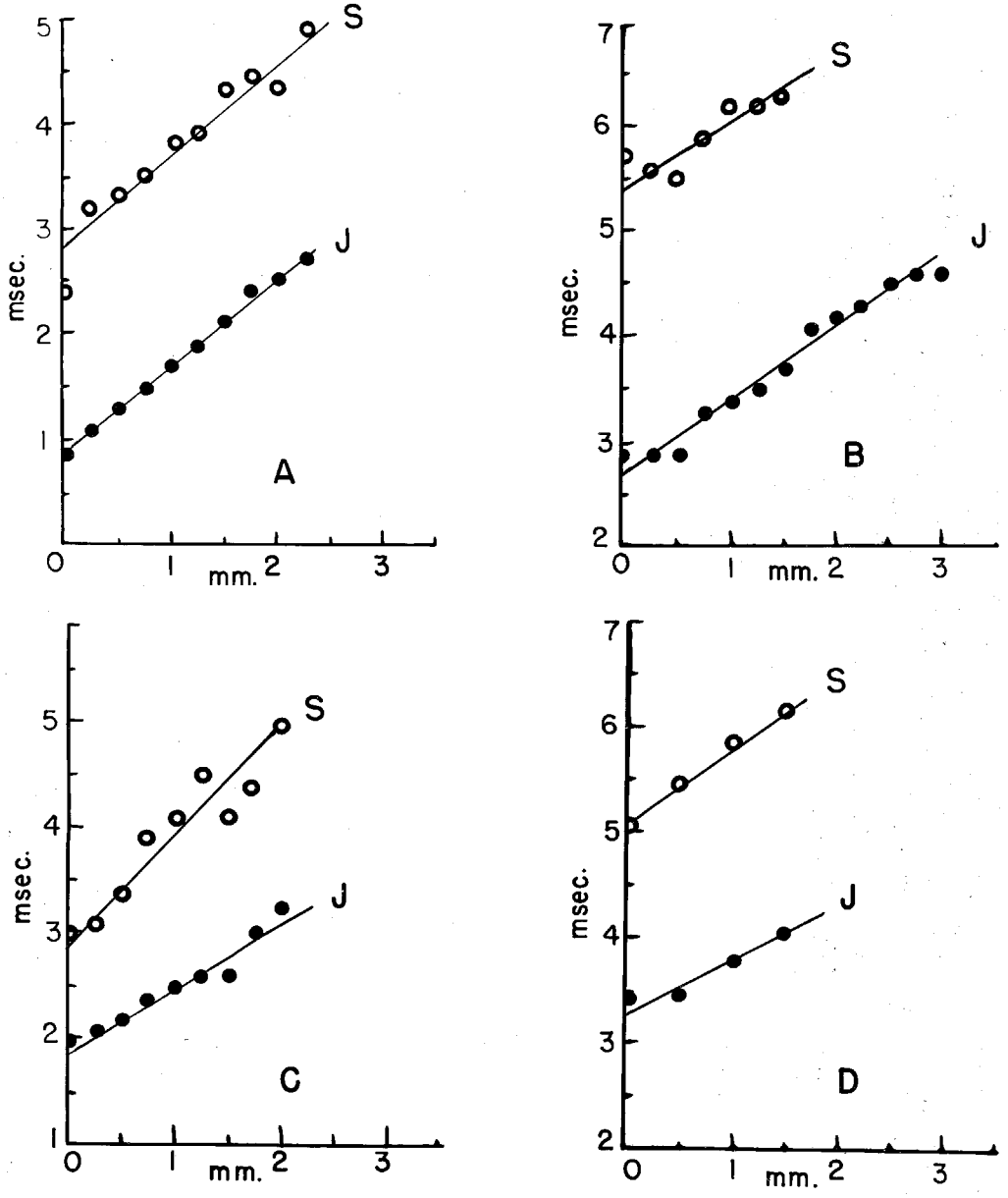


Fig. II. The increase of latency with distance along fiber: J, of the junctional potential (base) and S, of the spike (peak). Four preparations of the RM2 muscle fiber. Abscissae: distance from first electrode position. Ordinates: delay time, from shock.

The observations presented thus far may be recapitulated as follows:

(i) Crustacean muscle fibers exhibit neuromuscular junctional potentials which are capable of summation, and except for their presence throughout the fiber, resemble other postsynaptic potentials. When they exceed a certain level of depolarization, a spike-like response results.

(ii) Although the spike potential is followed by refractoriness, some component of an immediately ensuing response can summate with its declining phase.

(iii) The spike is often not all-or-nothing. It can vary in size at one point on a fiber with different patterns of nerve stimulation, and be of different size at various places on the fiber following a given nerve stimulation. The height of the junctional potential is one of the factors determining the size of the spike.

(iv) The spike progresses along the muscle fiber at about the same rate as the junctional response.

A reasonable inference from (iii) and (iv) is that the spike is not normally conducted in the muscle fiber membrane, but is elicited at the various points on the fiber by the junctional potential at that point.

Polyneuronal innervation:

The next attribute of crustacean muscle to be considered is the innervation of single muscle fibers by more than one axon. The experiments were performed on two types of muscle,

those receiving (a) two motor axons and (b) four motor axons.

Muscles with two motor axons:*

Although all previous evidence had indicated the presence of polyneuronal innervation, it was felt that the prominence of this phenomenon in much of the reasoning about crustacean muscles warranted a more critical test of it. This was provided by the use of the recently developed technique for intracellular electrical recording, combined with the older procedure of isolating single motor axons. It was thus possible to record the electrical response of individual muscle fibers to separate stimulation of the axons innervating them.

For several reasons, which will be considered below, a thorough sampling of the fibers of any one muscle was not performed. Nevertheless, muscles from a variety of species were used. The result may be stated as follows. With a few exceptions, every sampled fiber of muscles receiving two motor axon exhibited an electrical response, in the direction of depolarization, to stimulation of either of those axons.

The muscles and species used, together with the number of successful preparations of each, are as follows: Astacus trowbridgii, claw closer - 3; Cambarus clarkii, claw closer - 3; Pachygrapsus crassipes, walking-leg "closer" - 3; P. crassipes, claw bender (propodite flexor) - 1; Cancer productus, walking-leg "closer" - 2; C. nudus, walking-leg "closer" - 1.

---

*Most of the experiments on this class of muscles were performed in collaboration with Professor C. A. G. Wiersma at the Hopkins Marine Station of Stanford University, at Pacific Grove.

With the exception of the bender, all of the above muscles are homologous. In four of the preparations the two motor axons, fast and slow, were completely isolated as single nerve fibers. In the others, one or both of the axons was left surrounded by a small bundle of sensory axons. This practice facilitated the dissection as well as yielding hardier preparations. The location of the inhibitory axon was unknown in these cases. Stimulation of the inhibitor would not be expected to affect the present results, however, since the muscles used do not show reduction of the action potential during inhibition (38).

Fig. 12 shows the paired fast and slow muscle potentials recorded from three fibers of a claw closer of Cambarus. The left (a, c and e) and right (b, d and f) columns depict the fast and slow responses, respectively. The fast axon was stimulated, in each case, by a single shock, while a frequency of 30 shocks per sec. was used for the slow axon. These stimulation parameters were employed to avoid fatiguing the fast response and to make the slow response detectable. The latter was usually below the noise level following a single nerve impulse, and very small with frequencies under 15 shocks per sec. It is apparent that the size of the response varied from fiber to fiber. In this example the variation was more pronounced for the slow potential, but it was also typical for the fast response. It is also evident that the size ratio of the fast and slow potentials was not constant among different fibers. All six responses were junctional poten-

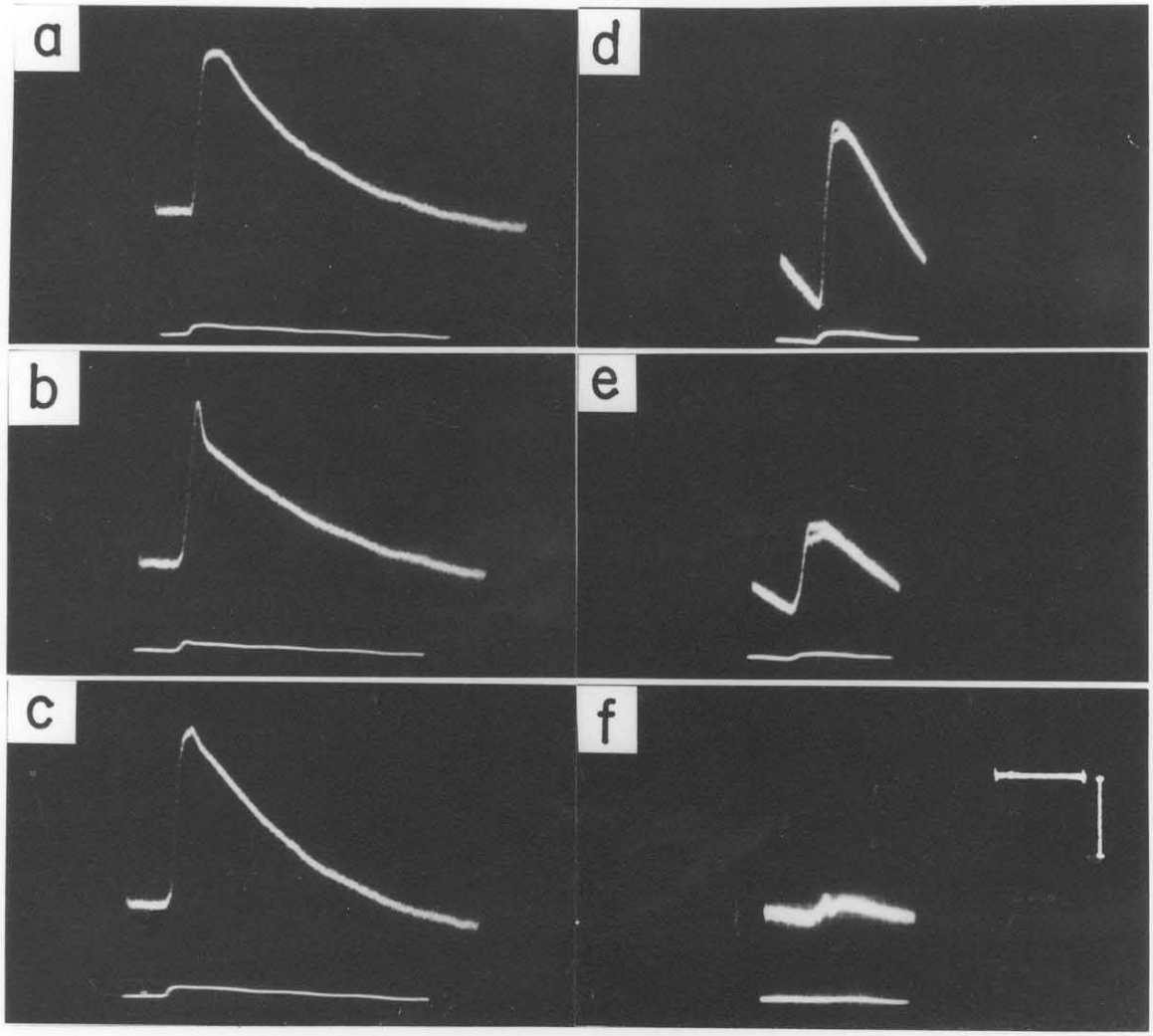


Fig. 12. Fast and slow responses in the same muscle fibers of the claw closer of Cambarus. The upper beam shows the same response as the lower one in each case, but at 18x amplification. The left column shows the fast responses of three fibers to a single nerve shock each. The paired slow responses to 30 nerve shocks per second are opposite in the right column. Calibrations: time, 20msec; voltage, 1mv. for upper beam.

tials and were considerably below the spike threshold. The spike-like appearance of c and the notch in f are believed to result from proportionately large responses in muscle fibers adjacent to the impaled one. Evidence supporting this contention will be presented below, with the experiments on the Panulirus main flexor muscle.

Results similar to those shown from Cambarus were also obtained with the other preparations, but specific differences in the character of some of the responses were observed. In the preparations from the grapsoid crabs, the variation, from fiber to fiber, in the size of the fast potential was very pronounced. The amplitude of the slow potential, although also variable, was consistently very small (mostly below 0.5 mv.). Wiersma and Ellis (38), using external recording electrodes also recorded very small slow potentials in Pachygrapsus. The present experiments indicate that this observation is attributable to small responses in almost all fibers, rather than large ones in a few fibers. The variation in the size of the fast response in the grapsoid muscles was not entirely random. Fibers further from the exposed surface of the muscle and nearer to its central end, typically showed larger fast potentials. The more internal and central fibers were thus the ones most commonly exhibiting spikes in response to a single nerve impulse. Although exceptions to the above generalization were common, it was useful experimentally in attempts to find fibers giving spikes following single shocks.

In Cancer the fast responses were, in general, smaller than in the other species and those elicited by a single nerve shock often resembled the slow potentials evoked by 30 shocks per sec. No fibers were found which exhibited spikes following single stimuli. In the Cambarus claw closer, both large fast and slow responses were found. Although it was difficult to sample this muscle, because of the large contractions evoked by a single shock to the fast axon, many of its fibers showed spikes following such stimulation.

Muscles receiving four motor axons:

Decapod limbs contain one muscle which receives five efferent axons, four motor and one inhibitory. It is the main flexor of the carpopodite and it has previously been studied by van Harreveld and Wiersma (41). They were able to isolate the individual motor axons and found that each evoked a contraction which differed from the others both in strength and time course. The action potentials recorded with external electrodes were also different from one another.

In the present experiments the distribution of the axons to the individual muscle fibers was studied. The only species in which the dissection was successfully completed was Panulirus interruptus and all of the following experiments were performed on the muscle of that animal. A total of fourteen successful preparations were made, in which all four motor axons functioned. In about an equal number, one or more of the axons did not evoke any responses; but some of these preparations were useful for other observations.

The criterion for accepting that an axon innervated a muscle fiber, was the same as that used above. Namely, that having made a suitable entry into a fiber, a microelectrode recorded an action potential (usually a junctional potential) in the direction of depolarization following stimulation of the axon in question. The determination of an entry as "suitable" was rather subjective. It depended upon the size of the resting potential measured and the rate of the transition from zero potential to the resting potential during the impaling process. This transition was very rapid in the best entries and a resting potential of over 60 mv. was usually considered "suitable."

The first point that was tested was the conjecture (41) that each of the fibers of this muscle would be innervated by all of the axons. In every preparation it was found that, whereas some muscle fibers did receive four motor axons, a very large percentage of them did not. Most of the fibers were innervated by one, two or three of the axons. Of 421 fibers sampled, 38% received only one of the motor axons; 26% received two; 29% three; while only 7% were innervated by all four. Too much emphasis must not be placed upon these percentages, however, for two reasons. As will be shown below, the distribution of axons to muscle fibers is not random. Fibers receiving some of the axons tend to occur in grouped clusters and there are at least two regions of the muscle in which the fibers are innervated by a characteristic axon complement. Since the number of regions (i.e. fibers

under a particular surface point) explored in any one preparation was small, the samples were probably not chosen representatively from among these different regions. Further, a number of the potentials observed were diphasic (for reasons to be discussed), and some of the decisions in these cases, based on the criterion of depolarization, were arbitrary. Thus, the above percentages have been given merely to demonstrate that this muscle is a heterogeneous population of fibers with respect to the number of axons it receives; and that all four categories are represented. (Muscle fibers with no motor innervation were not encountered).

Then the question arises whether or not within a particular category, the different possibilities which constitute it occur equally often. For example, among those fibers receiving only one axon, are there equal numbers innervated by each one of the four axons. The latter question can be immediately answered in the negative. Almost all of the fibers with only one motor axon were innervated by the same particular one. This axon was readily identified, for almost all of the fibers on the inner surface of the muscle (the surface facing the extensor muscle and which is first approached by the microelectrode) received it exclusively. This was also true of the several layers of fibers lying just beneath those on the inner surface. There are several reasons for believing that this axon is the one evoking the "type I" response of van Harreveld and Wiersma (41), and it will sub-

sequently be referred to as axon I. The identification is based on the facts that in the present experiments axon I most commonly evoked the largest responses and had the most widespread distribution of the four. The type I potentials of van Harreveld and Wiersma (as recorded with external electrodes) were also the largest and evoked the largest and fastest contractions. Axon I was, in addition, the one most readily evoking spikes, which would contribute to the rapid contraction rate. It was also the only axon consistently evoking a visible muscle potential following a single shock. This fact correlates with the short latency of the type I contraction.

As intimated above, axon I not only supplied almost all of those fibers receiving only one axon, but was also the most common participant in the innervation of fibers receiving two and three. It has been estimated that about 90% of all muscle fibers sampled (377 out of 421) were activated by axon I. The uncertainty of the estimate arises from the difficulty of detecting small potentials in a muscle fiber, if neighboring fibers are simultaneously exhibiting large potentials. This point can be better considered after the presentation of some additional results.

Unfortunately, the present data do not allow similarly conclusive identification of the other three axons in terms of the contraction and potential types of van Harreveld and Wiersma. One of the factors contributing to this difficulty was the heterogeneity of the response. As in the case of

muscles with double motor innervation, there were large variations in the size of the potentials evoked in different muscle fibers by stimulation of any one of the four axons. It was especially true, however, for the axons other than axon I. Table 2 shows the results of a typical experiment and serves to illustrate this, as well as several other phenomena commonly observed. The four axons are arbitrarily indicated by letters A through D. The four columns headed "Size of Potential" give the magnitude of the responses evoked by each of the four axons in 33 muscle fibers. The potential sizes were read directly from the calibrated face of the oscilloscope and are only approximate (to within about 0.1 mv. for small potentials, up to about 1 mv. for larger ones). All of the responses were to axon stimulation at a frequency of 45 shocks per sec. The small letters, associated with the groups of readings enclosed between solid lines, refer to points on the exposed surface of the muscle below which the responses of successively deeper fibers were serially recorded. Point a was the most distal and successive points were progressively more proximal. The two dotted lines indicate places where difficulty in impaling a fiber was encountered so that fibers 25 and 30 were probably not immediately beneath 24 and 29, respectively. Before entering fiber 19, the electrode tip broke and several partial entries were made. It was found that fibers with axon C were present, but measurements were not made and the other responses were not tested. The figures enclosed in parentheses

Table 2

The Responses of Individual Muscle Fibers to Stimulation of the Four Axons

Position	Muscle Fiber	Size of Potential (mv.)			
	Axon	A	B	C	D
a	1	1.1 mv.	1.2 mv.	0	1.5 mv.
	2	0.5	1.1	?	3
	3	3	2	?	1.7
	4	1.7	2.5	?	1.6
	5	6	12	?	(-0.5)
	6	1.2	2	0.2	2
	7	.2	.3	0.7	1.7
b	8	0	0	0	10
	9	0	0	0.1	10
	10	0.1	0	0.1	9
	11	1.1	0	0.1	14
	12	0.5	0	1.2	6
	13	0	0	7	(0.6)
	14	0	0	0.5	3
	15	?	?	0.2	5
c	16	0.2	0.2	0	10
	17	0.7	0.3	?	10
	18	0.2	?	(-0.2)	13
	19	--	--	large	--
d	20	0	0	0	8
	21	0	0	0	8
	22	0.1	0	0.5	10
	23	0	0	0.3	14
	24	?	0	10	(0.7)
	25	0	0	8	(2)
e	26	0	0	0	8
	27	0	0	0	8
	28	0	0	0	7
	29	0	0	0	8
	30	1.2	0	0.2	12
	31	1.1	0	0.2	12
	32	1.2	0	0.2	10
f	33	0.2	0	0.1	5

indicate that those potentials were diphasic or showed large notches. The number then refers to the size of the major phase and the minus sign indicates that this phase was in the direction of hyperpolarization. The question mark signifies that some response was observed but that it was so close to the noise level that it was difficult to discern its size or direction. The zeros indicate no detectable response. There are several interesting points illustrated by this experiment which were also common in others. Axon D can be immediately recognized as axon I, because of its large size, widespread distribution and its tendency to constitute the sole innervation of surface fibers (fibers 8, 16, 20, 26, 33). This tendency was less pronounced in this preparation than in some of the others in which all surface fibers tested received only axon I. Region a differed from more proximal ones in several respects. Muscle fiber 1 was on the surface but showed responses of nearly equal size to three of the axons. It was also the only region containing fibers with large responses to axons A and B. In all preparations in which samples were taken from these most distal fibers, near the mero-carpopodite joint, these general features were also found. It was more common in other experiments, however, for all four of the axons to give rise to large potentials in this area, although not typically in the same fibers. Another observation which was common is illustrated by fibers 5, 13 and 24, which were the only ones showing a small axon I response. These fibers were also the only ones in which large

potentials were evoked by other axons. After the entry into fiber 24, the electrode broke, but partial entries into a number of additional fibers were made. Among the latter some showed large C and small D responses, while the reverse situation was present in the others. This tendency toward reciprocating exclusion of the responses of two axons, typically occurred in a compact group of neighboring fibers. There was, in fact, a general propensity for fibers showing a response to a particular axon to occur in groups. (See the responses of axons A-C in Table 2.)

One is tempted from the data of Table 2, to identify axons A-C with other of the contraction types (41). For example, axon B, because of the paucity of the responses it evokes, might well be assumed to elicit the type IV contraction. In a similar way, one might associate axons C and A with types II and III, respectively. It must be emphasized, however, that because of the tendency of responding fibers to occur in groups, and because of the small size of the sample in any one experiment, such identifications are likely to be in error.

A few observations on the interaction of responses evoked by different axons in a single muscle fiber, will conclude this section. All of the junctional potentials shown in Fig. 13 were recorded from the same single muscle fiber in response to stimulation of the four motor axons. In each record the upper trace shows the same response as the lower but amplified by a factor of 6x (a-c) or of 35x (d-f).

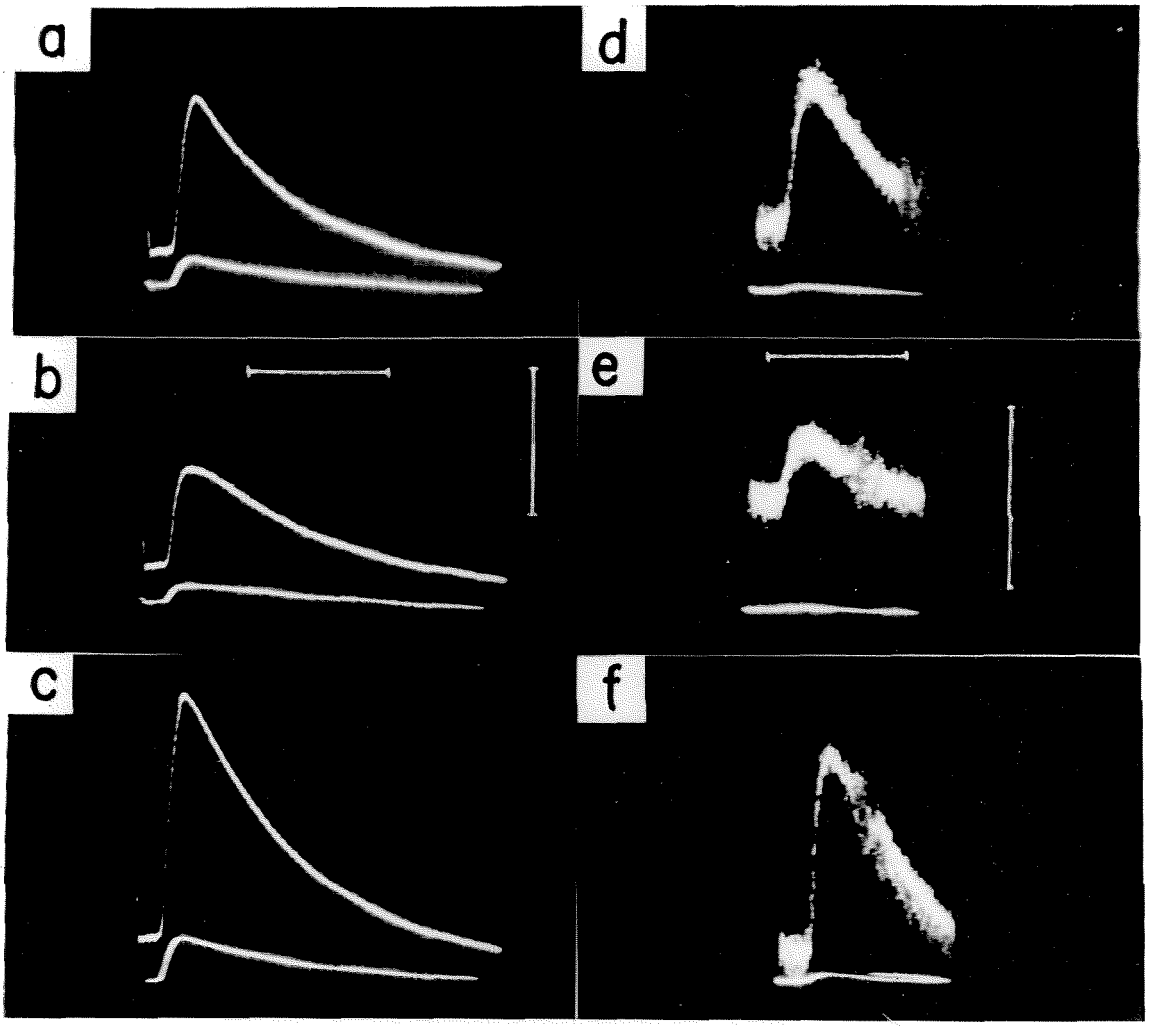


Fig. 13. Heterosummation of junctional potentials. All records from the same fiber of a main flexor muscle of Panulirus. See text for full description. In a-c upper beam is at 6x amplification of lower; in d-f at 35x. Calibrations: time, 20 msec.; voltage, for a-c 5 mv. and for d-f, 1 mv., both for upper beams.

Records a, b, d and e each depict the response to stimulation of a different one of the axons. c shows the result of simultaneous stimulation of the two axons giving the a and b responses, while f is analagous for the axons evoking the d and e responses. The stimulation in a through c was a single shock and in d through f was at a frequency of 45 shocks per sec. The axon of response a was identified as axon I, and that of response e was probably axon IV. No decision was possible as to the identification of the other two axons. It can be seen that responses c and f are close to being algebraic sums of responses a, b and d, e respectively. In this particular fiber other combinations of axon stimulation could not be tested because of the disparity in their sizes, but in other fibers the responses to three axons have been seen to summate.

It has also been possible to show that this type of heteroaxonic summation (heterosummation) can initiate spikes. Fig. 14 a shows the summated junctional potential which resulted from two shocks applied to axon I. The response was below the spike threshold in this fiber, although the dimple in the peak of the record probably indicates that spike activity was present in adjacent fibers. In b the response to stimulation of one of the other axons at 75 shocks per sec. is shown. In c the summation accompanying simultaneous stimulation of the two axons, each with its previous stimulating conditions, is seen to have resulted in a spike.

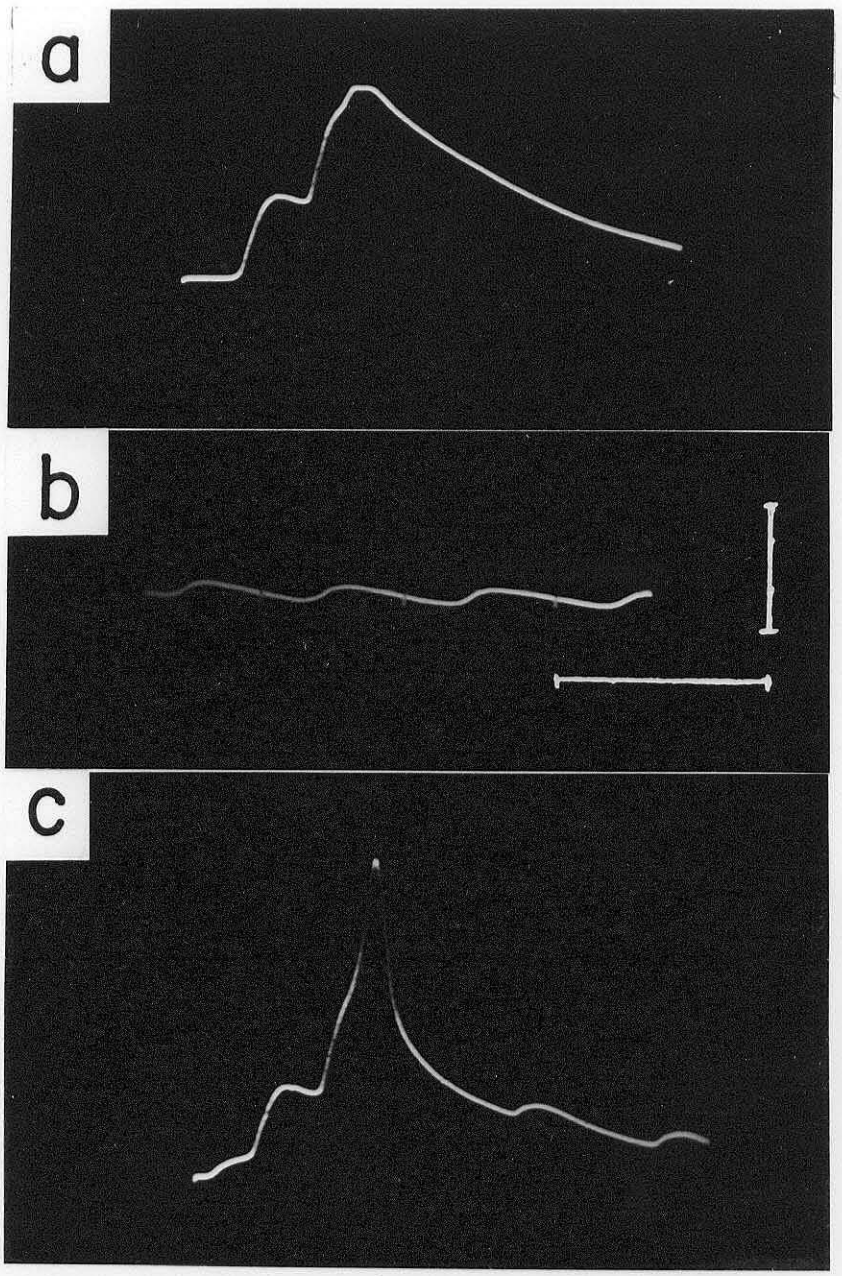


Fig. 14. The evocation of a spike by heterosummation of junctional potentials. All records are from the same fiber of a main flexor muscle of Panulirus. a shows the response to stimulation of one of the motor axons with two shocks separated by 6 msec. In b the response to stimulation of one of the other axons at 75 shocks per sec. is shown. c shows the effect of simultaneous stimulation of both of these axons, each with its previous stimulating conditions. Calibrations: 20 msec. and 20 mv.

D. Discussion.

Combining the concepts from older work with the recent results of Fatt and Katz (53,55) and those of the present experiments, one obtains a very complex picture of the crustacean neuromuscular mechanism. The two older views of this system, discussed in the introduction, must be reconciled as both being partially correct. Excitation is distributed to all parts of a muscle fiber by the nerve and at each of the numerous nerve endings local-type, summating muscle potentials are evoked. But it is also true that these junctional potentials can, in turn, elicit an additional process, a spike potential. This spike is often not all-or-nothing, is quite labile and in some cases is present in only a small fraction of a muscle's fibers. In addition, more than one motor axon can innervate a single muscle fiber and the fiber's response to each of its axons is usually different. If the different responses are junctional potentials, they can act in concert to give rise to a spike. The marked heterogeneity of crustacean muscle also adds to the complexity. Not only do the fibers of a muscle differ in the size of their responses to a particular axon, but can also vary with respect to the number and combination of axons they receive. The two kinds of variation may only be quantitatively different aspects of the same phenomenon, as is suggested by the very small responses (less than 0.1 mv.) often observed.

Some of these points require elaboration. The junctional potential of crustacean muscle resembles the vertebrate

endplate potential in the shape of its time course and its ability to summate and to evoke spikes. It has thus been called "endplate potential" (52) and "distributed endplate potential" (53) by other authors. The term "junctional potential" has been adopted in the present case as being more convenient than the latter and to emphasize its differences from the vertebrate response. For the junctional potential does not have the evocation of the spike as its only role, but can play an important part in eliciting contraction by itself. Further, discrete endplate structures have not been observed; and because of the large number of nerve endings, this potential occurs throughout the fiber, rather than at localized foci. The word "junctional," of course, refers to the nerve-muscle junction and has been used by Kuffler and Gerard (93) in the term "small-nerve junctional potential" (s.j.p.). This was with reference to the potentials evoked in the slow muscle fibers of the frog (which are innervated by small efferent nerve fibers).

The term "spike" has been used above to signify the additional membrane response evoked by supraliminal junctional potentials (see Fig. 2). This word is commonly used to describe regenerative responses in nerve and other electrically excitable structures. It has been used here mostly to avoid terms implying conduction, since at least in the muscle of the RM2 stretch receptor it does not appear to be actively self-propagating. Kuffler has also mentioned in his recent paper on the stretch receptors (92) that he has observed

spikes restricted to parts of the muscle strand. He also reports an interesting observation concerning the effect of stretch on the muscle. Apparently twitch contractions of RM2 only occur in the presence of spikes. (This has also been observed in the present studies.) Kuffler found that localized stretching of a portion of the muscle could cause that part to twitch, following a nerve impulse, when it had not previously done so; and one may infer that a spike had thus been evoked. In his study on locust muscle, Hoyle (64) reports that the form of the action potential, junctional potential ("e.e.p.") plus spike, was similar in different parts of the fiber, so that here also the spike does not seem to be self-propagating.

The crustacean muscle spike also differs from conducted action potentials in its graded behavior. Hoyle also reports that one of the nerve fibers to the extensor tibia of the locust, can set up spikes of variable size. The junctional responses of this particular nerve fiber (S_{1b}) showed facilitation with repetitive stimulation if they were initially small. In these cases the spike also grew as the junctional potential increased. Similar observations have been made in the present studies.

If the spike does not serve to conduct excitation along the muscle fiber, the question of its significance arises. Fatt and Katz (55) have suggested that the spike would serve to smooth out inequalities of the response along the length of the fiber. From the present experiments on the RM2 muscle

fiber, however, it appears that the longitudinal variation in the height of the spike may be even greater than that of the junctional potential. The observation mentioned above that the RM2 fiber only shows a twitch in the presence of a spike, suggests that the latter is capable of activating the contractile mechanism, while the junctional potential is not. Wiersma (94) has recently made some interesting observations on this point. Using external recording of electrical activity, he found that contraction only occurred when the electrical responses became diphasic, in limb muscles of the lobster, Homarus vulgarus. The diphasicity was interpreted to indicate the presence of spikes. Thus the contractile mechanism may be accessible to the spike, but not to the junctional response. Older experiments (44) on other muscles, however, have shown that contraction can be accompanied by monophasic, summating potentials. And further, it is known that stimulation of slow axons at frequencies at which the junctional responses remain significantly below the spike threshold, can evoke considerable contraction. Thus it appears that some types of junctional responses can activate the contractile mechanism, while others cannot. Another example of this is contained in the "paradox" (46) discussed in the introduction. These intriguing observations emphasize the necessity of experiments in which electrical and mechanical events are simultaneously recorded from single muscle fibers.

Fatt and Katz (55) have recently discovered another peculiarity of the crustacean muscle spike which it shares

with the action potentials of certain slowly conducting nerve fibers of the frog (see Introduction). The sodium ions in the external medium can be replaced by a number of quaternary ammonium compounds, as well as procaine-HCl, and the effect is an augmentation and prolongation of the spike rather than its diminution. Some of these effects were very striking. The form of the action potential changes to a long almost rectangular pulse which can last several seconds and overshoot zero potential (reversed membrane potential) during a considerable portion of this time. It therefore seems likely that the mechanism underlying the crustacean muscle spike is different in some important respect from that of the squid giant axon, for example. If such a basic difference exists, it is not surprising that some of the overt attributes of the two potentials are also at variance.

The experiments testing refractoriness after the spike were at first thought to reveal another unusual attribute of crustacean muscle. It was found that a component of a test response could add to the falling phase of a conditioning spike. This part of the spike thus seemed not to be absolutely refractory. Two articles have recently appeared in which a similar effect is reported for the electroplaque of the electric eel (95) and the endplate of frog sartorius muscle (96). Altimirano, Coates and Grundfest report that a neurally evoked postsynaptic response can add to the peak and declining phase of a conditioning spike potential in the electroplaque. They present evidence that the postsynaptic

response is not electrically excitable. Very strong anodal polarization, which completely blocked the directly evoked electrical response, did not abolish the postsynaptic potential; and a similar effect was obtained with several drugs. Further, the declining phase of the conditioning response was absolutely refractory for a directly evoked test response, but not for the neurally induced postsynaptic potential. On the other hand, the postsynaptic potential was capable of activating the electrically excitable components of the membrane and thus evoke a spike. One must therefore distinguish three types of potentials in this system: electrically inexcitable postsynaptic potentials; local, electrically excitable, subthreshold potentials; and spikes. In how far this analysis is applicable to the crustacean system remains to be studied.

del Castillo and Katz (96) report that in the endplate of frog sartorius, a neurally evoked endplate potential (e.p.p.) can add to the later part of the declining phase of a directly evoked muscle spike. If the e.p.p. is made to fall earlier on the muscle spike, a dimunition of the latter results.

With respect to the question of polyneuronal innervation of single muscle fibers, the experiments on the doubly motor innervated muscles will be considered first. These experiments were approached with the orientation of proving directly by use of microelectrodes, that it was possible for muscle fibers to receive more than one motor axon. It was not anticipated that the fibers might be heterogeneous with respect

to the number of innervating axons, until this was so clearly demonstrated by the experiments on the main flexor of the lobster. Thus the number of fibers sampled in any one muscle was smaller than is now considered desirable. Despite this difficulty, however, only several fibers lacking innervation by one of the axons were found and it seems likely that almost all of the fibers of these muscles do receive both axons. Additional experiments on this point would be desirable. In any case, these experiments did serve as an additional and convincing demonstration that a single muscle fiber can respond in two different ways to the stimulation of two separate axons. Fatt and Katz (53) in their paper on multiterminal innervation also report several cases of double motor innervation in fibers of Carcinus maenas muscle.

Concerning the experiments on the main flexor of the carpopodite, there are several difficulties which should be emphasized. Although the rock lobsters from which the limbs were taken, were kept in the laboratory at 10° C in a large volume of aerated sea water, they were not fed and some of them were in relatively poor condition. Probable evidence of this was the inability to evoke spikes in some preparations. Thus, the junctional potentials recorded in these cases were probably below the "normal" size. The qualitative aspects of the results were the same in all preparations, however, which included some that were judged to be in excellent condition and did show spike potentials in many fibers (with repetitive stimulation). Another difficulty lies in

the interpretation of some of the small diphasic, notched and "negative" potentials (hyperpolarizations) frequently observed. The microelectrode records a small component of the activity of fibers adjacent to the impaled one and would seem to be responsible for these phenomena. Brock, Coombs and Eccles (97) reported that the response measured with a microelectrode just outside a motoneuron in the spinal cord of cats was as much as 5% of that recorded intracellularly and as expected was in the opposite direction from the intracellular response. Easton (98) has recorded potentials in inactive frog muscle fibers due to action potentials in fibers adjacent to the impaled one. He refers to them as "extrinsic potentials" and found that they might account for attenuation of an internally recorded action potential to a maximum extent of about 8%, and that the attenuation would be greatest in the endplate region. The extrinsic potential was typically triphasic, except at the endplate region or the ends of fibers where it was diphasic. The attenuation may be attributed to two factors. Firstly, the recorded potential is the algebraic sum of the extrinsic potential and the impaled fiber's own membrane response; and secondly, the major phase of the extrinsic potential is usually recorded by the microelectrode as negative (as a "hyperpolarization").

Because of the heterogeneity of response size in crustacean muscle fibers, it was not possible to estimate percentage values for the attenuation due to the extrinsic potential.

If, however, in these muscles a microelectrode records potentials from fibers adjacent to the impaled one, to the extent of 5-10% of their actual membrane response, then the extrinsic potentials can become a source of error in attempting to determine whether or not a fiber receives a particular axon. This will only be true in the case of fibers giving a small response and which are surrounded by others showing much larger potentials. Examination of Table 2 shows that this is the only circumstance under which diphasic or negative potentials occur. In estimating the number of fibers receiving Axon I, it was assumed that such responses as those to axon D of fibers 5, 13, and 24 were due solely to extrinsic potentials and were not counted as receiving this axon. There is also another possible source of error in this situation of a fiber with a small response surrounded by others showing large potentials. In the method of sampling used, all of the fibers directly above the impaled one had been previously pierced by the microelectrode. The latter would be expected to measure injury potentials from such nearby damaged regions. The injury potentials are merely special cases of the extrinsic potentials in which the major phase would be positive (seen as a "depolarization") at the microelectrode. In the very specific situation described above, the extrinsic potentials and injury potentials could thus provide small artifacts of almost any shape. Fortunately this situation did not arise often and these factors probably provided no source of error in at least 90% of the fibers -

tested. A greater source of error probably lay in the small size of the sample as discussed in the Results section.

The main results of the experiments on the main flexor of Panulirus are twofold. Firstly, it was shown that a muscle fiber can receive as many as four motor axons. Although spikes were not observed in fibers receiving four axons, it is very likely that they can occur. Thus it would be possible for these fibers to exhibit five different electrical responses. In addition all muscle fibers apparently receive branches from the fifth axon, the inhibitor. This follows from the observation (41) that all four contraction types can be completely suppressed by the inhibitor axon. The second main result is the finding of extreme heterogeneity with respect to the number and combination of motor axons which the muscle fibers receive. The first result tempts one to study the contractions associated with each of these electrical responses, to determine in how far the two are correlated. That the result might be illuminating is suggested by the presence of the "paradox" (46) in these muscles. The second result cautions one of the necessity of performing such studies on single muscle fibers.

E. Summary.

Some of the attributes of the crustacean neuromuscular mechanism have been studied, by separately stimulating motor axons and observing the electrical response in individual muscle fibers. The intracellular recording technique was used to accomplish the latter. Several previously reported observations were reinvestigated by these more direct methods and shown to be correct; and some interesting phenomena were discovered.

(1). Junctional potentials. The first muscular response to nerve stimulation is a junctional potential resembling postsynaptic responses elsewhere observed. The resemblance extends to the time course and the ability of successive responses to summate. The crustacean junctional potentials differ, for example, from vertebrate muscle end-plate potentials, however, in their occurrence everywhere in the muscle fiber. This distribution of the junctional potentials is effected by the nerve.

(2). Spike potentials. If a junctional potential, or the summated response consisting of some number of them, attains a certain level of depolarization, an additional response is evoked. This has been termed the "spike potential," although it can differ in several respects from spikes elsewhere observed. The crustacean spike can be graded and in these cases its size depends upon that of the junctional response evoking it. This spike may be restricted to parts of a muscle fiber without spreading to others. The rate at

which the spike appears at successive points along an RM2 muscle fiber, is similar to that for the junctional potential. The apparent conduction of the junctional potential has been attributed to the conduction of the impulse in the motor axon which runs along the muscle fiber. It was concluded that the spike was evoked at each point on the fiber by the junctional potential at that point. Although the spike exhibits refractoriness, a small component of a test response can add to the declining phase of a conditioning spike.

(3). Polyneuronal innervation. The distribution of motor axons to individual muscle fibers was studied. Two types of muscles were investigated: those receiving two motor axons and those receiving four. It was shown that more than one axon can evoke a potential in a particular muscle fiber and that the response to each axon is typically different. It seems probable that almost all fibers of muscles receiving two motor axons are innervated by each of the axons. Muscles with four motor axons are more complicated in this respect. Fibers of these muscles can receive any number of motor axons from one to four. Only a small percentage, however, received all four. Aside from this heterogeneity of axon complement, the fibers of both types of muscles showed wide variations in the size of the response evoked by any one of the axons. The different responses elicited in a single fiber can interact, in the direction of summation, and in certain cases this heterosummation can give rise to spikes.

F. References.

1. van Harreveld, A. (1945). Am. J. Physiol. 144: 477.
2. Bors, E. (1926). Anat. Anz. 60: 415.
3. Eccles, J. C. and Sherrington, C. S. (1930). Proc. Roy. Soc. London B, 106: 326.
4. Kuffler, S. W., Hunt, C. C., and Quilliam, J. P. (1951). J. Neurophysiol. 14: 29.
5. Clark, D. A. (1931). Am. J. Physiol. 96: 296.
6. Kulchitsky, N. J. (1924). J. Anat., Lond., 59: 1.
7. Fulton, J. F. (1926). Muscular Contraction and the Reflex Control of Movement. Baltimore.
8. Cattell, McK. and Stiles, P. G. (1924). Am. J. Physiol. 69: 645.
9. Katz, B. and Kuffler, S. W. (1941). J. Neurophysiol. 4: 207.
10. Sandmann, G. (1885). Arch. Anat. Physiol. Lpz. 240.
11. Hunt, C. C. and Kuffler, S. W. (1954). J. Physiol. 126: 293.
12. Kuffler, S. W. (1952). Fed. Proc. 11: 87.
13. Brown, G. L. and Harvey, A. M. (1938). J. Physiol. 93: 285.
14. Kuffler, S. W. and Vaughan Williams. (1953). J. Physiol. 121: 289.
15. Kuffler, S. W. and Vaughan Williams. (1953). J. Physiol. 121: 318.
16. Barker, D. (1948). Quart. J. Micr. Sci. 89: 143.

17. Katz, B. (1948). Proc. Roy. Soc. London. B, 135: 506.

18. Lucas, K. (1907). J. Physiol. 35: 326.

19. Lucas, K. (1917). J. Physiol. 51: 1.

20. Wiersma, C. A. G. (1933). Z. f. vergl. Physiol. 19: 349.

21. Wiersma, C. A. G. and van Harreveld, A. (1934). Arch. Neerl. de Physiol. 34: 426.

22. Biedermann, W. (1887). S. B. Akad. Wiss. Wien. 95: 7.

23. Pantin, C. F. A. (1934). J. Exp. Biol. 11: 11.

24. Pantin, C. F. A. (1936). J. Exp. Biol. 13: 111.

25. Pantin, C. F. A. (1936). J. Exp. Biol. 13: 148.

26. Katz, B. (1936). J. Physiol. 87: 199.

27. van Harreveld, A. and Wiersma, C. A. G. (1936). J. Physiol. 88: 78.

28. Mangold, E. (1905). Z. allg. Physiol. 5: 135.

29. Hoffmann, P. (1914). Z. f. Biol. 63: 411.

30. van Harreveld, A. and Wiersma, C. A. G. (1936). Proc. Natl. Acad. Sci. 22: 11.

31. van Harreveld, A. and Wiersma, C. A. G. (1937). J. Exp. Biol. 14: 448.

32. d'Ancona, U. (1926). Trav. du lab. de rech. biol. de l'univ. de Madrid. 23: 393. (Cited from van Harreveld (34)).

33. Marmont, G. and Wiersma, C. A. G. (1938). J. Physiol. 93: 173.

34. van Harreveld, A. (1939). J. Comp. Neur. 70: 267 and 285.

35. Wiersma, C. A. G. (1941). J. Comp. Neur. 74: 63.

36. Wiersma, C. A. G. and Ripley, S. (1952). Physiol. Comp. et Oecol. 2: 391.

37. Wiersma, C. A. G. and Helfer, R. G. (1941). Physiol. Zool. 14: 296.

38. Wiersma, C. A. G. and Ellis, C. H. (1942). J. Exp. Biol. 18: 223.

39. Wiersma, C. A. G. (1949). Physiol. Comp. et Oecol. 1: 68.

40. Ripley, S. H. and Wiersma, C. A. G. (1953). Physiol. Comp. et Oecol. 3: 1.

41. van Harreveld, A. and Wiersma, C. A. G. (1939). J. Exp. Biol. 16: 121.

42. Wiersma, C. A. G. and van Harreveld, A. (1939). Physiol. Zool. 12: 43.

43. van Harreveld, A. (1939). J. Exp. Biol. 16: 398.

44. Wiersma, C. A. G. and van Harreveld, A. (1938). J. Exp. Biol. 15: 18.

45. Wiersma, C. A. G. (1951). J. Exp. Biol. 28: 13.

46. Wiersma, C. A. G. and van Harreveld, A. (1938). Physiol. Zool. 11: 75.

47. Ripley, S. H. (1953). Studies on Myo-Neural Mechanisms in Arthropoda. Thesis, Calif. Institute of Tech.

48. Foettinger, A. (1880). Arch. Biol. Paris 1: 279 (cited from Ripley (47)).

49. Marcu, O. (1929). Anat. Anz. 67: 369.

50. Wiersma, C. A. G. and Wright, E. B. (1947). J. Exp. Biol. 23: 205.

51. Holmes, W. (1943). Nature, Lond., 151: 531.

52. Katz, B. and Kuffler, S. W. (1946). Proc. Roy. Soc. B, 133: 374.

53. Fatt, P. and Katz, B. (1953). J. Exp. Biol. 30: 433.

54. Wiersma, C. A. G. (1952). Ann. Rev. Physiol. 14: 159.

55. Fatt, P. and Katz, B. (1953). J. Physiol. 120: 171.

56. Hodgkin, A. and Katz, B. (1949). J. Physiol. 108: 37.

57. Nastuk, W. L. and Hodgkin, A. L. (1950). J. Cell. Comp. Physiol. 35: 39.

58. Huxley, A. F. and Stämpfli, R. (1949). J. Physiol. 108: 315.

59. Lorente de No', R. (1949). J. Cell. Comp. Physiol. 33, Suppl., 1.

60. Pringle, J. W. S. (1939). J. Exp. Biol. 16: 220.

61. Roeder, K. D. and Weiant, E. A. (1950). J. Exp. Biol. 27: 1.

62. Hoyle, G. (1955). Proc. Roy. Soc. Lond., B, 143: 281.

63. Wilson, V. J. (1954). J. Exp. Biol. 31: 280.

64. Hoyle, G. (1955). Proc. Roy. Soc. Lond., B, 143: 343.

65. Hoyle, G. (1955). J. Physiol. 127: 1.

66. Hoyle, G. (1954). J. Exp. Biol. 31: 260.

67. del Castillo, J., Hoyle, G. and Machne, X. (1953). J. Physiol. 121: 539.

68. Winton, F. R. (1937). J. Physiol. 88: 492.

69. Fletcher, C. M. (1937). J. Physiol. 90: 415

70. Pumphrey, R. J. (1938). J. Exp. Biol. 15: 500.

71. Blashko, H., Cattell, McK. and Kahn, J. L. (1931). J. Physiol. 73: 25.

72. Ramsay, J. A. (1940). J. Exp. Biol. 17: 96.

73. Röchling, E. (1922). Z. f. wissensch. Zool. 119: 285.

74. Bayliss, L. E., Boyland, E. and Ritchie, A. D. (1930). Proc. Roy. Soc. Lond., B, 106: 363.

75. Lowy, J. (1953). J. Physiol. 120: 129.

76. Lowy, J. (1954). J. Physiol. 124: 100.

77. Niewenhoven, L. M. van (1947). An Investigation into the Structure and Function of the Anterior Byssal Retractor Muscle of Mytilus edulis. Doctoral Thesis, Utrecht. (Cited from Lowy (75)).

78. Jordan, H. J. (1938). Ergebn. Physiol. 40: 437.

79. Young, J. Z. (1938). J. Exp. Biol. 15: 170.

80. Prosser, C. L. and Young, J. Z. (1937). Biol. Bull. 73: 237.

81. Prosser, C. L. and Melton, C. E. (1954). J. Cell. Comp. Physiol. 44: 255.

82. Prosser, C. L., Curtis, H. J. and Travis, D. M. (1951). J. Cell Comp. Physiol. 38: 299.

83. Nicol, J. and Young, J. Z. (1946). Nature 158: 167.

84. Nicol, J. (1951). J. Exp. Biol. 28: 22.

85. Bullock, T. H. (1945). J. Neurophysiol. 8: 55.

86. Graham, J. and Gerard, R. W. (1946). J. Cell Comp. Physiol. 28: 99.

87. Ling, G. and Gerard, R. W. (1949). J. Cell. Comp. Physiol. 34: 383.

88. Bishop, P. O. (1949). Elect. Eng. 21: 332.

89. van Harreveld, A. (1936). Proc. Soc. Exp. Biol. and Med. 34: 428.

90. Schlatter, M. J. (1941). J. Cell Comp. Physiol. 17: 259.

91. Alexandrowicz, J. S. (1951). Quart. J. Micr. Sci. 92: 163.

92. Kuffler, S. W. (1954). J. Neurophysiol. 17: 558.

93. Kuffler, S. W. and Gerard, R. W. (1947). J. Neurophysiol. 10: 395.

94. Wiersma, C. A. G. (1955). Physiol. Comp. et Oecol. (in press).

95. Altimirano, M., Coates, C. and Grundfest, H. (1955). J. Gen. Physiol. 38: 319.

96. del Castillo, J. and Katz, B. (1954). J. Physiol. 125: 546.

97. Brock, L. G., Coombs, J. S. and Eccles, J. C. (1952). J. Physiol. 117: 431.

98. Easton, D. M. (1955). J. Neurophysiol. (in press).

APPENDIX*

ELECTRICAL MEASUREMENTS ON SEA-URCHIN EGGS

Lillie (99) originally suggested that the fertilization reaction and the block to polyspermy might take the form of a state of excitation passing over the surface of the egg; and that this excitation would in some way be analogous to the action potential of nerve and muscle. With this suggestion in mind, some of the pertinent attributes of eggs will be considered and an attempt to compare eggs with other excitable tissues will be made. The discussion will be limited almost entirely to echinoderm eggs.

A number of determinations of the concentrations of inorganic ions within sea-urchin eggs have been made. The most recent ones, by Rothschild and Barnes (100) are the only determinations in which an attempt was made to estimate the error due to ions in the small volume of residual suspending medium, after centrifugation of the eggs. There is wide variation in the values given by previous authors and only the results of Rothschild and Barnes will be considered. Their values for sodium, potassium and chloride, in the eggs of Paracentrotus lividus are given in the

---

* This problem constituted part of the work for a minor in Embryology.

following table, together with similar estimations of internal ion concentrations on the squid giant axon and frog muscle. The values for the latter two are taken from a review by Hodgkin (101).

Table

Ion	Concentration in millimoles per Kg. of H_2O		
	Paracentrotus Egg	Loligo* giant axon	Frog* Sartorius
Sodium	52 (485)	49(440)	15(110)
Potassium	210 (10)	410(22)	125(2.6)
Chloride	80 (566)	40(560)	1.2(77)

* Freshly dissected material.

The numbers in parentheses give the concentrations of the same ions in the fluid which normally surrounds these tissues. There are fairly steep concentration gradients for each of the above ions across each of the cell surfaces. The resting potential of nerve and muscle is usually attributed mainly to the potassium-ion gradient, while the sodium-ion gradient is held responsible for the conducted action potential. On this basis, it might seem possible for eggs to exhibit electrical excitation. It should be emphasized, however, that the concentration gradients are not sufficient in themselves to give rise to bioelectric phenomena. It is also necessary

that the internal ions be free to diffuse and that the cell surface be permeable to at least one of the ion species which has a gradient. Furthermore, if permeabilities to more than one of the ions exist, the former must be selective to the extent that the sum of the potentials thus produced is not zero.

In a brief paper, Gelfan (102) reported some pertinent experiments with the eggs of the starfish Asterias forbesii. He inserted a microelectrode, having a tip diameter of 1-3 μ, into the cytoplasm of an egg and recorded no potential difference across the egg surface. This was true of eggs of varying age, from immaturity to second cleavage. When the microelectrode entered the germinal vesicle, however, a potential was measured. The size of the potential varied between 4 and 21 mv. and seemed to depend upon the condition of the eggs, being higher when recorded from eggs in better condition. The average potential measured in about 100 eggs was 10 mv., the germinal vesicle always being positive with respect to the external sea water. With the microelectrode in the germinal vesicle, the same potential was measured whether the other electrode was in the sea water or inserted into the egg cytoplasm. The addition of KCl to the external medium reduced the potential. Bathing the eggs in a solution consisting of one-half sea water and one-half isotonic KCl, lowered the potentials to 10-20% of the controls. Gelfan does not specifically state if this was also true when the

"indifferent" electrode was in the cytoplasm. Additional comment on these results will be reserved for the Discussion.

In 1938, Lord Rothschild (103) impaled eggs of the sea urchin, Echinus esculentus, with glass microelectrodes of tip diameter 2-10 μ. The mature eggs of the sea urchin do not have a germinal vesicle, but rather a more compact nucleus and it seems unlikely that he inadvertently entered this nucleus. To this extent then, his results agree with those of Gelfan, for Rothschild measured no potentials. This was true of both fertilized and unfertilized eggs, some of which were fertilized with the micropipette inside the egg. He used a relatively slow-moving galvanometer to record potentials and might have missed rapid transients during fertilization. There was one abnormal circumstance in which Rothschild did measure potentials. If the eggs were placed in an ion-deficient medium consisting of 1 part sea water to 19 parts isotonic dextrose, they underwent cytolysis. During the cytolytic process a potential of about 8 mv. (inside negative) was observed. The potential fell to zero at the end of cytolysis which had a duration of 1-2 min.

In 1935, Peterfi and Rothschild (104) published a note on transient electrical changes during fertilization in the frog egg. They placed two small external electrodes at opposite poles of the egg and ejected sperm from a small pipette near the surface of the egg. The nature of the

electrical changes was not discussed except that they were apparently "irreversible." In a later paper (105), Rothschild states that due to technical difficulties, experiments on the electrical properties of frogs' eggs should not have too much value placed upon them until they are systematically repeated.

Very recently, Scheer, et al. (106), have confirmed the lack of a resting potential in the eggs of two species of sea urchin, Paracentrotus lividus and Arbacia lixula. They also inserted a glass micropipette into the egg to perform their measurements. Their electrodes had tip diameters under 5 μ. These authors were able, however, to measure some transient potentials accompanying fertilization. Although it is difficult to discern the character of the response from the records published, the potentials could apparently have the form of damped oscillations (Paracentrotus) or be irregular (Arbacia). The size of the potentials was of the order of 5 mv. or less and during the pulses the cytoplasm became positive with respect to the external medium. In Paracentrotus the oscillations were very rapid, a single wave seeming to last 0.2 - 0.3 msec. They were few in number. In Arbacia the waves were more numerous, as many as fifty having been observed during one fertilization. Although Scheer, et al., term these waves, action potentials, they point out that the egg potentials differ from those of nerve and muscle in several respects. The egg waves are not depolarizations in

as much as there is no resting potential. These authors also refer to an observation by Cole (107), that eggs exhibit no change in membrane resistance upon fertilization. This is an important point in attempting to assign a mechanism to the egg potentials. It is not obvious, however, that Cole's experiments are really pertinent to this point. His impedance measurements took several minutes to complete and would probably not have detected any transient changes in membrane conductance. Furthermore, as will be considered below, Cole's method was probably not sensitive enough to detect changes in impedance of the sort which accompany nerve action potentials.

There are also some other types of observations which are pertinent to a consideration of the way in which the fertilization reaction and the block to polyspermy spread over the egg. A spreading "cortical reaction" of some type has long been recognized. For example, Just (108) reported that the block to polyspermy spread over the egg preceding the elevation of the fertilization membrane. Moser (109) observed that a thin layer of cortical granules broke down on fertilization and that granule breakdown proceeded as a wave from the point of sperm entry. The point on the egg surface opposite to that of sperm entry was the last to show cortical-granule breakdown. Moser found that this wave took about 10 sec. to pass around the entire surface of the egg. In dark-field illumination, a bright layer at the

egg surface was seen to disappear on fertilization. In 1949, Rothschild and Swann (110) also observed a cortical change occurring on fertilization. They used dark-field illumination but found, contrary to Moser, a wave of brightness progressing around the egg surface from the sperm entry point. About 20 sec. was required for the wave to spread over the entire egg. This reaction preceded fertilization membrane elevation. The latter is, of course, also a reaction to fertilization and the lifting off of this membrane also seems to start at the point of sperm entry. That these comparatively slow reactions are not the primary block to polyspermy is indicated by some observations in the later work of Rothschild and Swann (111). For example, they used high sperm concentrations, with which all eggs were estimated to have been fertilized within 2 sec. after insemination; but in the succeeding several seconds only small fractions of the eggs became polyspermic (less than 10%?). It is also true, however, that much longer times were necessary for the block to polyspermy to become entirely completed. In fourteen experiments, an average of 63 sec. elapsed after insemination before the number of polyspermic eggs ceased to increase. Rothschild and Swann consider the possibility of two different mechanisms: a rapid initial block to polyspermy, which is incomplete, followed by a slower process which completes the block. They also mention the possibility that the block would spread rapidly to all parts

of the egg, but would not be all-or-nothing, and that it would increase in strength after it had spread.

Methods.

Glass microelectrodes of less than 1 μ tip diameter were used except where otherwise mentioned. The methods of preparing the microelectrodes and manipulating them was the same as described in section IIB with several exceptions. For most experiments L-shaped microelectrodes were used. They were affixed to a rod, carried by the micromanipulator, in such a position that the pointed arm of the "L" was almost parallel and close to the bottom of the vessel containing the eggs (in sea water). The other arm of the "L" projected vertically through the surface of the sea water and the lead to the cathode-follower was inserted into it. This arrangement allowed entries to be made using a horizontal motion which facilitated observation as well as holding of the eggs. The latter were secured by the following procedure. The end of a glass tube, which was carried by another micromanipulator, was drawn out to a fine capillary, the inner diameter of which was only slightly smaller than the diameter of the eggs.* The other end of the tube was connected to a screw-operated hypodermic syringe. With this arrangement it was possible to just suck an egg into the orifice of the capillary and keep it

*Adopted from a technique used by Mitchison and Swann (112) (suggested by Prof. A. Tyler).

there by making occasional adjustments with the screw. This procedure was important, in that it was not found possible to enter an egg without first securing it; and this method was the most satisfactory out of several that were attempted.

The recording procedure was similar to that described in section IIB. The microelectrode and Ag-AgCl bath electrode led into a differential cathode-follower input stage. The output of the latter was split, so that the two beams of the #322 Dumont oscilloscope registered the input signal at different sensitivities, one following d.c. amplification and the other after a.c. The sensitivity of d.c. recording was 25 mv./in. deflection of the beam. The other beam displayed the a.c.-amplified signal at usually 50 times that amplification. The microelectrodes were filled with 3M KCl.

The eggs and sperm were collected by standard procedures involving injection of isotonic KCl into mature urchins. In most experiments the jelly coats were removed from the eggs using acidified sea water. In later experiments, however, it was found that the jelly did not seem to impede the entry of the microelectrode if the egg was well secured; and the acid sea water treatment was omitted. Two species of sea urchins were used: Strongylocentrotus purpuratus and Lytechinus pictus. Most of the later experiments were performed with S. purpuratus eggs.

Results:

Considerable difficulty in entering the eggs was at first encountered, using a vertical descent of the microelectrode. The eggs tended to roll out from under it. The method of securing the egg in the tip of a capillary tube made apparent entries easily performed. The egg was usually slightly distorted by both the securing and entering processes, but the elevation of a membrane following fertilization was not inhibited. The inability to measure a resting potential, reported by several previous authors, was confirmed. Possible reasons for this will be considered. The transient potentials accompanying fertilization, reported by Scheer, et al., were not observed; but only five successful cases of fertilization with a microelectrode apparently inside an egg were obtained. A large increase in resistance between the recording electrodes was not observed following the impaling of an egg. Each of these points is considered in a little more detail below.

Although an exact tally was not kept, perhaps forty unfertilized eggs were impaled without measuring any potential difference between the electrodes. The maximum sensitivity of d.c. recording that was available (25 mv./in.) probably did not allow discrimination of steady potentials of less than 1-2 mv. It was difficult to make entries with the microelectrode and observe the oscilloscope simultaneously and in four of the trials a second observer watched the

oscilloscope during the process. It was possible to push an electrode into an egg and out the other side without detecting a potential. Because of the opacity of the egg, it was not possible to see the tip of the electrode within it. But it was possible to move the electrode slightly and observe localized movement of granules. Fertilized eggs were also entered. In these cases it was possible to enter the fertilization membrane and to observe the electrode tip in the space between membrane and egg. No potential was detected. The further traverse of the electrode tip into the fertilized egg, itself, likewise resulted in no measurement of a potential. The spherical shell formed by the fertilization membrane was much stiffer than the surface of the unfertilized egg and was more easily entered by the microelectrode.

A regrettably small number of successful attempts to observe electrical changes during fertilization were made. In five such cases, however, no potentials were observed, although in each of them a fertilization membrane was elevated. A drop of concentrated sperm suspension was placed near the impaled egg to effect fertilization. The time required for the sperm to reach the egg insured that fertilization did not occur before attention could be directed to the oscilloscope. Observation of the latter was continued for several minutes and then the egg was checked for the presence of a fertilization membrane. With the

additional sensitivity provided by the condenser-coupled preamplifier, transient potentials of less than 1 mv. could have been detected.

In the last two experiments performed, it was realized that there was a simple method for estimating a certain maximum possible transverse resistance of the egg surface. It is a method commonly employed for measuring the resistance of microelectrodes. The indifferent electrode was removed from the bath and a d.c. potential (50-100 mv.) was applied between that electrode and the bath. Then a known resistor, having a similar resistance to the microelectrode (10-20 megohms) was placed across the two input leads. The applied potential is then divided between the microelectrode and the known resistance and the amount of attenuation of the recorded d.c. potential then depends upon the relative values of the two resistances. If the procedure is now repeated with an egg impaled on the microelectrode, the egg resistance will be in series with the microelectrode resistance. Any apparent increase in the latter will then be equal to the egg resistance. It was empirically determined, by putting known resistances in series with the microelectrode, that the smallest increase in resistance which could be readily detected was about 1 megohm. Ten unfertilized and six fertilized eggs were impaled and tested in this way. In no case did the apparent microelectrode resistance increase after impaling an egg. This means that under the

conditions of the experiment the resistance of the entire egg surface must not have exceeded 1 megohm. The eggs used (S. purpuratus) were in the vicinity of 75 μ in diameter. The surface area of an egg was therefore about 1.8×10^{-4} $cm.^2$ and the apparent membrane resistance was less than 180 ohm $cm.^2$ ($1.8 \times 10^{-4} \times 10^6$). The microelectrodes used in these two experiments were larger than in others, being about 2 μ in external tip diameter.

Discussion.

The major problem associated with these and other similar experiments concerns the lack of a resting potential in echinoderm eggs despite the presence of ion concentration gradients. The problem seems very complicated and there are a number of possible explanations. One type of explanation considers that a potential would actually exist, but would not be measured because of experimental difficulties. Rothschild (103) has considered some of these possibilities and rejected them. For example, it might be that the microelectrode never actually enters the egg, but merely invaginates the surface. He reports that he could inject fluid from the micropipettes and see it diffuse through the cytoplasm. In the present experiments the electrode could be pushed into one side of the egg and out the other. It seems likely that in some of these cases the microelectrode entered the first side before leaving

the opposite one. Furthermore, although the egg surface does invaginate upon attempting to enter, only a partial withdrawal of the electrode serves to restore the spherical shape of the egg.

Another possibility is that the egg surface does not form a good seal about the microelectrode and that the membrane is short-circuited. This is also a possible explanation of the apparent low membrane resistance found in the present experiments. Assuming the specific resistance of sea water to be 25 ohm cm. (107), that the egg membrane is infinitely resistive, and is 0.1 μ in thickness, a hole in the membrane with an area of 0.025 μ^2 would account for the observed resistance (10^6 ohms). The assumption that the resistivity of the membrane is infinite is probably not valid. Cole (113) and Cole and Spencer (114) reported experiments which seemed to show that the echinoderm egg membrane did have an infinite resistance. Working with suspensions of eggs, and using an equation developed by Maxwell, they found that the non-conducting volume of the suspension was equal to the volume of the eggs. Cole (115) reported later, however, that the method was inadequate. A 2% error in the estimation of the volume of the eggs would allow membrane resistances as low as 3 ohm cm.^{2}, which is very low indeed. That the method was inadequate was also shown by the fact that, using an adaptation of it,

the membrane resistance of the squid giant axon was found to be not detectably different from infinity. The squid axon is now known to have a membrane resistance of the order of 1000 ohm cm.^{2}. The value of 0.1 μ chosen above for the thickness of the membrane is probably within an order of magnitude of the correct value. The calculation only shows that a small hole can provide an apparently low membrane resistance. Thus it does not seem unreasonable that the low upper limit of apparent membrane resistance, found in the present experiments (180 ohm cm.^{2}), is due to both the conductivity of the membrane and an incomplete seal around the electrode. Unfortunately, this equivocal answer does not help in determining the reason for the lack of a resting potential. Rothschild (103) reported an upper limit for the resistance of a sea-urchin egg membrane of 10^6 ohm cm.^{2}. This value, however, may also be in error because of an incomplete seal around the electrode. Gelfan's (102) experiments, mentioned in the introduction, might also seem to argue against an infinite membrane resistance. He found potentials across the germinal vesicle membrane, which were of similar size whether the "indifferent" electrode was in the surrounding sea water or in the cytoplasm. But in this case, as well, the possibility of electrical leaks about the microelectrodes existed. Experiments on the electrical properties of echinoderm eggs do not seem, then, to provide an answer concerning the infinite resisti-

vity of the membrane. Evidence from other sources, however, is pertinent. Brooks (116) reported in a Cold Spring Harbor Symposium lecture that with the eggs of Arbacia, Patiria, Pisaster, and Urechis..."any radioactive ions added to the sea water in which the eggs lie appear quickly in the eggs, their intracellular concentration reaching a maximum in about 2 to 15 minutes." Additional data for the echinoderm eggs was not given and a more extended treatment of these results could not be found. Brooks does state, however, that radioactive phosphate put in the external medium in low concentrations, comes to equilibrium in 1 to 2 hours in eggs of Arbacia and Asterias. And a graph is given showing rapid uptake of radioactive sodium by eggs of Urechis caupo. If these data can be accepted they argue against the impermeability of the egg membrane as the reason for the lack of a resting potential.

One might also think that no resting potential is present because the potentials due to the various ion gradients add algebraically to zero. In order to predict what potential should result from an assemblage of ions separated by a membrane, it is necessary to know the concentrations of each ion on either side of the membrane, and the membrane permeabilities to each. Since the permeabilities are unknown, it is not possible to make such a prediction. It seems unlikely, however, unless a special mechanism for this purpose were present, that the situation, of all potentials adding up to zero, would exist.

Another possible explanation for no resting potential is that the ions within the egg are bound and not free to diffuse. The rapid exchange of radioactive ions (116) also makes this idea unlikely. Gelfan's experiments also argue against it, for even if there were a hole in the membrane, the cytoplasm would have to be conducting for the germinal-vesicle potential to be measured.

One is hard-pressed to decide among the above possible explanations, and perhaps there are others. Of those presented, the explanation based on a short-circuiting of the membrane by an incomplete seal around the electrode seems to suffer the least objections.

Concerning the inability to confirm the presence of transient potentials during fertilization, it should be emphasized that the present experiments were few in number. Scheer, et al., took a number of precautions to guard against artifacts. They found differences in the character of the potentials between the two species of sea urchins used. It might be, then, that the discrepancy is attributable to interspecific differences. If this idea is correct, it perhaps detracts from the significance of the potentials as part of the mechanism of fertilization.

Little light has been shed upon the original suggestion (99) that the fertilization reaction would be propagated over the egg surface by a wave of excitation resembling a nerve or muscle action potential. It can be said, how-

ever, of the waves discovered by Scheer, et al., that the resemblance is obscure. The eggs showed no resting potential under the conditions of measuring these waves, nor did the form or lack of regularity of the latter suggest action potentials.

References for Appendix

99. Lillie, R. S. (1913). J. Exp. Zool. 15: 23.

100. Rothschild, Lord and Barnes, H. (1953). J. Exp. Biol. 30: 534.

101. Hodgkin, A. (1951). Biol. Rev. 26: 339.

102. Gelfan, S. (1931). Proc. Soc. Exper. Biol. Med. 29: 58.

103. Rothschild, Lord (1938). J. Exp. Biol. 15: 209.

104. Peterfi, T. and Rothschild, Lord (1935). Nature, 135: 874.

105. Rothschild, Lord (1946). Nature, 157: 720.

106. Scheer, B. T., Monroy, A., Santangelo, M., and Riccobono, G. (1954). Exper. Cell Res. 7: 284.

107. Cole, K. S. (1928). J. Gen. Physiol. 12: 37.

108. Just, E. E. (1919). Biol. Bull. 36: 1.

109. Moser, F. (1939). J. Exp. Zool. 80: 423.

110. Rothschild, Lord and Swann, M. M. (1949). J. Exp. Biol. 26: 164.

111. Rothschild, Lord and Swann, M. M. (1952). J. Exp. Biol. 29: 469.

112. Mitchison, J. M. and Swann, M. M. (1954). J. Exp. Biol. 31: 443.

113. Cole, K. S. (1935). J. Gen. Physiol. 18: 877.

114. Cole, K. S. and Spencer, J. M. (1938). J. Gen. Physiol. 21: 583.

115. Cole, K. S. (1940). Cold Spring Harbor Symp. Quant. Biol. 8: 110.

116. Brooks, C. S. (1940). Cold Spring Harbor Symp. Quant. Biol. 8: 171.