

Correlated Electrophysiological and Ultrastructural Studies of a Crustacean Motor Unit

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ABSTRACT Structural and functional interrelationships between the pre- and postsynaptic elements of a singly motor innervated crab muscle (stretcher of *Hyas araneus* L.) were examined using electrophysiological and electron microscopic techniques. Excitatory postsynaptic potential (EPSP) amplitude at 1 Hz was found to be inversely related to the extent of facilitation, and directly related both to the amount of transmitter released at 1 Hz and the muscle fiber input resistance (R_{in}). The extent of facilitation (F_e), taken as the ratio of the EPSP amplitude at 10 Hz to that at 1 Hz, was inversely related to muscle fiber R_{in} , τ_m , and sarcomere length. Sarcomere length was directly related to R_{in} and τ_m . The excitatory nerve terminals of low F_e muscle fibers had larger neuromuscular synapses than did those of high F_e fibers. Inhibitory axo-axonal synapses were more often found in low F_e muscle fibers. These structural features may account for the greater release of transmitter at low frequencies from the low F_e nerve terminals as well as provide for a greater amount of presynaptic inhibition of low F_e muscle fibers. The implications of these findings for the development and physiological performance of the crustacean motor unit are discussed. It is proposed that both nerve and muscle fiber properties may be determined by the developmental pattern of nerve growth.

Many crustacean motor and inhibitory axons possess synapses which differ physiologically in such properties as facilitation and quantal content of transmitter release (Atwood, 1967 *a*; Bittner, 1968 *a*; Atwood and Bittner, 1971). So far, there has been no study of the ultrastructural features of the physiologically different synapses of these axons. One aim of the present study was to correlate the ultrastructural features of crustacean excitatory neuromuscular synapses with their physiological performance. We used the stretcher muscle of the spider crab *Hyas araneus* for this study, because of the

pronounced physiological differences among the neuromuscular synapses of the single motor axon supplying this muscle.

In some crustacean muscles, for example the opener muscle of the spider crab *Chionoecetes tanneri* (Atwood, 1965), the physiological properties of the neuromuscular synapses are matched with the electrical and mechanical properties of the muscle fibers. Electrically excitable, rapidly contracting fibers are supplied by neuromuscular synapses with marked facilitation, while less excitable, more slowly contracting muscle fibers receive poorly facilitating synapses. In other crustacean muscles with relatively more uniform fiber populations, for example the opener muscle of the crayfish *Procambarus clarkii*, such a correlation is less certain, although there may still be a correlation between presynaptic properties (amount of facilitation) and membrane electrical properties of the postsynaptic elements (Bittner, 1968 *b*; Atwood and Bittner, 1971). The question arises: How general is the matching of pre- and postsynaptic properties in crustacean muscles? The stretcher muscle of *Hyas* provided a test for the "matching phenomenon," because in spite of the synaptic diversity all fibers of this muscle appear to be of the slowly contracting, electrically inexcitable type. Nevertheless, we were able to show that there is significant correlation of pre- and postsynaptic physiological and structural features. The mechanisms underlying the "matching phenomenon" are of potential importance for consideration of the development of nerve circuits.

METHODS

The stretcher muscle (reductor of the propodite) in walking legs 1-3 of the spider crab, *Hyas araneus* L., was used throughout; this muscle receives motor innervation from a single neuron which also innervates the opener (abductor) muscle of the dactylopodite (Wiersma and Ripley, 1952). The stretcher muscle was exposed by removing a semicircular piece of the exoskeleton overlying its outer surface. The leg nerve was exposed in the meropodite. The preparation was kept in physiological solution (Atwood and Dorai Raj, 1964) maintained at 11°-12°C by means of a Peltier battery.

The bundle of the leg nerve containing the stretcher-opener motor axon was stimulated with rectangular current pulses of 0.02-0.08 msec duration delivered through thin platinum wire electrodes, insulated except at the tips. Postsynaptic potentials (PSPs) of various muscle fibers were recorded with glass intracellular microelectrodes, filled with 3 M KCl and of 8-15 M Ω resistance. In each case, records of the excitatory postsynaptic potential (EPSP) were made while the motor axon was stimulated at 1/sec, 10/sec, and usually at several other frequencies. The facilitation index F_s of Atwood and Bittner (1971), i.e. the ratio of EPSP amplitude at 10/sec to that at 1/sec, was used to compare over-all synaptic properties of the motor innervation of different muscle fibers.

Most of the fibers sampled were located in the surface layer of the muscle. A few recordings from fibers in the second and third layers below the surface were made by pushing the electrode between surface fibers. No differences in EPSPs were seen

between the surface and deeper layers. Furthermore, no differences in fiber morphology were observed between these layers as seen in transverse sections of paraffin-embedded material. Thus the sample obtained in the present study probably is representative of the muscle as a whole.

Extracellularly recorded synaptic potentials were obtained from individual synaptic regions with glass microelectrodes filled with 2 M NaCl, of 2–4 M Ω resistance. Techniques described elsewhere (Dudel and Kuffler, 1961 *a*; Atwood and Johnston, 1968) were used in making these records, which served to relate the properties of individual synaptic regions to those of EPSPs recorded intracellularly. Use was made of signal averaging, as described by Dudel (1965), to compare extracellularly recorded synaptic potentials at 1 and 10/sec, especially for highly facilitating synapses. The Fabri-Tek 1072 Signal Averager (Nicolet Instrument Corp., Madison, Wis.) was used for this purpose.

Membrane electrical constants were determined for a number of muscle fibers with known EPSP properties. Methods of square pulse analysis (Hodgkin and Rushton, 1946; Fatt and Katz, 1953) were used. Rectangular hyperpolarizing current pulses (200–800 msec duration) were passed through an intracellular microelectrode at the tendon end of the fiber, while a second microelectrode was inserted at two or more locations along the fiber to record the membrane voltage responses. All fibers examined behaved as “short cables,” with length constants (λ) often about equal to the length of fiber. Equations for the “short cable” situation given by Weidmann (1952) have been applied to frog slow fibers by Stefani and Steinbach (1969) and to crustacean muscle fibers by Orkand (1962), Atwood (1963), and others. The length constant of a muscle fiber with “short cable” properties can be determined by measuring the potentials at the two ends of the fiber during injection of current (see Appendix).

Measurement of membrane time constant (τ_m) is different for the short cable situation compared to the simpler “infinite cable” model (Stefani and Steinbach, 1969). For the infinite cable, τ_m equals 0.84 of the time required for the electrotonic potential to reach its steady value when recorded close to the current electrode. For a short cable, this value lies between the extremes of 0.63 for a spherical core conductor and 0.84 for the infinitely long core conductor, as shown graphically by Stefani and Steinbach (1969: p. 390, Fig. 3). We used this graph in estimating membrane time constants for fibers used in the present study.

A second method of comparing τ_m in different muscle fibers was to measure the time required for the EPSP to decline to 0.37 of its peak amplitude. It was assumed that the fibers were uniformly depolarized by the EPSP. There was no appreciable difference between the mean values of τ_m obtained by the two methods. The τ_m values in Figs. 7 and 10 were measured using the latter method, because it permitted comparison of a larger number of fibers.

Most of the *Hyas* stretcher fibers were not perfectly circular in cross-section but instead more closely resembled ellipses. Therefore, calculations of cable constants were made assuming the fibers represented cylinders of elliptical cross-section. Fiber dimensions were measured with an ocular micrometer which was magnified so that the precision of the measurements was ± 0.074 mm. Measurements on dissected single

fibers were made after the entire muscle had been fixed *in situ* at resting length in 3% glutaraldehyde made up in physiological solution.

Muscle fiber sarcomere lengths were also measured after fixing the entire muscle as described above. Individual fibers were removed from the muscle and teased apart into a number of myofibril bundles. Five consecutive sarcomeres were measured in several (5–10) myofibril bundles. One-fifth the average value of these measurements was taken to represent the mean sarcomere length of the fiber. The precision of the measurements was $\pm 0.018 \mu\text{m}$.

Paraffin sections of the whole muscle and of single muscle fibers were prepared using routine histological methods (Sherman and Atwood, 1971). Fixation of muscle fibers for electron microscopy was done in two ways.

(a) Fibers were fixed for 2 hr at room temperature in a solution containing 4% glutaraldehyde, 2% NaCl, 0.2% CaCl_2 , and 0.1 M cacodylate buffer at pH 7.4. After a 6 hr wash in buffer plus salts with several changes of solution, the tissues were postfixed for 1 hr in a solution of 1% OsO_4 , the above salts, and buffer at pH 7.4. This method is similar to that used for lobster muscles (Sherman and Atwood, 1971).

(b) Fibers were fixed in a solution containing 1% glutaraldehyde, 2% formaldehyde, 3% NaCl, 4% sucrose, and 0.1 M NaPO_4 Sorenson phosphate buffer at pH 7.4 for 1 hr at room temperature. They were then washed in a solution containing 8% sucrose and 0.1 M phosphate buffer (pH 7.4) for 1–2 hr at room temperature, with several changes of solution. Postfixation was done in 1% OsO_4 , 4% sucrose, and 0.1 M phosphate buffer (pH 7.4) for 1 hr at room temperature. This method was suggested to us by a reviewer of this paper and is based on a method developed by Fahrenbach (1968).

Following fixation, the fibers were dehydrated in a graded ethanol: water series, cleared in propylene oxide, and embedded in Epon: Araldite. Thin sections were stained in ethanolic uranyl acetate and in lead citrate, and examined with a Philips EM 200 electron microscope.

Method (b) was generally superior to method (a) for this material: fewer artifacts (myelin figures, vacuolation in axons, etc.) were evident. Size and shape of synaptic vesicles were similar with both methods. Muscle structure was generally better preserved with method (b), although neither method gave completely satisfactory results.

RESULTS

Variation in EPSP Characteristics

EPSPs varied greatly in amplitude and duration from one muscle fiber to the next without any obvious intramuscular regional differentiation. The extent of variation of EPSP properties is illustrated in Fig. 1. In fiber A, the EPSPs were small at 1 Hz, but at 10 Hz they had facilitated as much as nine times. By contrast, the EPSPs recorded from fiber C were large at 1 Hz and did not facilitate appreciably when the frequency was raised to 10 Hz. The EPSPs of fiber B are intermediate between those of A and C.

We wish to emphasize that variation in EPSP properties was continuous, although for convenience it is easier to examine the extremes of the distribu-

tion. Any model which seeks to explain the mechanisms of synaptic variation must take into account the continuous spectrum of EPSP types.

The facilitation index, F_e , was used as a convenient measure to compare the extent of facilitation seen in different muscle fibers. This index appeared to represent well the facilitation characteristics of most fibers, since the major increment of facilitation usually occurred between 1 and 10 Hz. Furthermore, most fibers did not contract appreciably at these frequencies, and injury to the impaled fibers was minimized.

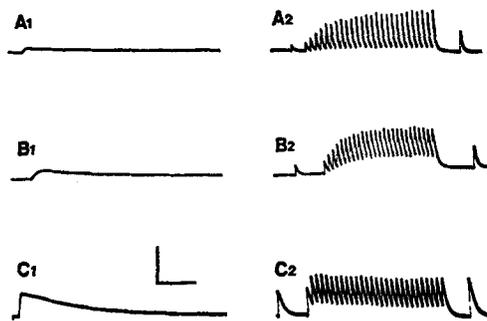


FIGURE 1. Excitatory postsynaptic potentials (EPSPs) recorded from three different stretcher muscle fibers of *Hyas* to show the range in EPSP characteristics and facilitation that occurs in this singly motor innervated muscle. The stimulation frequency in A_1 , B_1 , and C_1 is 1 Hz; it was changed from 1 to 10 Hz and then to 1 Hz again in A_2 , B_2 , and C_2 . EPSP amplitude and duration were smallest in fiber A, intermediate for B, and largest for C. The extent of facilitation (F_e) was in the reverse order, i.e. greatest in A and smallest in C. Vertical calibration: 7.5 mv in A_1 and A_2 ; 15 mv in B_1 , B_2 , C_1 , and C_2 . Horizontal calibration: 30 msec in A_1 and B_1 ; 75 msec in C_1 ; 750 msec in A_2 , B_2 , and C_2 .

Those muscle fibers giving large EPSPs at 1 Hz often showed a decrease in EPSP amplitude at 10 Hz. Examination of voltage-current curves obtained from such fibers indicated that most of the decrease in EPSP amplitude was attributable to rectification (Fig. 2). The more rapid decay of the EPSPs at larger total membrane depolarizations, indicative of increased membrane conductance, suggested this also.

Poorly facilitating fibers usually showed nonlinear voltage-current plots, reflecting a decrease in membrane resistance, when depolarized more than 8–12 mv, which corresponds to the levels of depolarization attained during stimulation of the motor axon at 10 Hz. This would reduce the size of the EPSP produced by stimulation at 10 Hz, and hence the calculated value of F_e . Uncorrected F_e values below 2.2 had to be adjusted for muscle fiber membrane rectification, using information obtained from the voltage-current curves. In general, the correction was made by extrapolating the initial linear part of the voltage-current curve and adding the difference between this

extrapolated curve and the observed voltage-current curve to any nerve-evoked depolarizations falling on the nonlinear part of the observed voltage-current curve. The resulting corrected value represented the nerve-evoked depolarization that would occur in the absence of rectification. Thus, more accurate estimation of the EPSPs produced by stimulation at 10 Hz and a more accurate estimate of F_e were obtained. The corrected F_e 's were all

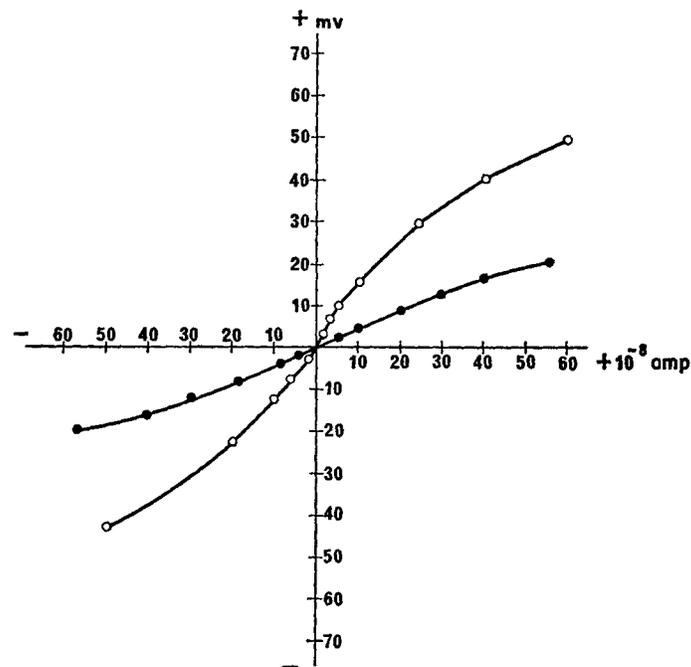


FIGURE 2. Current-voltage relationships for representative low and high F_e muscle fibers. The low F_e fiber (open circles, corrected $F_e = 2$) shows a nonlinear curve above 8 mv depolarization, indicating the occurrence of rectification. The curve of the high F_e fiber (closed circles, $F_e = 6$) is linear at depolarizations ranging up to 15 mv.

greater than 1.0 for the fibers in this study, although many of the uncorrected F_e 's fell below this value. Apparently, at least a small amount of facilitation occurred in all fibers of this muscle between 1 and 10 Hz.

No attempt was made to correct for the approach of the membrane potential at 10 Hz stimulation to the equilibrium potential for the EPSP, since we did not know the latter value. Such a correction would not have substantially affected the subsequent analyses.

Almost all fibers with observed F_e greater than 2.2 did not show appreciable rectification below 15 mv of depolarization (Fig. 2). The depolarizations obtained with 10 Hz stimulation were invariably less than this, so reasonably accurate values of F_e were obtained from these fibers without correction.

The above results serve to illustrate the fact that the muscle fibers differ in electrical properties, and that muscle fiber properties are "matched" with EPSP properties. We will discuss the "matching" effect in more detail in a later section.

Atwood and Bittner (1971) found that an inverse relationship exists between EPSP amplitude at 1 Hz and F_o in stretcher muscle fibers of the crab *Grapsus grapsus* and opener muscle fibers of the crayfish *Procambarus clarkii*. This relationship was confirmed for *Hyas* (Fig. 3). The correlation between

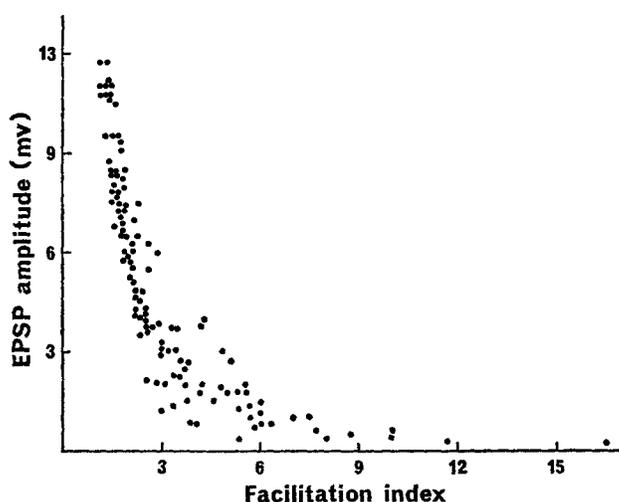


FIGURE 3. Relationship between EPSP amplitude at 1 Hz (Y , ordinate) and F_o (X , abscissa) for 120 muscle fibers. Correlation coefficient is 0.95 which is significant at the 1% level. Equation for the line best fitting these points is $Y = 17.03 (X^{-1.52})$.

these parameters is highly statistically significant, as determined by a least squares regression analysis.

The relationship between EPSP amplitude at 1 Hz and F_o was further examined by making extracellular recordings of synaptic potentials at individual synaptic regions. This method of recording provides an indication of transmitter release at individual synaptic regions (Dudel and Kuffler, 1961 *a*). At 1 Hz, large externally recorded synaptic potentials (ERSPs) were recorded from sites on poorly facilitating muscle fibers, whereas much smaller ERSPs were recorded from highly facilitating fibers (Fig. 4). At 10 Hz, the ERSPs recorded from low F_o fibers showed much less facilitation than those observed in high F_o fibers. Changes in ERSP amplitude and in EPSP amplitude were roughly parallel, when the frequency was increased from 1 to 10 Hz.

Lower quantum content at the high F_o fibers was indicated not only by the smaller ERSP size at low frequency, but also by the higher number of transmission failures. At 1 Hz, low F_o fibers seldom showed any transmission

failures, whereas in high F_e fibers they were common. Quantum contents of high F_e synapses, estimated from number of failures (del Castillo and Katz, 1954), ranged from 0.08 to 0.5 at 1 Hz.

In a few fibers, spontaneous miniature synaptic potentials were recorded extracellularly, although usually the frequency was extremely low. From

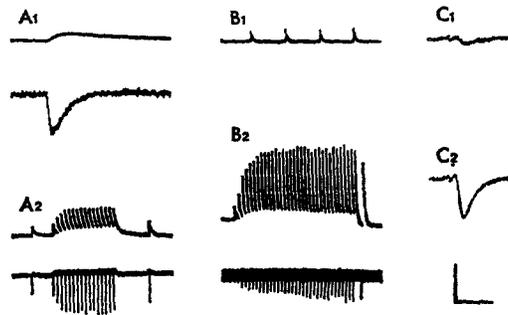


FIGURE 4. Records of internally recorded EPSPs and externally recorded synaptic potentials (ERSPs) from a low (A) and a high (B and C) F_e fiber. Fibers showing a large EPSP at 1 Hz (upper trace in A_1) are innervated by terminals releasing large amounts of transmitter at 1 Hz, as is evident from the large ERSP (lower trace of A_1). In A_2 , the same fiber was stimulated first at 1 Hz, then 10 Hz, and then 1 Hz again to examine the extent of facilitation. The increase in EPSP amplitude (upper trace) roughly parallels the increase in ERSP amplitude (lower trace, retouched); in this case, both increase two- to threefold. Fiber B showed a much smaller EPSP at 1 Hz (B_1), which facilitated greatly upon raising of the frequency to 10 Hz (B_2 , upper trace). C_1 shows the very small ERSP recorded at 1 Hz from a synaptic region on fiber B, using a computer of average transients (128 summations). There were frequent failures of transmission at 1 Hz. C_2 and the lower trace in B_2 show the ERSP recorded from fiber B at 10 Hz. In C_2 , 128 summations were used to obtain the record. In this fiber also the increases in EPSP and ERSP amplitudes are roughly parallel (five- to sixfold). Vertical calibration: 20 mv in upper traces of A_1 and A_2 ; 5 mv in B_1 and upper trace of B_2 ; 40 μ v in lower traces of A_1 and A_2 ; 200 μ v in lower trace of B_2 . Horizontal calibration: 10 msec in A_1 ; 1 sec in A_2 , B_1 , and B_2 ; 8 msec in C_1 and C_2 .

such measurements, it was estimated that quantum content at 1 Hz ranged from 1.5 to 3.4 at low F_e synapses compared with a range of 0.1–0.8 at high F_e synapses. These values compare well with those of Bittner and Kennedy (1970) for crayfish. They found mean quantal contents of 0.13 and 0.85 at 1 Hz for high and low F_e fibers, respectively. It should be noted that crab and crayfish synapses often differ in detail, although they are broadly similar (Atwood and Bittner, 1971).

All available measurements indicate that at 1 Hz the low F_e fibers receive a much greater output of transmitter at each synaptic region, as shown by the higher quantal content of the ERSP, than do the high F_e fibers (see Atwood, 1967 *a*; Atwood and Parnas, 1968; Bittner and Kennedy, 1970). Thus, F_e

can be used as an indicator both of facilitation and of synaptic transmitter output at low frequencies of stimulation.

Correlation between F_e and Muscle Fiber Electrical Properties

Previous work on the crab *Grapsus* and the crayfish *Procambarus* (Atwood and Bittner, 1971) showed that F_e , and EPSP amplitude at 1 Hz, were correlated with the input resistance (R_{in}) of the sampled muscle fibers. In the present study, a statistically significant relationship between EPSP amplitude and R_{in} also was found for the spider crab stretcher muscle, as shown in Fig. 5.

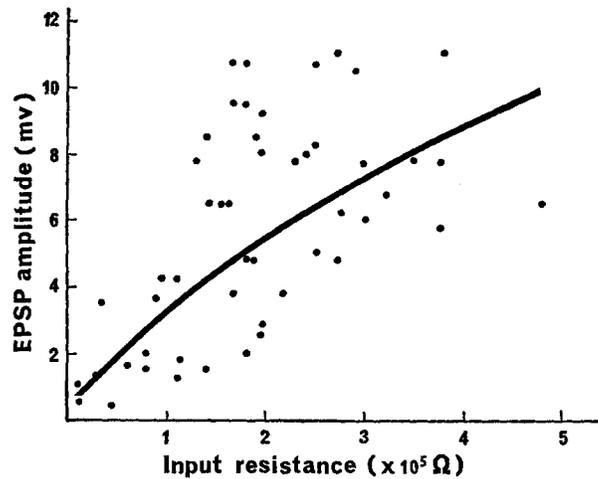


FIGURE 5. Relationship between EPSP amplitude at 1 Hz (Y , ordinate) and muscle fiber input resistance (X , abscissa) for 50 fibers. Correlation coefficient is 0.61, which is significant at the 1% level.

R_{in} values were calculated from the amount of current required to hyperpolarize the membrane by about 5 mv, when the stimulating and recording electrodes were within 50 μm of one another.

Since EPSP amplitude is correlated with both F_e and R_{in} , a multiple regression analysis was performed to determine if the correlation between EPSP and F_e is stronger than that between EPSP and R_{in} , as reported by Atwood and Bittner (1971). The "index of determination," i.e. the square of the correlation coefficient (Snedecor and Cochran, 1967, p. 176) obtained for the curve that best fits the observed data for EPSP amplitude and F_e was only slightly improved by the addition of R_{in} values to the analysis, from 0.895 to 0.930 (Table I). Therefore, even though R_{in} is significantly correlated with EPSP amplitude, the observed diversity in EPSP amplitudes in the spider crab stretcher is more closely related to differences in transmitter output between the various nerve terminals, as reflected by the F_e . This

conclusion supports that made earlier from observations on other species (Atwood and Bittner, 1971).

A more extensive examination showed that F_e was correlated with a number of the electrical properties of the muscle fibers. In Fig. 6, R_{in} values were plotted against F_e values for 50 different fibers. A highly significant correlation was found, indicating that the greatly facilitating terminals occur on muscle fibers having the lower values of R_{in} ; conversely, poorly facilitating terminals innervate the higher R_{in} fibers.

A similar relationship was found between F_e and membrane time constant (τ_m) as shown in Fig. 7 for a sample of 95 muscle fibers. These values were significantly correlated, indicating that muscle fibers having the shorter τ_m were innervated by the high F_e terminals, while the muscle fibers having the longer τ_m were innervated by the low F_e fibers. It is noteworthy that a 10-

TABLE I
ANALYSIS OF THE REGRESSION OF EPSP AMPLITUDE AT 1 Hz ON F_e AND R_{in}

Equation*	Solution	Index of determination
$Y=C_1(X_1^{D_1})$	$Y=3.371 (X_1^{0.499})$	0.375‡
$Y=C_2 (X_2^{D_2})$	$Y=17.031 (X_2^{-1.624})$	0.899‡
$Y=A_0+B_1X_1+B_2X_2$	$Y=-0.466+0.166 X_1+0.875 X_2$	0.930‡

* Conditions: Y = EPSP amplitude; $X_1 = R_{in}$; $X_2 = F_e$; A , B , C , and D = constants.

‡ Significant at the 1% level.

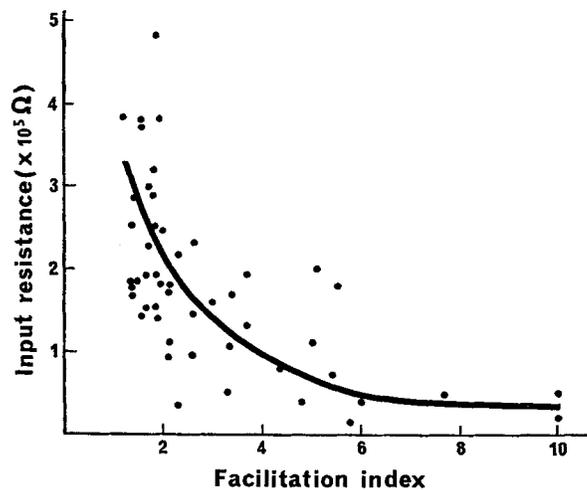


FIGURE 6. Relationship between muscle fiber input resistance (Y , ordinate) and F_e (X , abscissa) for 50 muscle fibers. Correlation coefficient is 0.59, which is significant at the 1% level.

fold range in τ_m values was seen, which corresponds with the large differences in the EPSP durations observed between high and low F_e fibers.

For a relatively small sample of fibers, determinations were made of length constant (λ), specific membrane resistance (R_m), and membrane capacitance (C_m). Although the sample was not large enough to establish firm correlations, certain trends were apparent.

The length constants obtained for all fibers were relatively long. For a group of six high F_e fibers the mean λ was 4.59 mm, while for a group of eight low F_e fibers the mean λ was 4.77 mm (Table II). These means were not significantly different as judged by t test.

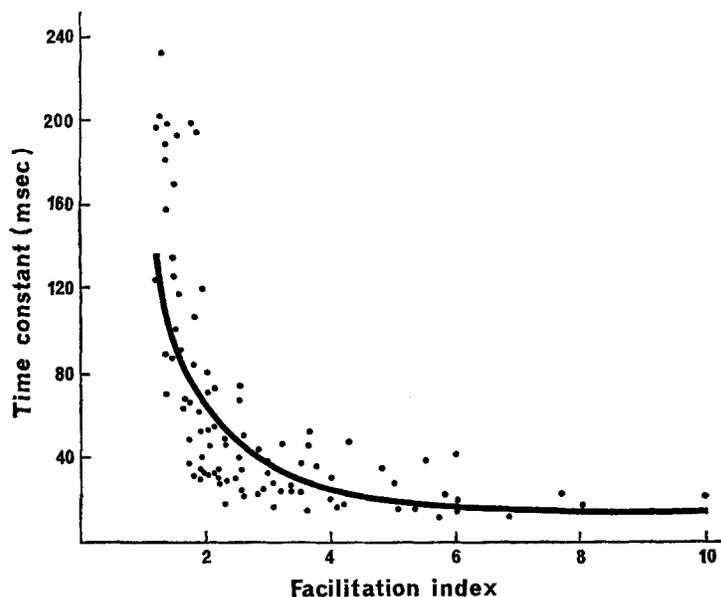


FIGURE 7. Relationship between muscle fiber time constant τ_m (Y , ordinate) and F_e (X , abscissa) for 95 fibers. Correlation coefficient is 0.67, which is significant at the 1% level.

TABLE II
MEMBRANE ELECTRICAL CONSTANTS OF LEG STRETCHER MUSCLE FIBERS OF
THE SPIDER CRAB, *HYAS ARANEUS* (LINN.) ($\bar{X} \pm SD$).

	Low F_e fibers (8)*	High F_e fibers (6)*
F_e	1.77 \pm 0.32	5.32 \pm 1.05
Sarcomere length, $\mu\ddagger$	13.18 \pm 1.08	10.59 \pm 0.87
Length constant, mm	4.77 \pm 0.90	4.53 \pm 1.01
Specific resistance, $ohm\text{-}cm^2$	3100 \pm 2468	1872 \pm 1033
Capacitance, $\mu F/cm^2$	39 \pm 11	31 \pm 18

* Denotes number of fibers examined.

‡ Denotes significant difference at the 1% level between means as judged by t test.

The specific resistance (R_m) of the fiber membrane was greater for low than for high F_e fibers, but the difference was not statistically significant (Table II). This was due in part to the high degree of variability encountered, as reflected by the standard deviation. Likewise, the membrane capacitance (C_m) was not significantly different between low and high F_e fibers (Table II). Lack of significant correlation between F_e and either R_m or C_m may only reflect inadequate sampling in the face of considerable variation, for many more fibers were examined for R_{in} and τ_m than for R_m and C_m due to the relative ease in measuring the former parameters and the errors inherent in calculating the latter.

Correlation of Muscle Fiber Sarcomere Length with F_e and with Membrane Electrical Constants

The muscle fibers of the *Hyas* stretcher all appeared to be of the long-sarcomere, slowly contracting type. However, since some of the electrical properties (τ_m , R_{in} , etc.) showed considerable variation, it was of interest to determine whether any structural features of the fibers were correlated with the fiber electrical properties, or with F_e . For this purpose, sarcomere measurements were made on as many of the sampled muscle fibers as possible.

The relationship between sarcomere length (SL) and the facilitation index, F_e , is shown in Fig. 8. The correlation between these values, as indicated by the correlation coefficient, is statistically highly significant. According to the pattern revealed by Fig. 8, the fibers with shorter SL's are preferentially innervated by the nerve terminals which produce small, highly facilitating EPSPs, whereas the fibers with longer SL's are supplied by the low F_e ter-

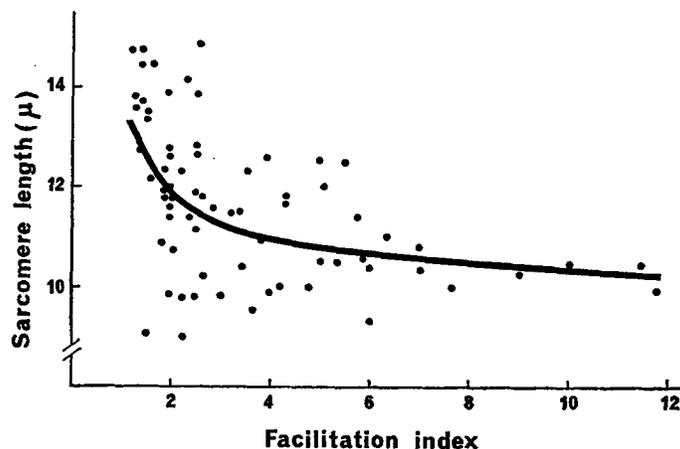


FIGURE 8. Relationship between sarcomere length (Y , ordinate) and F_e (X , abscissa) for 70 fibers. Correlation coefficient is 0.48, which is significant at the 1% level.

minals. Thus, although the sarcomere lengths of the different fibers in this muscle are not greatly different, they actually show significant variation which is related to the type of innervation they receive.

Plots of SL against R_{in} and τ_m also showed significant correlations (Figs. 9 and 10). Long-sarcomere fibers tend to have higher R_{in} and longer τ_m than short-sarcomere fibers. In the present small sample, no statistically significant correlations were established between SL and the specific membrane constants R_m and C_m .

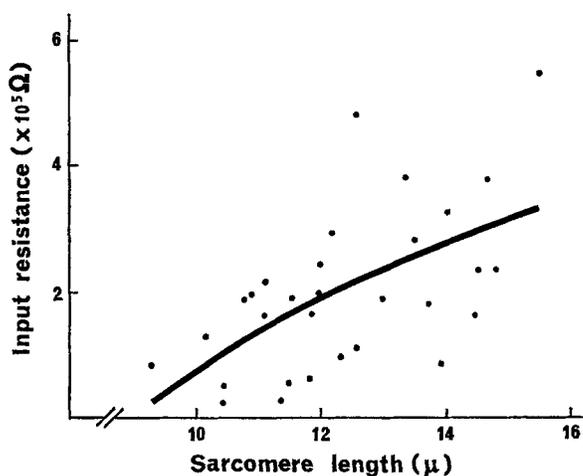


FIGURE 9. Relationship between muscle fiber input resistance (Y , ordinate) and sarcomere length (X , abscissa) for 30 fibers. Correlation coefficient is 0.45, which is significant at the 5% level.

Structural Features of the Muscle Fibers

The general appearance of the muscle fibers, as viewed in transverse sections of paraffin-embedded material, was similar for all fibers regardless of their F_e . The shape of the fibers varied somewhat, but all of them resembled other crustacean slow muscles in that they were extensively subdivided by surface invaginations (Fig. 11 A). Muscle ultrastructural features also were generally similar for all fibers, and again they resembled other slow crustacean fibers. As shown in Fig. 11 B, C, and D, slow muscle features such as wide A bands, thick uneven Z lines, absence of a well-defined H zone, and high thin-to-thick filament ratios (6:1) were observed.

In addition to differences in length of A band and sarcomere, the low and high F_e fibers showed a difference in thickness of the Z line, with low F_e fibers having a thicker, less regular Z line than high F_e fibers (Fig. 11 C and D). There was also a possible difference in the numbers of dyadic contacts per sarcomere. In the fibers examined, the fibers with shorter sarcomeres had

more dyads per sarcomere than those with longer sarcomeres. However, dyads were not very abundant in any of these fibers and no attempt was made to quantify these differences.

Structural Features of Synaptic Regions

Synaptic regions were associated with local enlargements of the sarcoplasm and sarcolemma at a point where the nerve branches reached the muscle fiber. The sarcoplasm in this region was granular in appearance and devoid of contractile filaments; it was extensively penetrated by axon terminals and

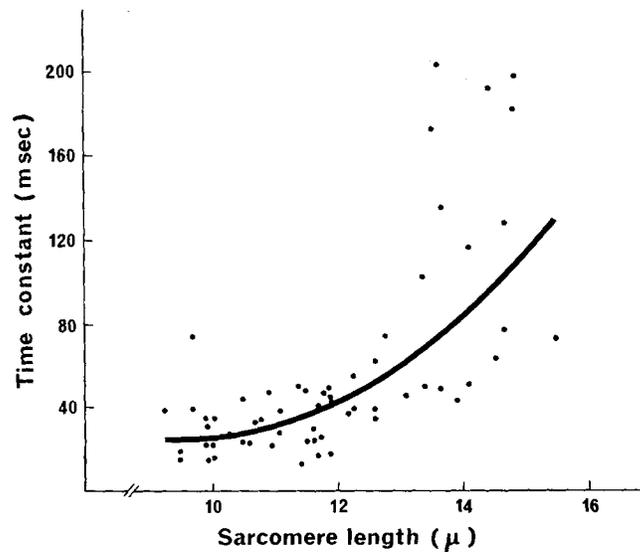


FIGURE 10. Relationship between muscle fiber time constant (Y , ordinate) and sarcomere length (X , abscissa) for 60 fibers. Correlation coefficient is 0.60, which is significant at the 1% level.

by extensions of glial cells (Figs. 12–14). This extensive glial penetration has not been observed in crayfish material studied previously (Atwood and Morin, 1970). Mitochondria were densely packed into parts of the subsynaptic region.

Two types of synapse were seen: neuromuscular and axo-axonal. The neuromuscular synapses were characterized by the close apposition of axon and sarcoplasmic membranes, which always were denser at the synapse than at nonsynaptic areas, and by the clusters of presynaptic vesicles (Figs. 12 and 14). The axo-axonal synapses occurred much less frequently than the neuromuscular synapses. They were characterized by the close apposition of thickened pre- and postsynaptic axonal membranes at a region where glial cell processes were absent, and by the presence of synaptic vesicles on at least one side of the region of contact (Fig. 13).

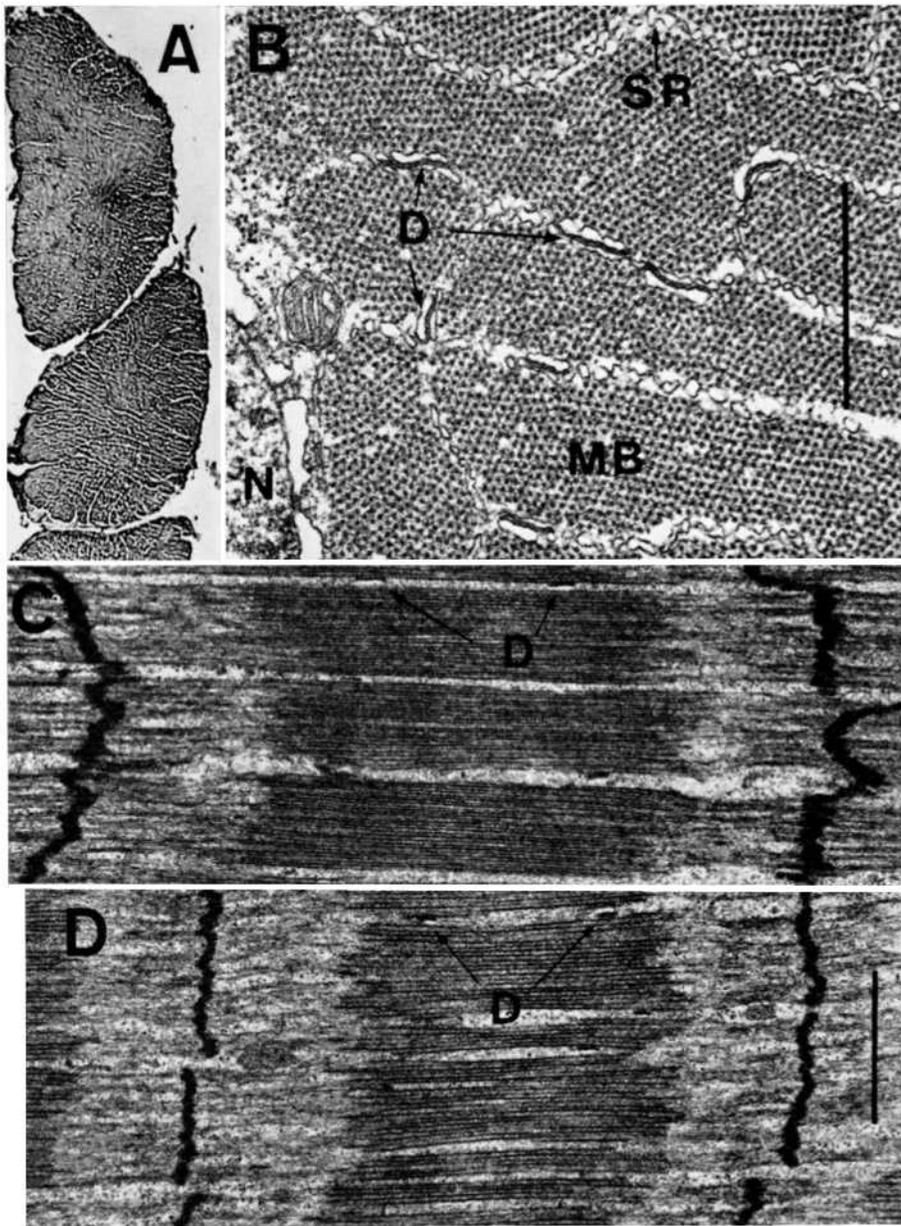


FIGURE 11. Structural features of stretcher muscle fibers. (A) is a transverse section of paraffin-embedded material showing the subdivision of the fibers by deep invaginations of the sarcolemma. Note the irregular shape of the fibers. (B), (C), and (D) are electron micrographs showing: a region of a high F_e muscle fiber in transverse (B) and longitudinal (D) views, and a low F_e fiber in longitudinal view (C), at the same magnification as (D). Features typical of crustacean slow muscles are evident, namely broad A bands, thick, irregular Z lines, and relatively few dyads. Note the longer A band and thicker Z line of the low F_e fiber (C). *D*, dyads; *MB*, myofibril bundle; *N*, nucleus; *SR*, sarcoplasmic reticulum. Calibration: the line in (B) shows 0.5 μm for (A) and 1 μm for (B); the line in (D) shows 2.5 μm for both (C) and (D). $\times 65$ (A), $\times 31,000$ (B), $\times 850$ (C) and (D).

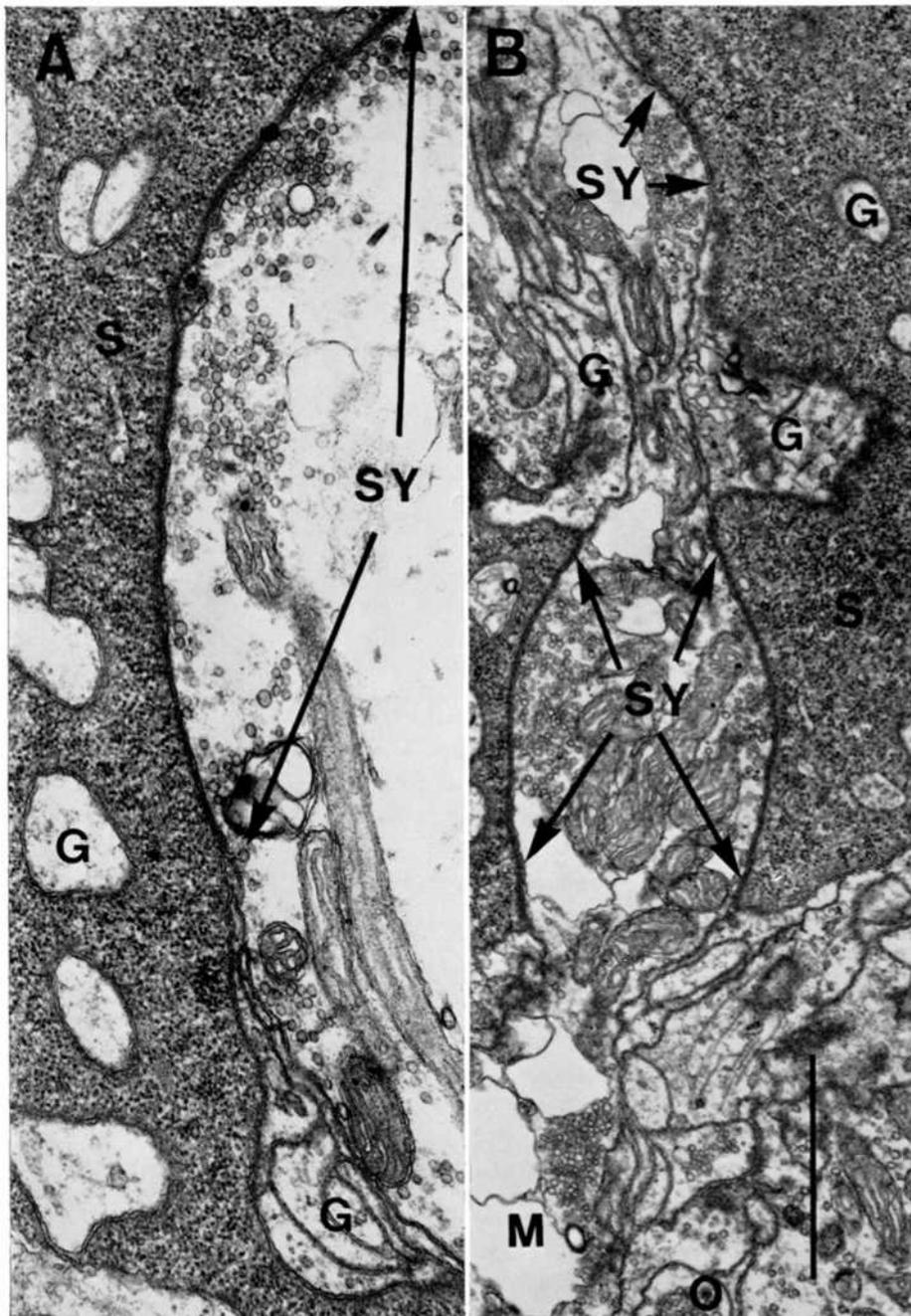


FIGURE 12. Electron micrographs of large excitatory neuromuscular synapses in synaptic regions of a low F_e muscle fiber. (Fixation method [a] was used.) An extremely large synapse (SY) formed by a large axon branch ($8 \mu\text{m}$ diameter) cut transversely is shown in (A). In (B), the side arm of a main axon branch (M) is shown, in longitudinal view, to form extensive synapses along its length. The granular sarcoplasm (S) is devoid of contractile filaments and is extensively penetrated by axon terminals and extensions of glial cells (G). The neuromuscular synapses (SY) are characterized by the apposition of dense nerve and muscle plasma membranes and by the presence of synaptic vesicles. Both agranular (clear) and granular (dense) vesicles are present; one granular vesicle is circled at the lower edge of (B) and another is present near the upper arrow in (A). Calibration: $1.1 \mu\text{m}$ in (A) and $1.4 \mu\text{m}$ in (B). $\times 30,000$ (A), $\times 21,500$ (B).

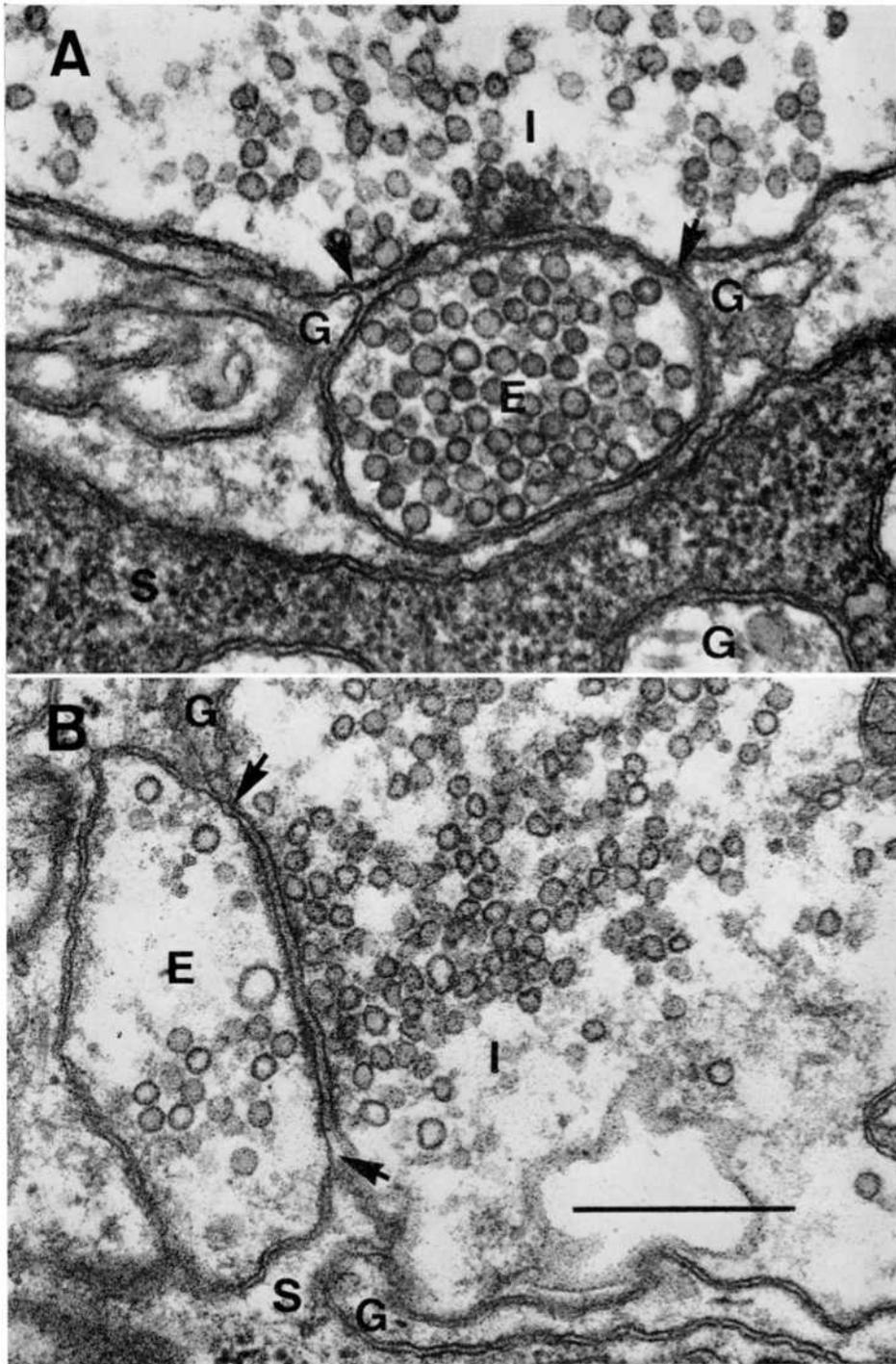


FIGURE 13. Electron micrographs of two inhibitory axo-axonal synapses located in synaptic regions of low F_e muscle fibers. (Fixation method [a] was used.) The synapses, denoted by arrows, are characterized by the close apposition of thickened axonal plasma membranes at a region where glial elements (G) are absent. The presynaptic element is believed to be an inhibitory axon (I); the synaptic vesicles are irregular in shape and clustered at the synapse. The postsynaptic element (E) is believed to be excitatory, and has uniformly round vesicles and no vesicle clustering at the synapse. S , granular sarcoplasm. Calibration: $0.45 \mu\text{m}$ in both (A) and (B). $\times 68,000$.

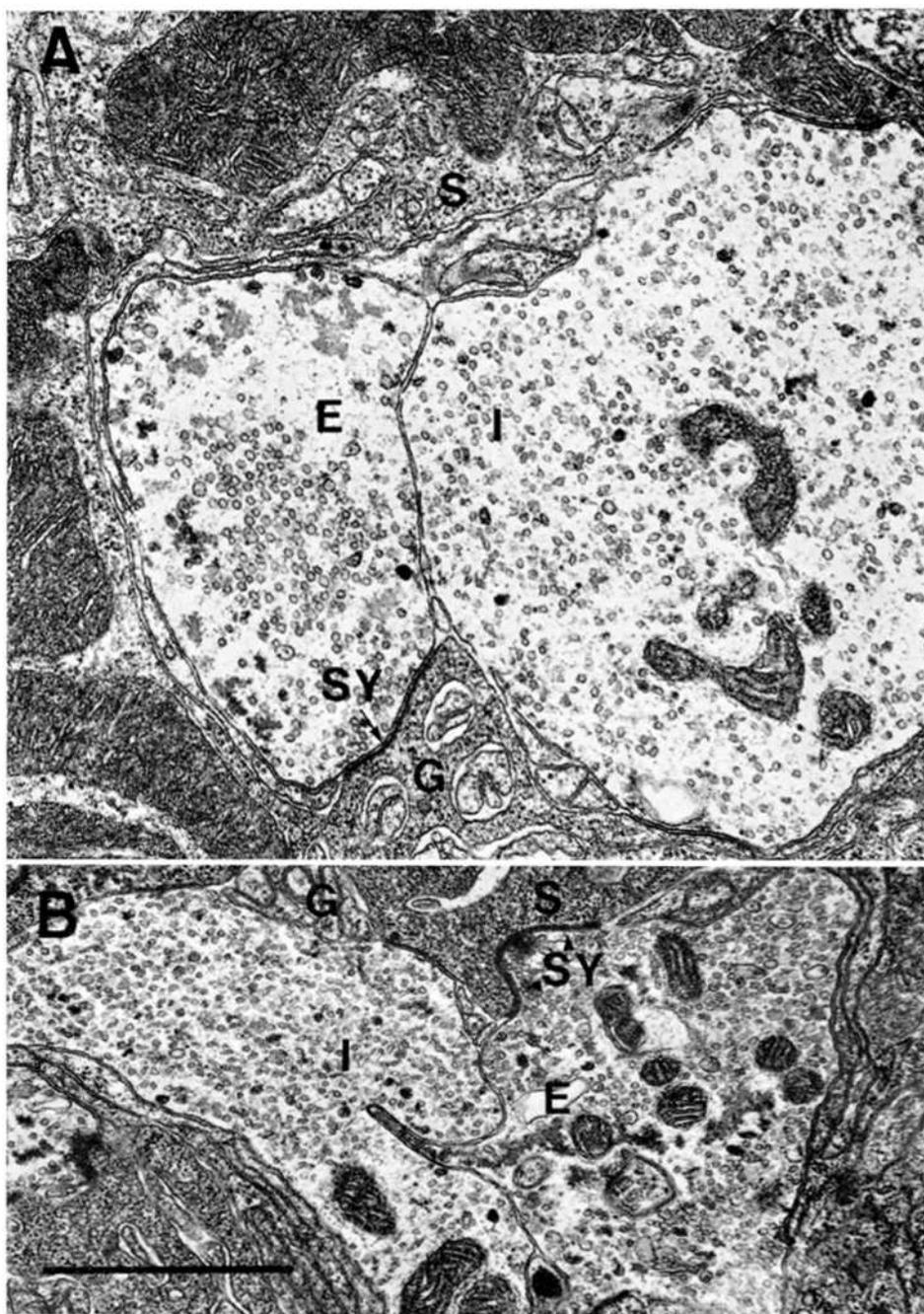


FIGURE 14. Electron micrographs of nerve terminals from high F_e muscle fibers. (Fixation method [6] was used.) In both cases, axon pairs are shown; one member of each pair was identified as excitatory (E), and the other as inhibitory (I), on the basis of vesicle morphology. No axo-axonal synapses appear in these sections, but excitatory neuromuscular synapses (SY) are present; note their limited extent compared with those of Fig. 12. The granular sarcoplasm (S) contains glial elements (G) and numerous mitochondria in (A). Note also the mitochondria of the nerve terminals. Calibration mark: $1 \mu\text{m.} \times 34,000$.

The synaptic vesicles were nearly all agranular or clear cored; their greatest dimension averaged about 450 Å. Larger, granular or dense-cored vesicles were seen in a number of terminals (Fig. 12, circled). Only a few of these vesicles ever occurred in a given terminal; they were approximately 1200 Å in diameter.

An attempt was made to distinguish excitatory terminals from inhibitory ones on the basis of ultrastructural features. Previous authors have reported that the nerve terminals in crab muscles were uniform in structure (see Cohen and Hess, 1967; Hoyle and McNeill, 1968). However, inhibitory endings could be distinguished from excitatory ones in freshwater crayfish material on the basis of the size and shape of the synaptic vesicles. Excitatory endings contained spherical vesicles of uniform size and shape, whereas the vesicles in inhibitory endings were irregularly shaped and somewhat smaller than the excitatory ones (Uchizono, 1967; Atwood and Jones, 1967; Kosaka, 1969; Atwood and Morin, 1970).

Careful examination of different *Hyas* nerve endings revealed that the synaptic vesicles in some terminals were more irregular in shape than those in other endings. This difference was evident in terminals fixed by either the (a) or the (b) method. This was particularly true for the terminals forming axo-axonal synapses, where one of the pair contained uniformly round vesicles and the other less regularly shaped ones (Fig. 13). On the basis of the results obtained for crayfish, the endings containing irregularly shaped vesicles were identified as inhibitory, while those containing the more uniformly shaped vesicles were classed as excitatory. The clustering of the less regularly shaped vesicles at the axo-axonal contacts confirmed this interpretation.

To assess quantitatively the observed differences in vesicle shape, maximum and minimum diameters were measured for 15–30 vesicles contained in each of eight terminals forming four different axo-axonal synapses. Only agranular vesicles were included in the sample. The values for each terminal were averaged to give a mean for the terminal. The mean maximum diameter of the excitatory vesicles was not significantly different from that of the inhibitory vesicles, as determined by a paired *t* test (Table III). However, there was a highly significant difference between the mean minimum diameters of the vesicles in the two types of terminal, and between the ratios of the maximum to minimum diameter of vesicles in these same terminals (Table III).

The excitatory axon branches were identified using vesicle shape as a criterion. It was not possible to identify the two different inhibitory axons which supply most of these muscle fibers (Wiersma and Ripley, 1952).

Comparison of Synaptic Regions of Low and High F_e Fibers

Examination of physiologically identified muscle fibers revealed a number of differences between the ultrastructures of the synaptic regions of low and high

F_e fibers. These regions on low F_e fibers were larger in area and contained more endings than high F_e fiber synaptic regions.

Another difference concerned the size of the axon terminals. A great diversity in the diameter of endings was observed in both fiber types, but a greater range in size occurred in the synaptic regions of low F_e fibers. Terminal sizes for the low F_e fibers ranged from very large ($8.0 \mu\text{m}$) to very small ($0.3 \mu\text{m}$); the large terminals were few in number, while the small ones were often numerous. Examples of the terminals seen in low F_e synaptic regions are shown in Fig. 12. In some synaptic regions it was observed that two or three large axon branches were sectioned at the edge of the synaptic complex, while additional numerous small branches appeared further in. Apparently, most of the small branches are given off by the larger ones and are closely

TABLE III
MAXIMUM AND MINIMUM DIAMETERS OF SYNAPTIC VESICLES
IN FOUR AXO AXONAL SYNAPSES (IN ÅNGSTROM UNITS)

	Presumed excitatory ($\bar{X} \pm \text{SE}$)	Presumed inhibitory ($\bar{X} \pm \text{SE}$)	Level of significance*
			%
Maximum	448 \pm 20	460 \pm 24	20.0
Minimum	418 \pm 20	362 \pm 18	0.5
Max:min	1.07 \pm 0	1.27 \pm 0	0.1

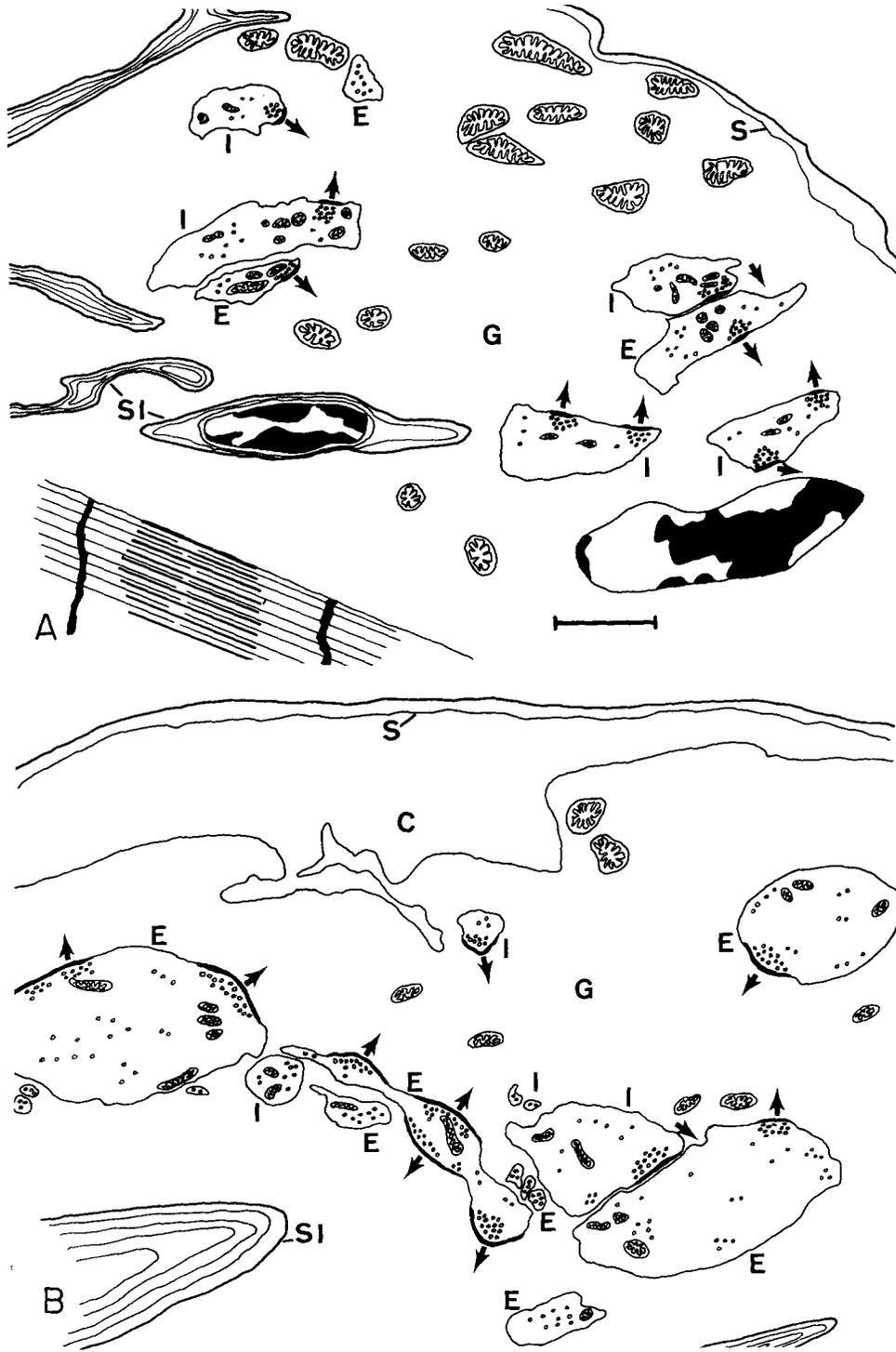
* Paired *t* test.

associated with them. This is illustrated further by the tracing of Fig. 15 B, made from a montage of low-power electron micrographs of a low F_e synaptic region.

High F_e synaptic regions showed a more limited axon size distribution, with axons ranging from 4.5 to $0.5 \mu\text{m}$ in diameter. In addition, fewer small-diameter terminals were present. The tracing of Fig. 15 A shows these features.

A third major difference concerned the size of the individual synapses. Terminals innervating low F_e muscle fibers often made very large synapses. In some instances, a terminal formed several synapses, some of which extended for a considerable distance along the terminal (Fig. 12 B). Such large synapses were seldom encountered on high F_e fibers, where more moderately sized synapses prevailed (Fig. 14). We could not compare synaptic areas precisely because we did not get complete serial sections of entire synaptic regions. However, representative cross-sections through high and low F_e synaptic regions showed consistently larger synaptic contact areas on the major excitatory axon branches of low F_e regions (Figs. 15 and 16). However, release points were usually more numerous on high F_e terminals.

The number of axo-axonal synapses encountered in synaptic regions of low F_e fibers was also greater than in high F_e fibers. Axo-axonal synapses were



seen in all of the low F_o synaptic regions examined, while they were rare or not observed in the synaptic regions of high F_o fibers. (The axon pairs of Fig. 14 were closely associated, but they do not exhibit axo-axonal synapses.) This observation suggests that there is more presynaptic inhibition of low F_o muscle fibers than of high F_o fibers (see Atwood and Bittner, 1971).

All terminals of the motor axon, except the very fine ones, contained large numbers of mitochondria in addition to the numerous synaptic vesicles. The density of mitochondria and of vesicles was much higher in this axon than in the "fast" crab axon studied by Atwood and Johnston (1968). Thus, it is likely that a correlation exists between physiological features, especially the rate of fatigue, and the density of vesicles and mitochondria.

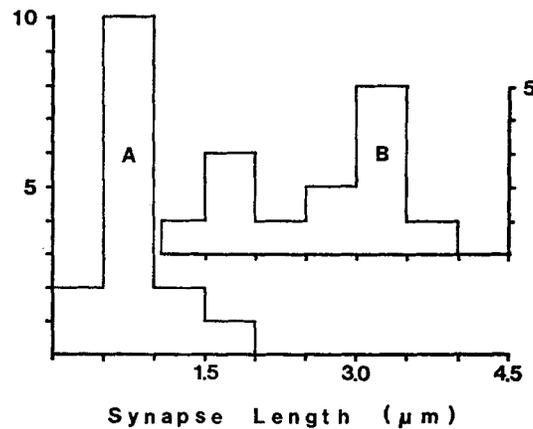


FIGURE 16. Histogram of synapse lengths on main excitatory axon branches for two high F_o synaptic regions (A) and two low F_o synaptic regions (B).

DISCUSSION

Relation of Structure to Function in the Nerve Terminals

The stretcher muscle of *Hyas* is innervated by a single motor axon, but contains muscle fibers which vary considerably in morphological and electrical properties. Furthermore, the synapses of the single motor axon show a

FIGURE 15. Outline drawing of nerve terminals in high F_o (A) and low F_o (B) synaptic regions. The drawings were traced from montages of low-power electron micrographs; not all of the nuclei, mitochondria, etc., are shown. Nerve terminals are identified as excitatory (E) or inhibitory (I) on the basis of vesicle morphology. Synaptic release points are shown by thickened axonal membranes and by arrows. Note that synapses on main branches are larger in (B), and that numerous small side branches occur near the main branches in (B). S, surface layer of sarcolemma; G, glial and granular sarcoplasm layer; SI, sarcolemmal invaginations; C, complex surface layer of sarcolemma, glial cells, and other structures. Calibration mark: 3 μm .

continuous range of variation, as judged by the synaptic potentials they produce. The system is thus rather different from the neuromuscular systems of vertebrates, in which the terminals of a given motor axon are, as far as we know, similar, and found on muscle fibers of uniform type (Burke et al., 1971). We are faced with the problem of accounting for the differences in synapses and muscle fibers, and explaining how these differences become established during development in the absence of qualitatively different motor neurons such as are found in vertebrates.

Morphological differences in synaptic terminals may help to account for some of the differences in performance. We examined terminals at the two ends of the spectrum of possibilities, hoping to obtain a clear-cut comparison.

The electron microscopic observations of the present study indicate that structural differences occur between poorly facilitating (low F_e) and highly facilitating (high F_e) terminals. The low F_e terminals consist of some relatively large-diameter axon branches which in turn give off a large number of much smaller branches. The latter are often packed closely together. The large branches have numerous extensive synapses associated with them. The synapses formed by the small branches are considerably less extensive in area. Large and small branches are closely associated, often occurring adjacent to each other or within a few microns. The high F_e terminals consist of a number of intermediate-sized branches which give rise to fine branches, but not as many as in low F_e regions. The very large synaptic contact areas characteristic of large-diameter branches in low F_e synaptic regions were not seen in the high F_e synaptic regions; instead, numerous less extensive synaptic contact areas were present.

Can the morphological features explain any of the variation in physiological performance, especially differences in transmitter output at low frequencies of stimulation, and in facilitation?

It is possible that the more extensive synaptic contact areas on large axon branches of low F_e fibers provide for a relatively large output of transmitter at low frequencies of stimulation, thus generating the typical large EPSP of the low F_e fibers. Conversely, the less extensive contact areas on the main branches of high F_e synaptic regions could limit the quantal output of these terminals at low frequencies of stimulation. A similar relationship between morphological and physiological features has been proposed for frog neuromuscular junctions by Kuno et al. (1971).

In the present study, we were not able to estimate total synaptic contact area per muscle fiber in high and low F_e types. This would have required a new specific staining method for synapses or extremely laborious serial sectioning of large fibers.

Differential Invasion Hypothesis

A differential invasion of the nerve terminals by the action potential has been suggested to explain the differences in transmitter output observed between crustacean nerve terminals (Atwood, 1967 *a*; Atwood and Johnston, 1968; Bittner, 1968 *a*). According to this hypothesis, terminals releasing a large

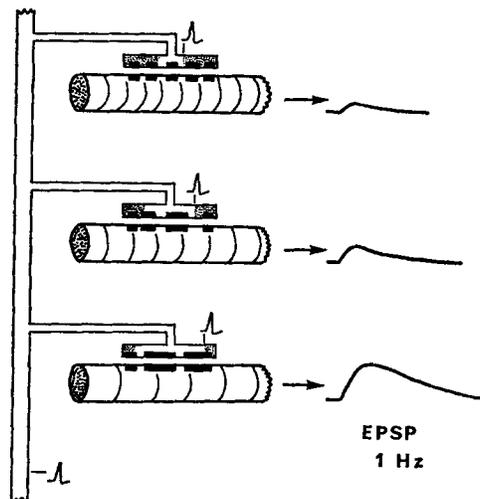


FIGURE 17. Diagram to illustrate the model for differential invasion of nerve terminals by the action potential, and the "matching" of nerve terminal and muscle fiber properties. Three muscle fibers are shown (cylinders), each one giving a different-sized EPSP at 1 Hz. An action potential initiated in the motor axon (lower left) is conducted past more synapse-bearing regions of one of the terminals (lower example) than the others. This terminal releases more transmitter substance than others, thereby producing the largest EPSP. A considerable range in EPSP sizes can result, depending on the extent of invasion by the action potential of the synaptic regions of the terminals. Although a motor axon is diagrammed, the same model applies to peripheral inhibitory neurons. The dark bars represent synapses and the stippled areas terminal regions not invaded by the fully propagated action potential. The uppermost fiber has the shortest sarcomeres and is supplied by terminals releasing relatively little transmitter at low frequencies. The lower two fibers show progressively longer sarcomeres and larger synaptic release points.

amount of transmitter at low frequencies of stimulation (low F_c terminals) are more completely invaded by the action potential than terminals releasing a small amount of transmitter. (See Fig. 17 for a diagram of this model.) The differential invasion hypothesis requires that the action potential becomes decremental at some point away from at least some of the synapse-bearing regions of the nerve terminals. This hypothesis is based on the work of Dudel (1963, 1965), who obtained evidence for the decremental invasion of (high F_c) crayfish nerve terminals. He found that the externally recorded multi-

phasic potential changes which are associated with the action potential gave way to much smaller monophasic positive potentials closer to the synaptic regions. This positive potential was interpreted to result from the passively spreading electrotonic remnant of the action potential. The positive potential increased in amplitude and duration as the stimulation frequency was raised, providing a possible basis for at least some of the facilitation of transmitter release (Dudel, 1971).

Evidence that low F_e terminals are more fully invaded by the action potential than high F_e terminals is derived in part from examination of externally recorded nerve terminal potentials. Most of the nerve terminal potentials recorded so far from low F_e synaptic regions in crabs have been of the multiphasic type; few purely positive ones have been seen (Atwood, 1967 *a*; Atwood and Jones, 1967). Some of these potentials are relatively quite large. At high F_e terminals, smaller potentials, sometimes monophasic, are recorded. The recordings are consistent with the idea that a large electrical event (perhaps a fully propagated spike) occurs in low F_e terminals, while a smaller event (perhaps an incompletely propagated spike) occurs in high F_e terminals. The evidence for this hypothesis is not yet conclusive.

Putting together the differential invasion hypothesis and the morphological features, we can speculate on the reasons for differences in transmitter output. The larger synapses of low F_e fibers may effectively increase the probability of transmitter release for individual impulses. This, coupled with a more complete invasion of the terminal regions, could lead to a near maximal output of transmitter at low frequencies, and relatively little facilitation of release at high frequencies. Conversely, it is possible that the transmitter output of high F_e fibers at low frequencies is limited by the degree of invasion of the action potential, and at high frequencies by the total area available for transmitter release.

The critical factor in transmitter output at low frequencies is postulated to be the relative positions of synaptic release point and the point at which the action potential becomes decremental (Fig. 17). Before the differences in facilitation can be completely explained, however, it will be necessary to know more about calcium mobilization in the different terminals and about transmitter supply and mobilization.

Inhibitory Axo-Axonal Synapses

Peripheral inhibition of many crustacean muscles occurs both pre- and postsynaptically (Dudel and Kuffler, 1961 *b*; Kennedy and Evoy, 1966; Atwood, 1968). At low frequencies a major part of the inhibition occurs pre-synaptically, while postsynaptic inhibition becomes more pronounced at higher levels of activity (Atwood and Morin, 1970; Atwood and Bittner, 1971).

The inhibitory inputs to a given muscle are often matched in physiological properties with the excitatory inputs, so that muscle fibers showing large EPSPs also show large inhibitory postsynaptic potentials (IPSPs) and vice versa (Atwood and Bittner, 1971). This was true of the *Hyas* stretcher muscle as well as of others studied previously. Furthermore, the EPSP of low F_0 fibers is more effectively reduced by presynaptic inhibition than is the EPSP of high F_0 fibers (Atwood and Bittner, 1971). From these observations, the low F_0 fibers should possess more inhibitory axo-axonal synapses than higher F_0 fibers. This was confirmed in the present study, for axo-axonal synapses were seen more often on low F_0 fibers.

Correlation Between Pre- and Postsynaptic Properties

The correlation between SL and R_{in} , and between SL and τ_m adds further support to the idea that SL is a basic physiological parameter in crustacean muscles, as well as a basic structural property. SL previously was shown to be correlated with speed of contraction, with membrane excitability, and with ultrastructural features such as number of dyadic contacts and thin-to-thick myofibril ratios (Atwood, 1967 *b*; Hoyle, 1967; Jahromi and Atwood, 1967, 1969; Law and Atwood, 1971). From the ultrastructural features, it is possible to predict that membrane capacitance (C_m) should be greater for short-sarcomere fibers, and membrane resistance (R_m) lower (see Falk and Fatt, 1964; Adrian and Peachy, 1965; Law and Atwood, 1971). The present results, particularly the correlations between SL, R_{in} , and τ_m , support the prediction relating to membrane resistance. There is not enough evidence in the present study to substantiate the prediction relating SL to membrane capacitance. For this, it would be desirable to study fibers with greater differences in sarcomere length.

The selective matching of pre- and postsynaptic properties could result in two general ways. First, the postsynaptic properties of the entire population of muscle fibers may be uniform before innervation, and the fibers become differentiated as a result of the innervation they receive. Such neural control of muscle fiber properties has been well established in vertebrates. For example, a mammalian fast muscle can be converted to a slow one by directing slow muscle motor nerves into the fast muscle after it has been denervated (Buller et al., 1960 *a, b*; Close, 1969; Barany and Close, 1971). Alternatively, in crustaceans the muscle fibers may be differentiated before innervation occurs, and by means of chemical gradients, or some other mechanism, somehow "attract" the appropriate axon branches.

The correlations between F_0 and the postsynaptic properties of R_{in} , τ_m , and SL add additional support for a "deterministic" mechanism for the development of synaptic connections in crustacean neuromuscular systems, as argued by Atwood and Bittner (1971). The observation that the largest sized axon

branches occur on low F_e fibers suggests that these fibers may be innervated first during development. It seems likely that these large terminals may represent the initial branching of the motor neuron; further branching of some or all of the initial branches probably occurs, and these smaller branches may then innervate the high F_e fibers. In this manner a large range of F_e 's could result, depending on the type of axon branches supplying each muscle fiber during development. Size of synaptic region may be partly determined by time of innervation. The electrical and structural properties of the muscle fibers may also depend on time of innervation during development.

It is easier to conceive of a progressive branching of the motor nerve as a deterministic mechanism in development than to evoke some postsynaptic mechanism such as a chemical gradient. Neuron branching is commonplace in the multiterminally innervated crustacean muscles, whereas chemical gradients in muscle fiber development have not been shown conclusively to occur. Furthermore, the variety of different patterns of regional differentiation of muscle fibers that have been reported (Dorai Raj, 1964; Bittner, 1968 *a, b*) could be explained on the basis of different branching patterns of the motor neuron. Even more compelling are the recent findings of Atwood (unpublished), who observed that, in regenerating crab limbs, the stretcher muscle fibers innervated first are those that show poorly facilitating EPSPs, i.e. they are low F_e fibers. Fibers showing highly facilitating EPSPs occur later in regeneration. This finding is in perfect agreement with the ultrastructural observations presented in this study. Further experiments on regenerating crustacean limbs at different stages of development and involving electrophysiological and ultrastructural techniques should provide additional information concerning the development of synaptic connections in crustaceans.

Implications for Muscle Performance

The differences in transmitter release and facilitation shown by the nerve terminals of the stretcher motor neuron provide for the progressive recruitment of fibers as the level of neuronal activity increases. Since the longer-sarcomere fibers are innervated by terminals that produce large depolarizations at low frequencies of activity, they contract first. As the frequency of presynaptic activity increases, the shorter-sarcomere fibers are progressively activated as a result of facilitation. Since sarcomere length is inversely related to speed of shortening, contractions of the stretcher muscle are slowest at low levels of activity, but become progressively faster as the frequency is elevated. This provides for quicker and stronger contractions of the stretcher muscle at high levels of activity.

It appears likely from the above discussion that the longer-sarcomere fibers are used for postural movements, while the shorter-sarcomere fibers are

activated relatively infrequently when more rapid movements are required, such as in escape reactions.

APPENDIX

In a "short cable," the length constant (λ) can be determined by measuring the potential changes set up at each end of the cable by current injected at one end. The equation given by Weidmann (1952) for potential distribution along a "short cable" is

$$V_x = V_o \cosh (L - x/\lambda) / \cosh (L/\lambda) \quad (1)$$

where

V_x is the potential at any point x measured from the end of the cable where current is applied;

V_o is the potential at $x = 0$;

L is the length of the cable;

λ is the length constant.

By substituting m for L/λ and $1 - u$ for x/L , equation 1 becomes

$$V_x/V_o = \cosh mu / \cosh m. \quad (2)$$

When the potential at the end of the cable remote from current application is recorded, $x = L$ and $u = 0$. Let V_x/V_o (with $x = L$) be r . Equation 2 then becomes

$$r = 1/\cosh m. \quad (3)$$

The solution for m in equation 3 is

$$m = \ln [1/r^2 + \sqrt{(1/r^2 - 1)}]. \quad (4)$$

Since L is known, λ can be found from L/m . This solution provided a convenient method for determining the length constant of muscle fibers in the stretcher muscle of *Hyas*.

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