CHANGES IN THE FINE STRUCTURE OF THE NEUROMUSCULAR JUNCTION OF THE FROG CAUSED BY BLACK WIDOW SPIDER VENOM

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ABSTRACT

Application of black widow spider venom to the neuromuscular junction of the frog causes an increase in the frequency of miniature end-plate potentials (min.e.p.p.) and a reduction in the number of synaptic vesicles in the nerve terminal. Shortly after the increase in min.e.p.p. frequency, the presynaptic membrane of the nerve terminal has either infolded or "lifted." Examination of these infoldings or lifts reveals synaptic vesicles in various stages of fusion with the presynaptic membrane. After the supply of synaptic vesicles has been exhausted, the presynaptic membrane returns to its original position directly opposite the end-plate membrane. The terminal contains all of its usual components with the exception of the synaptic vesicles. The only other alteration of the structures making up the neuromuscular junction occurs in the axon leading to the terminal. Instead of completely filling out its Schwann sheath, the axon has pulled away and its axoplasm appears to be denser than the control. The relation of these events to the vesicle hypothesis is discussed.

INTRODUCTION

The vertebrate neuromuscular junction is a synapse that can be experimentally investigated with comparative ease and convenience. It has, therefore, received a great deal of attention for the information it has to yield about the biochemical, electrical, and morphological events of synaptic transmission. Almost 20 years ago, Katz and his collaborators established the quantal nature of the end-plate potentials and subsequently both physiologists and morphologists have speculated whether such quantal behavior has a structural basis.

When electron microscopists first looked at neuromuscular junctions, they saw that the nerve terminals were filled with small spheres of membrane, the synaptic vesicles (Palade, 1954; Robertson, 1954, 1956; Reger, 1958; Andersson-Cedergren, 1959; Birks, Huxley, and Katz, 1960). These were immediately advanced as the structural manifestations of the packets of transmitter which produced the miniature end-plate potentials (min.e.p.p.)[1] (De Robertis and Bennett, 1954, 1955; del Castillo and Katz, 1955; Palay, 1956). Evidence for this guess has been inconclusive.

If the assumption of equivalence between the quantum, min.e.p.p., and synaptic vesicle is correct, then an increase in the rate of quantum release signaled by an increase in min.e.p.p. frequency could be reflected by a decrease in the population of synaptic vesicles in the nerve terminals. Maneuvers such as tetanic stimulation,

[1] *Abbreviations:* BWSV, black widow spider venom; min.e.p.p., miniature end-plate potentials.

using solutions with elevated concentrations of potassium ion, or subjecting the nerve-muscle preparation to hypertonic media have led to inconclusive results (De Robertis, 1958; Thies, 1960; Birks, Huxley, and Katz, 1960; Jones and Kwanbunbumpen, 1970 *a*; Hubbard and Kwanbunbumpen, 1968). On the other hand, degeneration of the terminal after section of its nerve leads to sharp reductions in min.e.p.p. frequency and the number of synaptic vesicles (Birks, Katz, and Miledi, 1960; Miledi and Slater, 1970). Jones and Kwanbunbumpen (1970 *a*) have shown that tetanic stimulation of the nerve in combination with a bathing solution containing hemicholinium, an inhibitor of choline uptake, significantly reduces the number of synaptic vesicles.

An alternative to the vesicle hypothesis is to reject the involvement of synaptic vesicles in the release of quanta. Instead, the vesicles may only serve as storage depots for acetylcholine. The acetylcholine actually involved with the release of quanta might be free in the cytoplasm of the terminal or else associated with the presynaptic membrane. Experimental evidence for such a theory can be obtained from the work of Whittaker (1969). In his analysis of acetylcholine associated with nerve terminals isolated from brain, he has found a "labile" and a "stable" fraction. The labile acetylcholine may be free in the terminal cytoplasm, while the stable fraction is associated with the synaptic vesicles. This alternative hypothesis has a disadvantage for the electron microscopist in that the structural mechanism responsible for quantal release of the labile fraction might not be demonstrable with the instrument. Of course, it would explain the apparent failure of experiments designed to alter the number of synaptic vesicles in the nerve terminal.

A new experimental tool, black widow spider venom (BWSV), may be useful in determining the role of the vesicles. Application of small amounts of the venom to the frog neuromuscular junction causes an avalanche of min.e.p.p. activity and an unambiguous reduction in the number of synaptic vesicles (Longenecker et al., 1970; Clark et al., 1970). In earlier experiments on another preparation, D'Ajello et al. (1969) observed that venom caused a block of transmission in the sixth abdominal ganglion of the cockroach. These authors hypothesized that the block was due either to depolarization of the nerve cell bodies as was found on the nerve cell body of the crayfish stretch receptor by Obara and Mauro (unpublished observations) or to an effect exerted directly on the presynaptic terminals as in the frog nerve terminal. It is interesting that in both these instances the venom did not affect the axons of the nerve cell, just as in the frog it leaves untouched the ability of the axon and muscle fiber to conduct action potentials. In the rat superior cervical ganglion, Paggi and Rossi (1971) have shown that the venom causes block of transmission and release of tagged acetylcholine with no impairment of axonal conduction.

More recently, Chen and Lee (1970) have found that a fraction of the venom of the banded krait, *Bungarus multicinctus*, has an effect much like BWSV on the neuromuscular junctions of the mouse. In addition, venom from the South American rattlesnake, *Crotalus durissus terrificus*, (Brazil and Excell, 1970) contains components that provoke a great increase in min.e.p.p. frequency.

The following paper describes in detail the structural changes that occur in frog neuromuscular junctions after administration of BWSV.

MATERIALS AND METHODS

Nerve-Muscle Preparation

The muscle used in this study was the cutaneous pectoris of the frog, *Rana pipiens*. Braun and Schmidt (1966) introduced the use of this muscle and it possesses several virtues. The muscle and its nerve are easy to dissect. The muscle is very thin so that finding and impaling junctions can be done with relative ease. Finally, the nerve takes up osmium to a greater extent than the muscle fibers so that under transmitted light the nerve and most of its branches are distinct, facilitating the search for junctions to view with the electron microscope.

Monitoring min.e.p.p.

After a muscle was dissected, it was mounted in a Lucite chamber (10 ml) and bathed in solutions containing either 115 mM NaCl, 2.10 mM KCl, 1.00 mM $MgCl_2$, and 1.91 mM $CaCl_2$, or 110 mM NaCl, 2.50 mM KCl, and 4.0 mM $MgCl_2$ at room temperature (approximately 25°C). A muscle fiber was impaled near its end-plate region with glass micropipettes (10–20 MΩ) filled with 3 M KCl (Fatt and Katz, 1951). Min.e.p.p. were suitably amplified and displayed on a cathode ray oscilloscope, where they were photographed.

Preparation of BWSV

Italian black widow spiders, *Latrodectus mactans tridecimguttatus*, were kindly supplied by N. Frontali, A. Grasso, and S. Bettini, Istituto Superiore di Sanita, Rome. California black widow spiders, *Latrodectus mactans hesperus*, were kindly supplied by Mr. Y. Ito and Mr. J. Flowers, Manteca, Calif. In our experience, venom from the Italian spiders is more potent. The venom glands of four spiders (eight glands) were macerated in 1.0 ml of the bathing solution. Depending on the potency of the venom, 10–500 μl of venom solution were added to the bath directly over the impaled junction.

Electron Microscopy

At the termination of the experiment, the impaling electrode was withdrawn from the muscle and the bathing solution was quickly replaced with one at room temperature containing 1% formaldehyde, 1% glutaraldehyde, 2% sucrose, and 0.1 M sodium phosphate buffer at pH 7.4 (Fahrenbach, 1968). The muscle stiffened after 10 min in the fixative and it was removed from the chamber, trimmed, and placed in fresh fixative for a total time of 60 min. The muscle was then rinsed with several changes of 0.1 M sodium phosphate buffer, pH 7.4, with 3% sucrose added. It could then be stored for several days at 4°C or else postfixed at once with 1% OsO_4 and 2% sucrose in 0.1 M sodium phosphate buffer, pH 7.4, for 60 min. Some muscles were stained en bloc with aqueous uranyl acetate according to the method of Karnovsky (1967). The muscles were then embedded in Epon 812 according to standard methods. Sections were stained with lead citrate and uranyl acetate and viewed on a Philips EM 200 electron microscope.

Control

There were three types of controls. In the first type, one of the two cutaneous pectoris muscles from a single animal was soaked in the bathing solution while the other muscle was being experimented upon. Both muscles were fixed at the same time and subsequently treated in an identical way. In the second type of control, a muscle was mounted in the Lucite chamber, an end plate was impaled with a micropipette, and the min.e.p.p. frequency was monitored for periods of up to 5 hr before fixation. Finally, muscles were mounted in the chamber, an end plate was impaled with a micropipette, and min.e.p.p. frequency was monitored for periods of up to 5 hr after the addition of 0.5–2.0 ml of BWSV inactivated by storage at 4°C for more than 4 wk.

RESULTS

Control Neuromuscular Junctions

The min.e.p.p. frequency of impaled control end plates varied from 0.5–5 sec for periods of up to 5 hr. A small portion of a typical record is shown in Fig. 1.

The morphology of the control preparations corresponds closely to the description of normal neuromuscular junctions in the frog by Birks, Huxley, and Katz (1960). The nerve terminals contain neurofilaments, neurotubules, elements of smooth endoplasmic reticulum, mitochondria, coated and dense-core vesicles, and large numbers of synaptic vesicles. Although most of the synaptic vesicles are spherical with an average diameter of 450 A, many of them are pleomorphic. Some are flattened, while others appear to be cup shaped as though one side had been pushed in (Figs. 3 and 4).

Groups of synaptic vesicles are associated with

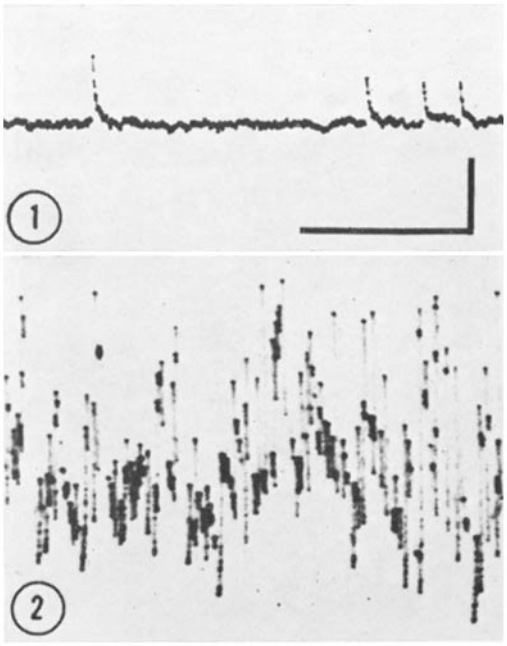


FIGURE 1 Intracellular recording from a control neuromuscular junction showing the miniature depolarizations of the end-plate membrane. Calibration is for 500 μv and 0.25 sec.

FIGURE 2 Intracellular recording from a neuromuscular junction 10 min after the onset of the min. e.p.p. avalanche caused by BWSV. Time and voltage settings are the same as for Fig. 1.

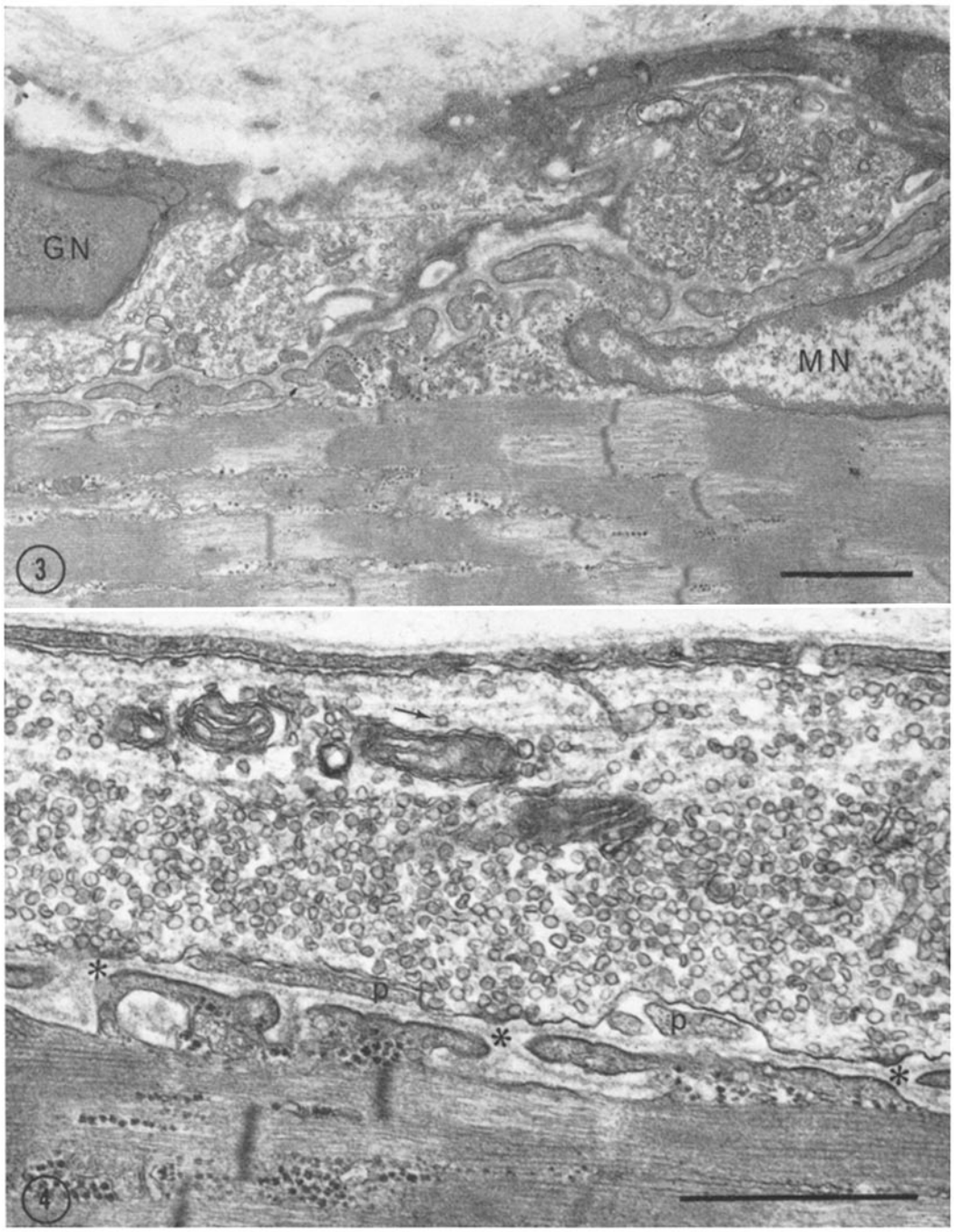


FIGURE 3 Electron micrograph showing a portion of a neuromuscular junction from a control preparation treated with inactivated BWSV for 5 hr. A muscle fiber nucleus (*MN*) is often associated with the end-plate region. There are also junctional folds characteristic of the end-plate membrane. The nerve terminal contains numerous mitochondria, neurotubules, neurofilaments, elements of smooth endoplasmic reticulum, and synaptic vesicles. The terminal for the most part is covered over by a Schwann cell although there is an interruption in the middle of the figure which we cannot explain. The Schwann cell nucleus (*GN*) is visible and the cytoplasm contains mitochondria, microtubules, and ribosomes. × 19,200. 1 μ.

FIGURE 4 Electron micrograph showing a portion of a neuromuscular junction from a control preparation treated with inactivated BWSV for 5 hr. At higher magnification, the pleomorphic shape of many of the synaptic vesicles can be seen. Active zones (✳), densities on the presynaptic membrane associated with a cluster of synaptic vesicles, are visible opposite the openings of the junctional folds. A coated vesicle (arrow) can also be seen. Finger-like projections (*p*) of the glial cell are interposed between the terminal and the end-plate membrane. × 35,000. 1 μ.

densities on the inner surface of the presynaptic membrane. First observed by Birks, Huxley, and Katz (1960) and described in greater detail by Couteaux (1961) and Couteaux and Pécot-Dechavasine (1970), these "active zones" are located directly opposite the openings of the postsynaptic folds (Fig. 4).

Each terminal is covered over by a Schwann cell whose nucleus contains condensed chromatin and whose cytoplasm contains free ribosomes, mitochondria, and microtubules. The covering of Schwann cell cytoplasm varies in thickness, becoming extremely attenuated in places. In addition, as observed by Birks, Huxley, and Katz (1960), finger-like projections of the Schwann cell cytoplasm extend for some distance under the terminal, between it and the basal lamina of the end-plate membrane (Figs. 3 and 4).

Experimental Neuromuscular Junctions

5–7 min after the addition of BWSV directly over impaled end plates, we usually observed an initial burst of min.e.p.p. activity with frequencies well above 100/sec for periods of 0.5–1 min. The min.e.p.p. frequency then fell to control rates for approximately a minute, and then once again rose to rates that could approach 1000/sec (Fig. 2). After reaching a peak, the frequency gradually returned to control levels or below after 30–40 min. Longenecker et al. (1970) have given a detailed description of these events.

The most striking initial changes in venom treated junctions are seen 10–15 min after the onset of the min.e.p.p. avalanche. Junctions on the surface of the muscle may show maximal structural alteration. Junctions that lie deeper in the muscle either show the initial structural changes caused by the venom or appear to be normal.

The initial structural alterations appear to be of two types and can occur in adjacent regions of the same terminal. In the first (Fig. 5), the presynaptic membrane invaginates as narrow channels and curls back on itself to form structures that resemble myelin figures (Fig. 5). The second type is characterized by a lifting of the presynaptic membrane (Fig. 6). Examination of these lifts or arches reveals that they occur over the ridges of the end-plate membrane. The active zones, those portions of the presynaptic membrane with densities and associated synaptic vesicles, remain in position over the openings of the junctional folds as though they were preferentially adhesive (Fig. 6).

Careful examination of the invaginations and arches reveals profiles of synaptic vesicles fusing with the presynaptic membrane (Figs. 5–10, arrows).

In some terminals, the lifting of the presynaptic membrane is so extensive that the peak of the arch reaches the side of the terminal away from the end plate. These terminals contain few vesicles and often contain little or no terminal cytoplasm at the peak of the arch (Figs. 9 and 10). Apparently free-floating blebs of membrane are frequently encountered in the spaces where the terminal used to be (Figs. 9 and 10). These blebs may be derived from the deep infoldings of the presynaptic membrane mentioned before. As the infoldings curl back on themselves and enlarge, they appear to be pinched off. Whether or not they actually are detached can only be determined by serial sectioning which we have not done. A potential bleb still attached to the presynaptic membrane is seen in Fig. 9.

During the period just described, the folds of the end-plate membrane appear to be enlarged (Figs. 5, 6, 9, and 10). The folds are not widened in either the controls or in experimental preparations where the min.e.p.p. frequency has returned to low levels after the application of BWSV (Figs. 11 and 12).

If the nerve-muscle preparation is fixed approximately 30 min after the onset of the min.e.p.p. avalanche, many of the nerve terminals in junctions near the surface of the muscle appear to be undergoing a return of their presynaptic membranes. Spaces still persist, but many of them appear to be enclosed within the terminal. These vacuoles may open out into the junctional space outside the plane of the particular section (Fig. 11). The cytoplasm of the terminal contains mitochondria, elements of smooth endoplasmic reticulum, neurotubules, and neurofilaments but virtually no synaptic vesicles. The cytoplasm also appears not to be as dense as that of the control terminal although this may be a function of the lack of vesicles.

2 hr after the application of venom, the presynaptic membrane of almost every terminal in the muscle has returned to its original position close to the end plate (Fig. 12). The cytoplasm of these terminals is characterized by the same com-

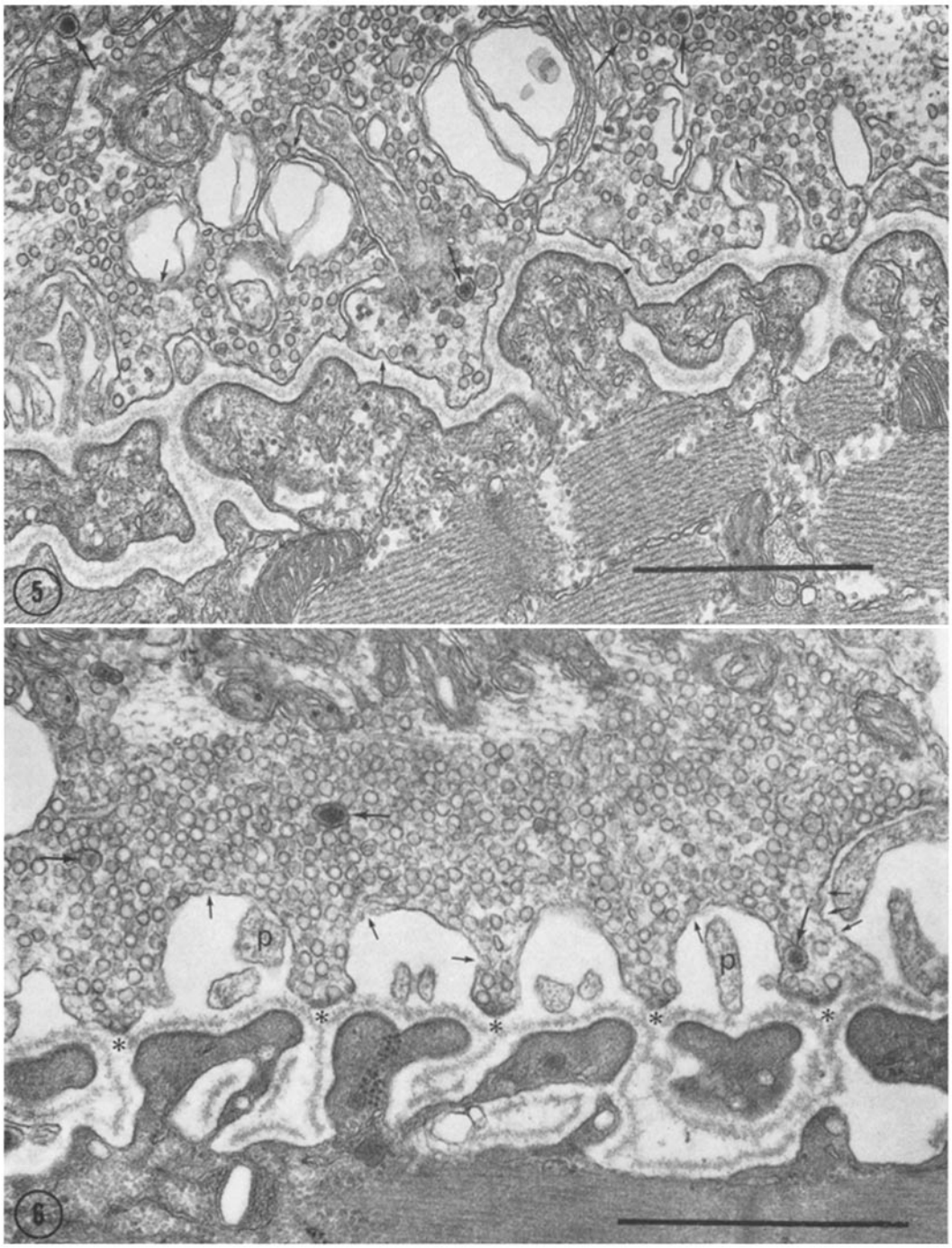


FIGURE 5 Electron micrograph showing an oblique section through a neuromuscular junction 15 min after the onset of the min.e.p.p. avalanche caused by BWSV. There are extensive infoldings of the presynaptic membrane, some of which have curled back on themselves and resemble myelin figures. Large arrows indicate vesicles with dense cores. Small arrows indicate synaptic vesicles in various stages of fusion with the presynaptic membrane. × 35,000. 1 μ.

FIGURE 6 Electron micrograph showing a longitudinal section through a neuromuscular junction 15 min after the onset of the min.e.p.p. avalanche caused by BWSV. This junction is from a different preparation than the one shown in Fig. 5. Lifting of the presynaptic membrane into arches is seen. The arches have formed between adjacent active zones (*). Small arrows indicate synaptic vesicles in various stages of fusion with the presynaptic membrane. (*p*, projections of the glial cell; large arrows, vesicles with dense cores.) × 42,500. 1 μ.

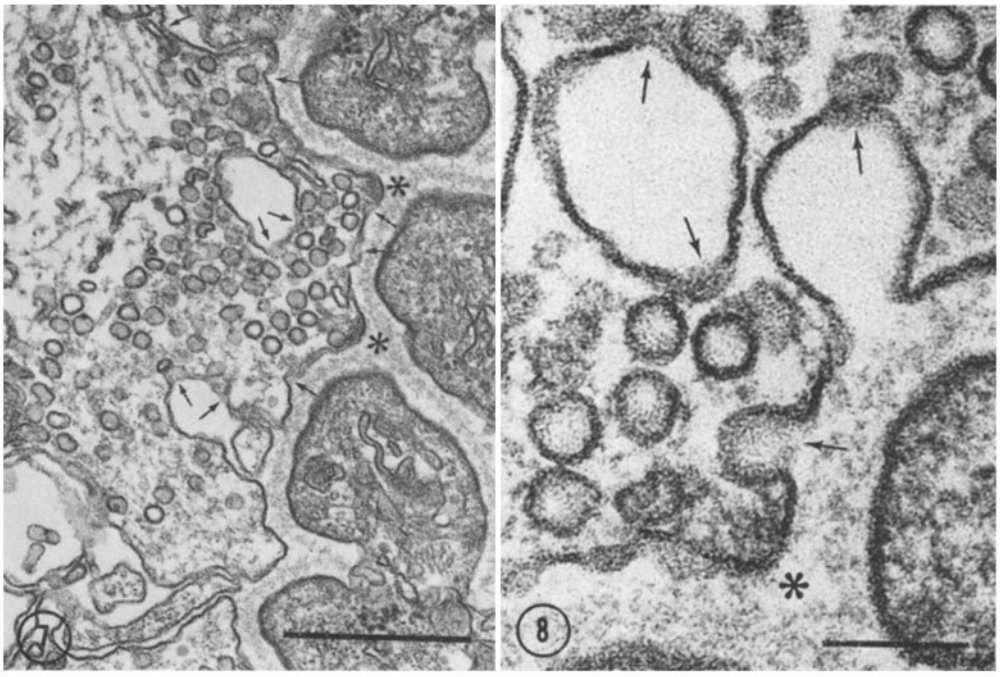


FIGURE 7 Electron micrograph showing a portion of a neuromuscular junction 15 min after the onset of the min.e.p.p. avalanche caused by BWSV. Arrows indicate vesicles in various stages of fusion along the presynaptic membrane. (*, active zones) × 49,500. 0.5 μ.

FIGURE 8 Electron micrograph showing a portion of a neuromuscular junction 15 min after the onset of the min.e.p.p. avalanche caused by BWSV. Arrows indicate synaptic vesicles in various stages of fusion with the presynaptic membrane. Figs. 7 and 8 are from different preparations. (*, active zones) × 192,000. 0.1 μ.

ponents found in the control with the exception of the synaptic vesicles. Coated vesicles, which can be found in all control terminals with careful examination, are especially obvious here since they are no longer hidden among their more numerous relatives. Associated with the uptake of protein in other systems (Roth and Porter, 1962), coated vesicles may perform a similar function in nerve terminals. As with the 30-min terminals, the cytoplasm appears to be less dense than in the controls (Figs. 13 and 14). Furthermore, it is our impression that the terminals are greater in diameter than the controls, although we have made no attempts to quantify this observation.

During this time, the other structures that make up the neuromuscular junction or are in the vicinity, such as the Schwann cell, the muscle fiber, fibroblasts, and capillaries, undergo no detectable structural changes, with the exception of the axon leading to the nerve terminal. In control preparations, the proximal portion of the axon appears to be perfectly normal, completely filling out the Schwann sheath (Fig. 15). In venom-treated preparations, however, especially after 2 hr, almost every axon has pulled away from some portion of its sheath (Fig. 16). In addition, the axoplasm appears to be much denser, although it still contains its normal complement of mitochondria, neurotubules, neurofilaments, and elements of smooth endoplasmic reticulum.

DISCUSSION

The neuromuscular junction has been used as model synapse because it is readily accessible to experimental analysis and it appears to have many properties in common with synapses found in other locations. One of the properties of synapses in the central nervous system is the adhesiveness of the presynaptic membrane to the postsynaptic membrane. The adherent region is usually associated with a thickening of either or both mem-

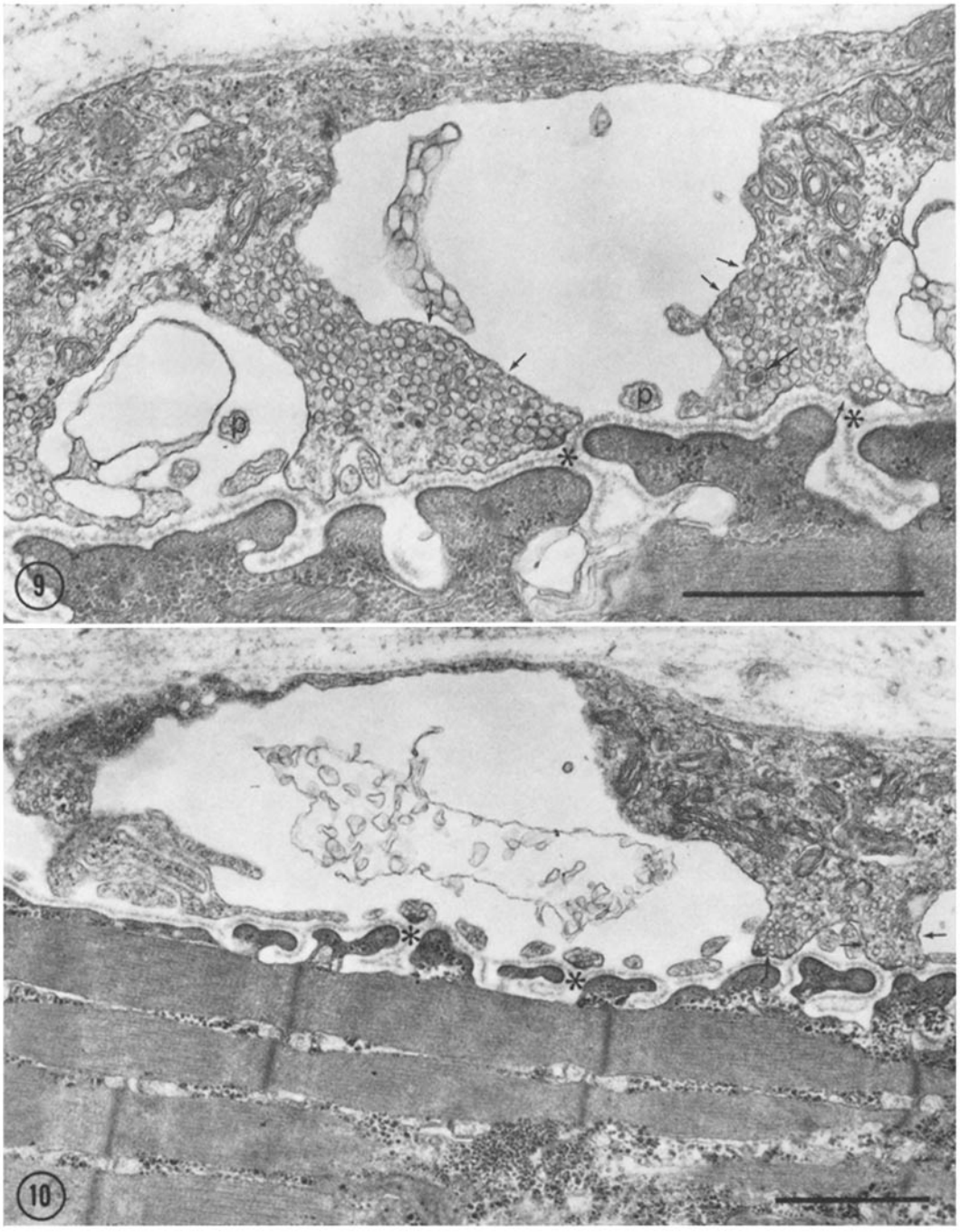


FIGURE 9 Electron micrograph showing a longitudinal section through a neuromuscular junction 15 min after the onset of the min.e.p.p. avalanche caused by BWSV. In the central arch, it appears that at the top there is no terminal cytoplasm. The arch at the left may represent a later stage of the infoldings shown in Fig. 5. What may be a potential bleb of membrane is seen still attached to the presynaptic membrane. Small arrows indicate synaptic vesicles in various states of fusion with the presynaptic membrane. A large arrow points to a dense-core vesicle. (*p*, projections of the Schwann cell; ✳, active zones) × 35,000. 1 μ.

FIGURE 10 Electron micrograph showing a longitudinal section through a neuromuscular junction 15 min after the onset of the min.e.p.p. avalanche. Where the terminal cytoplasm used to be, there is a space with a bleb of membrane apparently floating free within it. A thin roof of glial cytoplasm covers the space. Figs. 9 and 10 are from the same preparation. Small arrows indicate synaptic vesicles fusing with the presynaptic membrane. (✳, active zones) × 22,600. 1 μ.

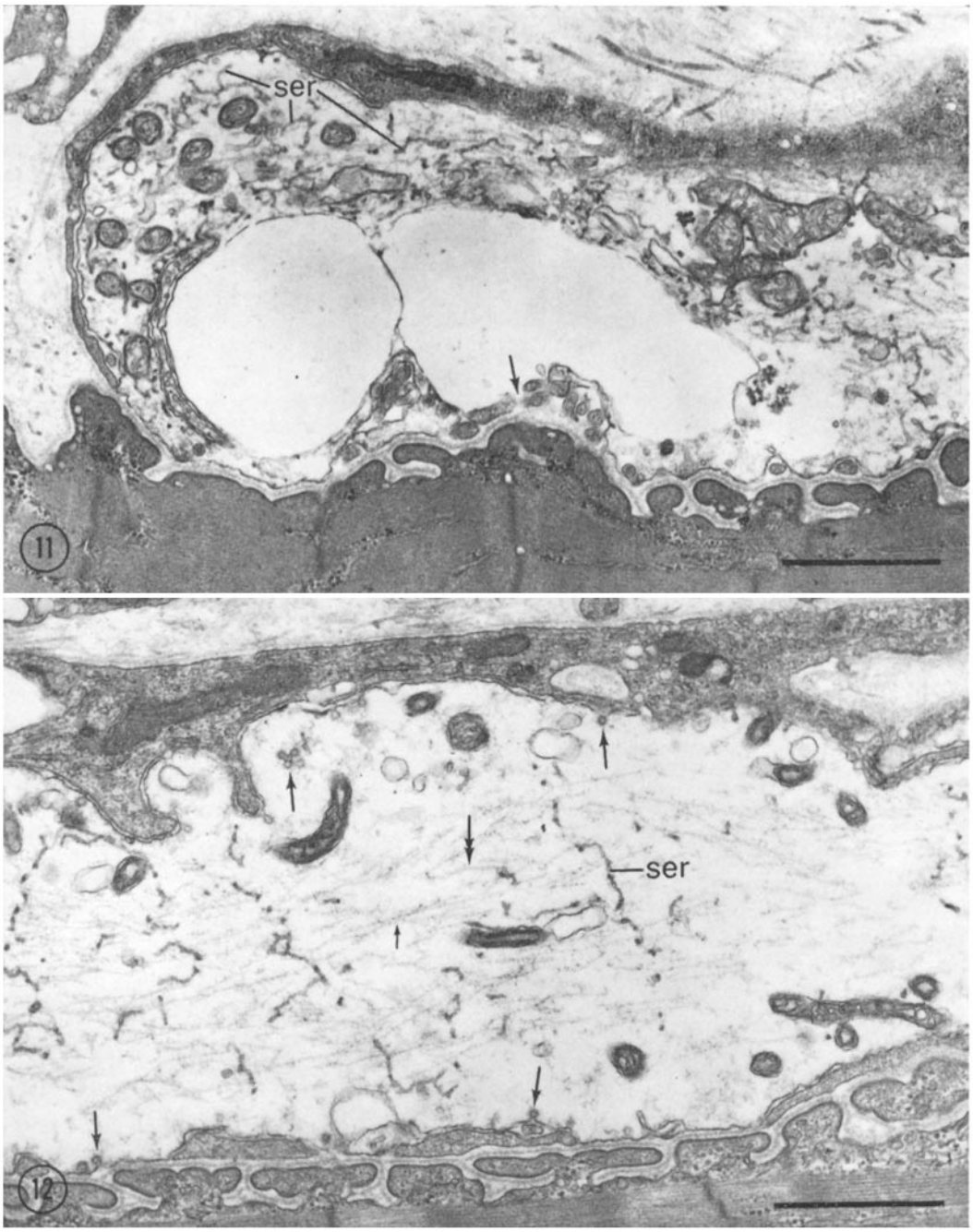
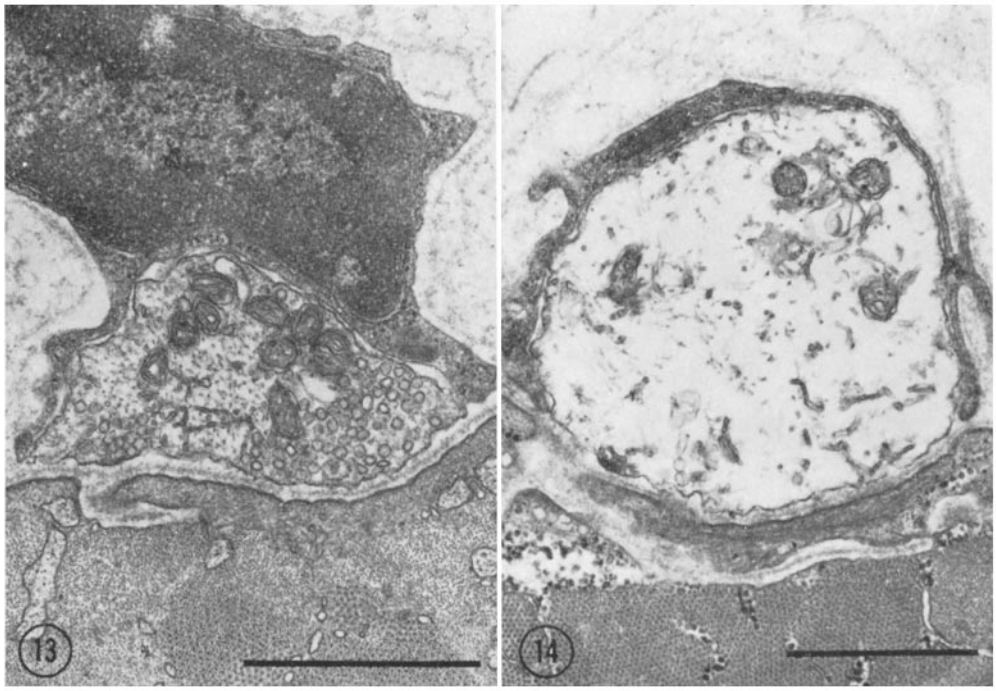


FIGURE 11 Electron micrograph showing a longitudinal section through a neuromuscular junction 30 min after the onset of the min.e.p.p. avalanche. At this point, the presynaptic membrane appears to be returning to its original position opposite the end-plate membrane. Large vacuoles are present and they may communicate with the junctional space (arrow). Without synaptic vesicles, the elements of smooth endoplasmic reticulum present in the terminal are more obvious (*ser*). The cytoplasm of the terminal appears to be less dense than that of the control, and the terminal itself appears to be larger than the control. × 22,300. 1 μ.

FIGURE 12 Electron micrograph showing a longitudinal section through a neuromuscular junction 2 hr after the onset of the min.e.p.p. avalanche caused by BWSV. The presynaptic membrane appears to have returned to its original position opposite the end-plate membrane. The synaptic vesicles are gone, revealing the other components of the terminal cytoplasm. Coated vesicles (arrows) are obvious, as are elements of the smooth endoplasmic reticulum (*ser*). The terminal cytoplasm appears to be less dense, and it is our impression that the terminal is larger. Both the muscle fiber and the glial cell appear to be normal (double arrow, neurotubules; small arrow, neurofilaments). × 24,000. 1 μ.

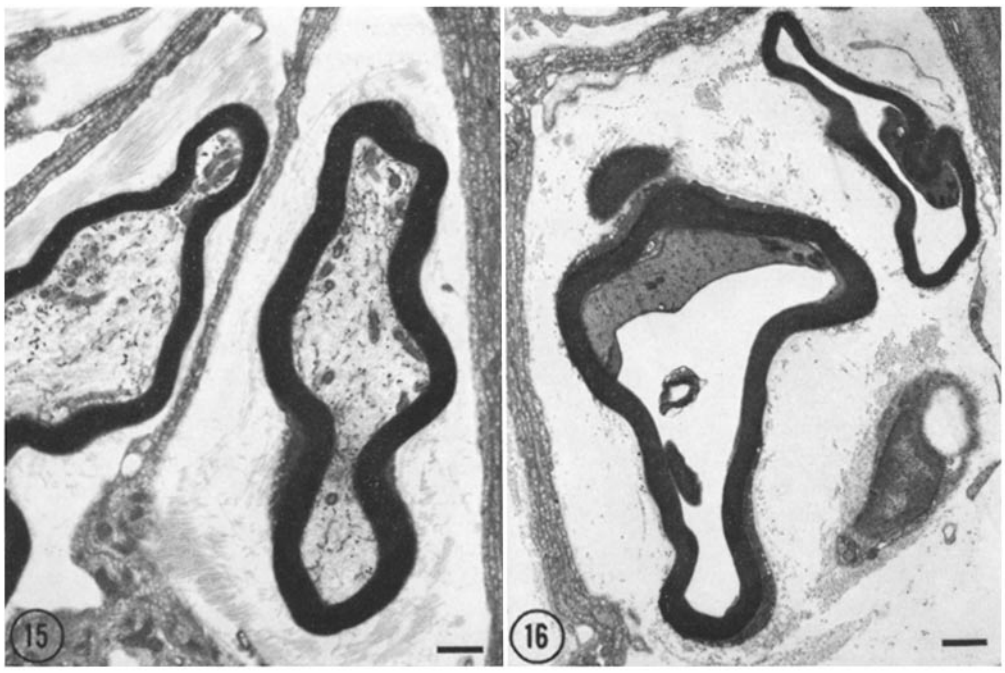


FIGURES 13 and 14 Electron micrographs showing transverse sections through a control neuromuscular junction (Fig. 13) and through a neuromuscular junction 2 hr after the onset of the min.e.p.p. avalanche caused by BWSV (Fig. 14). Fig. 13 is comparable to the junction shown in Fig. 1, while Fig. 14 is taken from the same preparation as Fig. 12. Fig. 13, × 31,000; Fig. 14, × 25,000. 1 μ.

branes (Gray and Guillery, 1966) and it regularly survives homogenization and centrifugation in a sucrose density gradient. The active zones of the neuromuscular junction appear to be comparable synaptic regions since they remain attached at the end plate during the lifting of the presynaptic membrane caused by black widow spider venom (Figs. 6, 9, and 10).

Lifting of the presynaptic membrane appears to be the result of the apparently irreversible fusion of synaptic vesicles with it. The most direct line of evidence for this interpretation consists of actual images of fusions. These have been presented, although they are not as numerous as we initially expected. Having assumed that the fusion rate was identical with min.e.p.p. frequency, we naively looked for the presynaptic membrane to be one long line of fusing vesicles in preparations fixed when min.e.p.p. frequency was maximal (300/sec and above). However, after a calculation of the surface area of the presynaptic membrane, it became obvious that our expectations were unrealistic.

Birks, Huxley, and Katz (1960) have estimated a typical nerve terminal in the frog to be a cylinder approximately 1 mm long and 1 μ in radius. If we assume that one-third of the surface of the cylinder is the actual presynaptic membrane, that is to say, the membrane directly opposite the end-plate membrane, then there are 670 $\pi\mu^2$ (i.e., $\frac{1}{3}$ of 2000 $\pi\mu^2$) of membrane with which the vesicles can fuse. If the vesicles do fuse with this membrane at the peak rate observed, 1000/sec, then there should be approximately 0.5 fusions/μ^2 every second. The thin sections we use are approximately 700 A thick (silver by reflected light) so that along every 1 μ of presynaptic membrane we should see 0.03 fusions every second. Furthermore, Birks, Huxley, and Katz (1960) estimated there were 3×10^6 vesicles in a terminal. At the maximal rate of 1000/sec, only 1 vesicle out of 3000 would be fusing each second. These calculations suggest that we see as many fusions as we do either because the assumed dimensions of the terminal are incorrect or because the fused vesicles disappear relatively slowly. Another factor



FIGURES 15 and 16 Electron micrographs showing oblique sections through control axons (Fig. 15) and through a comparable pair of axons 2 hr after the onset of the avalanche of min.e.p.p. activity caused by BWSV (Fig. 16). The control axons have the normal complement of mitochondria, neurotubules, neurofilaments, and elements of smooth endoplasmic reticulum. The axoplasm fills out the myelin sheaths. The axoplasm of the experimental axons has pulled away from the sheaths and is much denser than that of the controls. × 5900. 1 μ.

about which we know nothing is the amount of time it actually takes to fix a terminal, although fixation probably is not an instantaneous process. Admittedly approximate, these calculations suggest that the number of fused vesicles we observe is not too low to account for the increased min.e.p.p. frequency.

If BWSV does cause the synaptic vesicles to fuse with the presynaptic membrane, there should be an increase in the surface area of the nerve terminal and in its potential volume. The changes observed in the preterminal axons after treatment with BWSV suggest that such increases may take place. It is clear that there is a marked reduction in axoplasmic volume (Figs. 15 and 16). The question is: where did the axoplasm go?

If we turn again to the cylindrical terminal proposed by Birks, Huxley, and Katz (1960), the surface area of such a terminal is 2000 $\pi\mu^2$ while the volume is 1000 $\pi\mu^3$. They have estimated that the number of synaptic vesicles in such a terminal is 3×10^6. If we take the average diameter of the vesicles to be 450 A and we assume that they are spherical, the surface area of the synaptic vesicles is 6060 $\pi\mu^2$. Assuming that all the vesicles fuse with the terminal after treatment with BWSV, the terminal, then, would have a surface area of 8060 $\pi\mu$. During the addition of this membrane, the length of the terminal probably would remain the same, while the radius would increase to 4 μ. A cylinder 1 mm long with a radius of 4 μ has a volume of 16,000 $\pi\mu^3$. The volume of 3×10^6 vesicles with a diameter of 450 A is approximately 45 $\pi\mu^3$. If all these vesicles fused with the presynaptic membrane, the reduction of the total volume (1000 $\pi\mu^3$) of the terminal would be small. In contrast, the addition of their membrane to the terminal membrane would increase the potential volume of the terminal by a substantial amount (× 16). The reduction of the proximal axoplasm perhaps can be accounted for by the increase in potential terminal volume. Although we have not attempted to quantify it, our impression is that the terminal axoplasm is less

dense (Figs. 13 and 14). In this connection it is interesting to note that the proximal axoplasm in the experimental nerves appears to be denser than the axoplasm in either the control nerves or the experimental terminals (Figs. 15 and 16, Figs. 14 and 16). The proximal-distal shift is in the proper direction for axoplasmic flow and may only involve a shift in the aqueous phase of the axoplasm.

During the period when min.e.p.p. frequency is highest, the folds of the end-plate membrane enlarge (Figs. 5, 6, 9, and 10), indicating that there may be a change in the osmolarity of the medium surrounding the nerve terminal. Increases in osmolarity greatly increase the min.e.p.p. frequency (Liley, 1956), so that the changes of the end-plate membrane may be an indirect reflection of the action of BWSV on the nerve terminal.

Fusion of synaptic vesicles with the presynaptic membrane also occurs in the resting terminal. Using conventional thin-section electron microscopy, Couteaux and Pécot-Dechavasine (1970) find fusing vesicles in close relation to active zones that are clearly different from fusing coated vesicles. In addition, Nickel and Potter (1970) have used freeze-etching, a technique that requires no chemical fixation, to examine the electric organ of *Torpedo*. Since the nerve terminals in this organ apparently contain few or no coated vesicles, the fusions they observe must predominantly involve synaptic vesicles.

The question of vesicle fusion is of interest for what it may or may not say about the vesicle hypothesis. Direct proof for the hypothesis has been difficult to obtain since the investigator must somehow show that in the normal, physiological state the release of a quantum of acetylcholine from the nerve terminal is the result of a fusion of a synaptic vesicle with the presynpatic membrane. The normal excited state probably can best be experimentally approached by tetanic stimulation of the nerve. Unfortunately, this approach has had a long history of frustration (Thies, 1960; Birks, Huxley, and Katz, 1960) culminating in the report by Jones and Kwanbunbumpen (1970 *a*) that such a maneuver leads to an *increase* in the number of vesicles in the terminal, although the diameter of the vesicles apparently is smaller. An underlying assumption for most of these investigations has been that the fusion of the vesicle with the presynaptic membrane is more or less permanent. In the face of these results, it seems more likely that, if the vesicle hypothesis is true, then the connection between the vesicle and the presynaptic membrane is highly transient (Eccles, 1964).

Conservation of synaptic vesicles apparently must take place in some invertebrate neuromuscular junctions. Bittner and Kennedy (1970) have analyzed the quantal content of opener-stretcher motor neurons in crayfish and they have concluded that the loss of synaptic vesicle membrane or its permanent incorporation into the terminal is unlikely. The metabolic load placed on the neuron to resynthesize its lost transmitter *and* membrane would be too great for the cell to sustain.

It appears to be well established in vertebrate neuromuscular junctions that there can be massive release of quanta with no detectable reduction in the number of synaptic vesicles. Most recently, Heuser and Miledi (1970) have found that bathing nerve-muscle preparations in Ringer's solution in which lanthanum ion has been substituted for calcium ion causes an increase in min.e.p.p. frequency of 10^4 and no obvious immediate changes in the structure of the nerve terminal if the treatment is restricted to less than 1 hr. Their results are consistent with those from experiments utilizing tetanic stimulation, hypertonic media, and depolarization of the terminal with potassium ion that have already been mentioned.

So far, there appear to be only three procedures that correlate an increase in min.e.p.p. frequency with a reduction in the number of synaptic vesicles. Jones and Kwanbunbumpen (1970 *a*, *b*) have tetanically stimulated nerve-muscle preparations bathed in solutions containing hemicholinium. Under these conditions, both the number of vesicles and the amplitude of the min.e.p.p. are reduced. The other two ways of obtaining a reduction in the number of vesicles are to treat junctions either with beta bungarotoxin (Chen and Lee, 1970) or with BWSV. All of these procedures are abnormal, although the one utilizing hemicholinium is more intriguing from a physiological point of view since it suggests that if the vesicle hypothesis is true the rate-limiting step for reformation of the vesicles may be the resynthesis of acetylcholine.

Another instance of the reduction and disappearance of synaptic vesicles (dense-core) occurs in sympathetic nerve terminals after the adminis-

tration of 6-hydroxydopamine. Tranzer and Thoenen (1968) have found that cats when first injected with the drug showed signs of intensive sympathomimetic stimulation. 3 days later, the adrenergic terminals in all the tissues they examined showed signs of extensive degeneration and contained few if any dense-core vesicles. Unfortunately, they make no mention of the appearance of the terminals immediately after the injection of the drug, and so we have no way of knowing whether the liberation of noradrenalin is connected with the disappearance of the vesicles. Experiments utilizing reserpine have shown that liberation of the transmitter from the dense-core vesicles can take place without disappearance of the vesicles or their fusion with the presynaptic membrane (Pellegrino de Iraldi et al., 1965).

The same may be true of BWSV. The avalanche of min.e.p.p. activity and the fusion of the synaptic vesicles with the presynaptic membrane may be two separate and unrelated events. According to this interpretation, the disappearance of the vesicles is a secondary convulsion of the terminal, the result of some side effect of the venom. Although we believe that the time course of the appearance of structural changes in the terminals precludes this possibility, definite proof awaits fixing and viewing the same terminals in which min.e.p.p. frequency is being monitored. We are attempting to do this at the present time, but even if we are successful it would not prove that in the normal state the release of a quantum is the result of a fusion of a vesicle with the presynaptic membrane.

Direct, visual proof of the vesicle hypothesis may not be possible. Every one of the experimental results on quantal release mentioned above can be explained in terms of a labile fraction of acetylcholine that is the source of the quanta and is unassociated with synaptic vesicles. Instead, it may be more productive to determine the reality of the labile fraction and to see whether or not it corresponds to anything in the normal, intact nerve terminal.

If strong inference is the only recourse, there are two facts that should be considered. Synaptic vesicles do fuse with the presynaptic membrane in resting terminals, and both Couteaux and Pécot-Dechavassine (1970) and Nickel and Potter (1970) have demonstrated this. Much greater numbers of synaptic vesicles fuse with the presynaptic membrane in nerve terminals treated with BWSV. The direct connection of these images to the release of quanta and the frequency of min.e.p.p. has yet to be made.

Addendum

An article by M. Okamoto, H. E. Longenecker, Jr., W. F. Riker, Jr., and S. K. Song entitled "Destruction of mammalian motor nerve terminals by black widow spider venom" (1971, *Science (Washington)*, **172**:733) appeared after this paper had been submitted for publication. We do not see the sort of "destruction" of frog nerve terminals seen by these authors in mammalian terminals. The difference in reaction may be due to the difference in species.

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