

# DEPLETION OF VESICLES FROM FROG NEUROMUSCULAR JUNCTIONS BY PROLONGED TETANIC STIMULATION

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## ABSTRACT

Curarized cutaneous pectoris nerve muscle preparations from frogs were subjected to prolonged indirect stimulation at 2/sec while recording from end plate regions. At the ends of the periods of stimulation, the curare was removed and the preparations were fixed for electron microscopy or treated with black widow spider venom to determine the degree to which their stores of transmitter had been depleted. After 6–8 hr of stimulation the nerve terminals were almost completely depleted of their stores of transmitter and of their population of vesicles. Most of the transmitter release occurred during the first 4 hr of stimulation, and after this time most (about 80%) of the fibers were depleted of about 80% of their transmitter. The organization of the nerve terminals in 4-hr preparations appeared normal and the terminals still contained many vesicles. When peroxidase was present in the bathing medium, terminals from stimulated preparations showed many vesicles that contained peroxidase, whereas the rested control preparations showed few such vesicles. The fact that after 4 hr the total number of vesicles is not markedly changed while a large fraction (up to 45%) contained peroxidase suggests that in our experiments vesicles were continuously fusing with and reforming from the axolemma.

## INTRODUCTION

Several workers have tried to deplete neuromuscular junctions of their stores of transmitter and of their synaptic vesicles by tetanic stimulation of the nerve (1, 2, 3, 4). Depletion of transmitter has been obtained only when synthesis was inhibited by hemicholinium (2, 3) and, under this condition, a reduction in the number of vesicles occurred only in the regions of the axoplasm immediately adjacent to the axolemma (4). In these previous works the preparations were stimulated for from several minutes to a few hours at frequencies of 10/sec or more. We have stimulated a neuromuscular preparation from the frog for 6–9 hr at a rate of 2/sec in the absence of hemicholinium and have successfully depleted the terminals of their store of transmitter and of their population of vesicles.

## MATERIALS AND METHODS

The cutaneous pectoris muscle of the frog, *Rana pipiens*, was used. The muscles were mounted in the chamber described previously (5) and maintained at about 22°C in a Ringer's solution that contained 116 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, and 2 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.0). End plate regions were impaled with micropipettes filled with 3 M KCl. Conventional recording equipment was used and photographic records of the end plate potentials (e.p.p.s) and miniature end plate potentials (m.e.p.p.s) were obtained. The nerve was stimulated with square pulses 0.1 msec in duration and amplitude three to four times threshold.

The muscle twitch was blocked by adding curare to the bath at a concentration of  $3 \times 10^{-6}$  g/ml, an

end plate region was impaled, and 10–20 min later stimulation was begun at a rate of 2/sec. In our initial experiments the stimulation was interrupted every few hours and the preparation rested for 10–30 min in order to minimize the probability of conduction block developing in the nerve. As an experiment progressed the amplitudes of the e.p.p.s declined and the concentration of curare was reduced until after 6–8 hr the preparation was being stimulated in Ringer's solution. The preparation was rested for 10–20 min and the responses to single shocks were tested. If there was no twitch, or only a weak twitch involving a few muscle fibers, either black widow spider venom (BWSV) was applied or the preparation was fixed for electron microscopy. In other experiments the preparations were stimulated continuously for 2–4 hr before they were washed in Ringer's and then either fixed or treated with venom. In some of these latter experiments horseradish peroxidase (Sigma type VI; Sigma Chemical Co., St. Louis, Mo) was added to the medium at a concentration of 0.4% during the last 2 hr of stimulation.

When BWSV is applied to unstimulated nerve terminals, it evokes the spontaneous release of several hundred thousand m.e.p.s and completely depletes the terminals of their vesicles (6, 7). We used the venom-induced discharge of m.e.p.s as a measure of the store of transmitter remaining in the terminal. When the discharge was small we inferred that the terminal was depleted. The venom was prepared by grinding eight venom glands from four spiders (*Latrodectus mactans tredecimguttatus*) in 1.0 ml of 120 mM NaCl and was applied by adding 50–100  $\mu$ l of the crude homogenate to the 3–4 ml of Ringer's in the bath.

Two solutions were used to fix the muscle for electron microscopy. One contained 2% OsO<sub>4</sub> in 0.13 M phosphate buffer (pH 7.4); the other contained 1.0% glutaraldehyde and 2% sucrose in 0.1 M cacodylate buffer (pH 7.4). The solution in the recording chamber was replaced by the fixative and the muscle was cut into small pieces and placed in fresh fixative at 4°C for a total fixation time of 2 hr. Specimens fixed in glutaraldehyde were postfixated for 1 hr in 2% OsO<sub>4</sub> in 0.1 M cacodylate buffer. The peroxidase-treated muscles were fixed in glutaraldehyde as described and then treated according to the procedure of Graham and Karnovsky (8) to demonstrate sites of peroxidase activity. All specimens were embedded in Epon 812, cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Hitachi 11B electron microscope.

## RESULTS AND DISCUSSION

Fig. 1 shows some records of e.p.p.s taken during an experiment in which a preparation was stimulated continuously at 2/sec for 4 hr, and Fig. 2

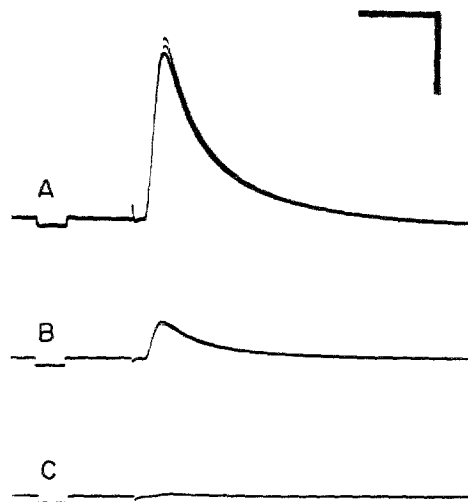


FIGURE 1 Records of e.p.p.s recorded at various times during continuous stimulation at 2/sec in curare,  $3 \times 10^{-6}$  g/ml. Each record is a superposition of four successive responses recorded: A, at the beginning of stimulation; B, after 20 min; C, after 4 hr. Calibration: 5 mv and 5 msec.

shows the time course of the change in the amplitude of the e.p.p. during a depletion experiment. After 8 hr of stimulation rather large e.p.p.s were evoked by single shocks in a dilute solution of curare, but the junction was unable to support a tetanus of 2/sec and the e.p.p. fell virtually to zero during 20 min of stimulation. The preparation was rested in Ringer's solution and BWSV was applied.<sup>1</sup>

When BWSV was applied to unstimulated preparations, the m.e.p.p. frequency rose to peak values greater than 500/sec and remained at levels above 50/sec for 30 min or more; about  $4 \times 10^5$  m.e.p.p. were released (6). These experiments with unstimulated preparations were carried out in solutions with low concentrations of Ca and high concentrations of Mg, for when the venom was applied to an unstimulated preparation in Ringer's solution, the muscle usually fibrillated violently. In contrast

<sup>1</sup> When the frequency of stimulation was increased to 5/sec during the plateau periods, the amplitude of the e.p.p. declined proportionately. This indicates that the rate of transmitter release during the steady state of the plateau periods was relatively independent of the rate of stimulation and it suggests that stimulation at high frequencies does not hasten the depletion of transmitter (9).

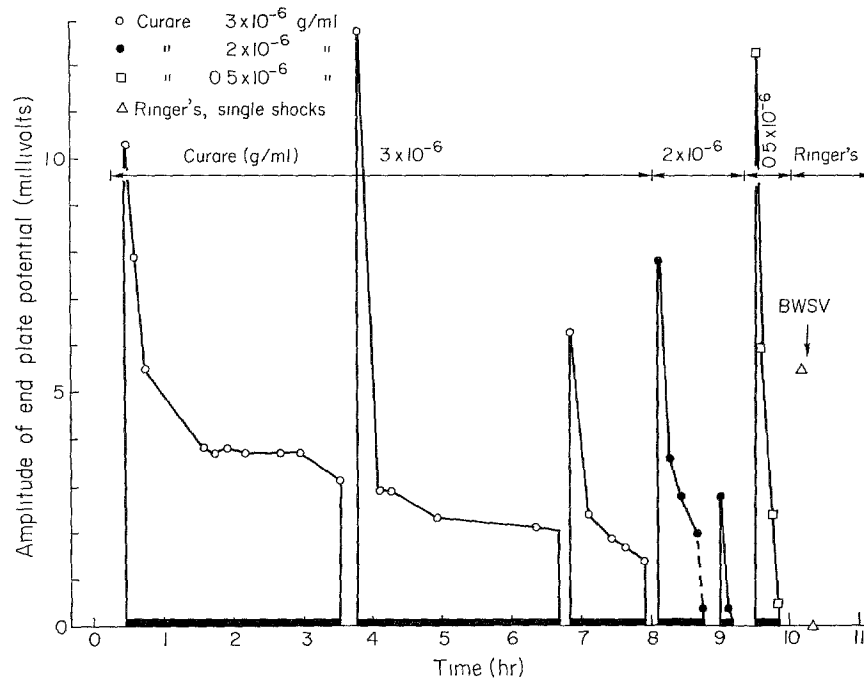


FIGURE 2 Time course of change in amplitude of e.p.p. during prolonged intermittent stimulation at 2/sec. Ordinate: amplitude of e.p.p. in millivolts. Abscissa: time in hours. The initial concentration of curare was  $3 \times 10^{-6}$  g/ml ( $\circ$ ). The base line is thickened to indicate the periods during which the preparation was stimulated at 2/sec. After stimulation was begun, the e.p.p. declined rapidly to a plateau level that was maintained for several hours. When stimulation was interrupted after about 3 hr the e.p.p. quickly gained its initial amplitude and then fell rapidly to the plateau level when stimulation was resumed. As the experiment progressed the plateau level gradually declined, the recovery of the e.p.p. during the rest intervals became incomplete, and the concentration of curare was reduced first to  $2 \times 10^{-6}$  g/ml ( $\bullet$ ) and then to  $0.5 \times 10^{-6}$  g/ml ( $\square$ ). During the period indicated by the dashed portion of the graph the rate of stimulation was raised to 5/sec, and the e.p.p. declined rapidly to zero. Later in the experiment (9.5 hr) relatively large e.p.p.s were evoked by single shocks, but the junction was unable to support a tetanus at 2/sec and the e.p.p. fell virtually to zero in 20 min. Stimulation then was stopped and the curare was removed. After about 10 min in Ringer's the e.p.p. had recovered to about 5 mv ( $\Delta$ ) and only a few fibers twitched. m.e.p.p.s were clearly visible at this time; their mean amplitude was 0.36 mv and their frequency was 11/sec. At the arrow 50  $\mu$ l of BWSV were added. 3 min later the m.e.p.p. frequency had risen to its peak value of 134/sec and it had declined to less than 1/sec 9 min later. About  $3 \times 10^4$  m.e.p.p.s occurred during the discharge. The nerve was stimulated 7 min after the venom had been added, and there was no e.p.p. The membrane potential of this muscle fiber varied between 70 and 90 mv and was lowest at the start.

to the violent reaction of unstimulated preparations, the stimulated preparations showed no fibrillation when the venom was added, and the m.e.p.p. discharge was small. In the fiber illustrated in Fig. 2, only about  $3 \times 10^4$  m.e.p.p.s were released by the venom. Thus, it is clear that for this fiber the long period of stimulation reduced the venom-evoked discharge of m.e.p.p.s by about 90% and we infer that the store of transmitter in this nerve terminal was severely depleted.

Preparations that had been stimulated at 2/sec

for 6–8 hr were uniformly depleted of transmitter. In several preparations, we explored the surface fibers of the muscle with the micropipette and we rarely found regions that exhibited m.e.p.p.s or e.p.p.s. In some muscles the 5–10 fibers nearest the lateral edge were impaled in Ringer's at the beginning of the experiment and the end plate regions were identified. When these same regions were reimpaled in Ringer's solution after prolonged stimulation, most of them showed neither e.p.p.s nor m.e.p.p.s. When active junctions were

found the e.p.p.s were small and the m.e.p.p. frequencies were low. BWSV was applied to 12 preparations while we recorded from known end plate regions. The most vigorous discharge observed was that from the fiber illustrated in Fig. 2; the other fibers gave almost no discharge (fewer than  $10^4$  m.e.p.p.s). Thus, it appears that after 6–8 hr of stimulation at 2/sec over 90% of the nerve terminals were depleted of over 90% of their store of transmitter.

However, it was difficult to characterize the physiological state of our preparations after periods of stimulation of 4 hr or less because of the great spread in the results. We recorded from single fibers in 13 preparations stimulated continuously for from 3 to 4.5 hr. The e.p.p.s had failed in five fibers, and in these fibers the venom-induced m.e.p.p. discharges were less than 10% of normal. In two fibers the e.p.p.s were 10% and 30% of normal and the m.e.p.p. discharges were in the normal range. In the other fibers the e.p.p.s had fallen to less than 10% of their initial amplitudes and the m.e.p.p. discharges were 10%–20% of normal. Thus, although there was a broad distribution in the results, it appears that about 80% of the terminals had been depleted of about 80% of their transmitter during 4 hr of stimulation at 2/sec.

We studied the ultrastructure of the neuromuscular junctions in preparations that had been stimulated for 3, 4, or 6–8 hr and in unstimulated controls that had been soaked up to 8 hr in Ringer's solution containing curare. The neuromuscular junctions from control preparations resembled those described in freshly fixed tissue (10). The nerve terminal contained neurofilaments, neurotubules, elements of smooth endoplasmic reticulum, mitochondria, large numbers of synaptic vesicles, and a few coated vesicles (Fig. 3).

After 6–8 hr of stimulation unequivocal changes appeared in all the terminals examined; the terminals were almost entirely depleted of vesicles and appeared to be swollen (Figs. 4 and 5). These changes were observed independently of the fixative used and were due to an absolute decrease in the number of vesicles and not to a simple dilution resulting from the swelling of the terminals. We can conclude, therefore, that the prolonged stimulation of the nerve ultimately resulted in the nearly complete depletion of both the neurotransmitter and the vesicles.

However, the release of transmitter as reflected in the electrophysiological data and the loss of

vesicles as seen in the ultrastructural studies were poorly correlated in time. The quantity of transmitter released from a terminal should be proportional to the area under a curve similar to that shown in Fig. 2. In most of our experiments most of the release of transmitter occurred during the first 4 hr of stimulation and, as noted above, most of the terminals were severely depleted of transmitter after 4 hr. However, at this time the general subcellular organization of most terminals still appeared normal and the reduction in the number of vesicles, though present, was not as marked as one would have expected from the physiological results (Fig. 6). This reduction occurred mainly in extensive regions of the terminal well removed from the prejunctional membrane and sometimes also in delimited regions of the terminal close to the prejunctional membrane and directly opposite the junctional folds (Fig. 6).

It is clear that the nerve terminal can secrete large quantities of transmitter without suffering a net loss of vesicles (10, 11). If vesicles must fuse with the axolemma to secrete transmitter, then during vigorous secretion the number of vesicles within the nerve terminal can remain relatively constant only if discharging vesicles are continuously replaced. Replacement of vesicles may occur by transport into the terminal from the axon, by production of new vesicles within the axoplasm of the terminal (possibly from elements of smooth endoplasmic reticulum), or by the formation of vesicles directly from the nerve terminal membrane. In the latter case, discharging vesicles may be recovered, or new vesicles formed by invagination of the plasmalemma of the terminal. Figs. 7–11 provide direct evidence that many synaptic vesicles are formed from the nerve terminal membrane. In these experiments horseradish peroxidase was added to the bathing solution and the figures show that the stimulated preparation contained many synaptic vesicles with reaction product (Figs. 9 and 10) while in the control almost none were found (Figs. 7 and 8) (12). Figs. 11 A, B, C show a series of stages in which vesicles opened to the junctional cleft have been penetrated by the marker. Taken together, Figs. 7–11 indicate that the release of a quantum of transmitter is not necessarily associated with the permanent loss of the vesicle membrane. It appears that upon fusion with the axolemma and release of transmitter (Fig. 11) the original vesicle, or a new vesicle derived from the axolemma, returns to the axoplasm (Figs. 9



FIGURE 3 Electron micrograph showing a portion of a neuromuscular junction from a control preparation soaked 8 hr in Ringer's solution containing curare at  $3 \times 10^{-6}$  g/ml. The axonal ending (*A*) contains numerous mitochondria (*m*), neurofilaments, elements of smooth endoplasmic reticulum, and synaptic vesicles (*v*). Active zones (\*), densities on the presynaptic membrane, are often visible opposite the openings of the junctional folds. Projections (*p*) of the glial cell are interposed between the terminal and the end-plate membrane. (*mf*, myofibrils).  $1 \mu$ ;  $\times 34,000$ .

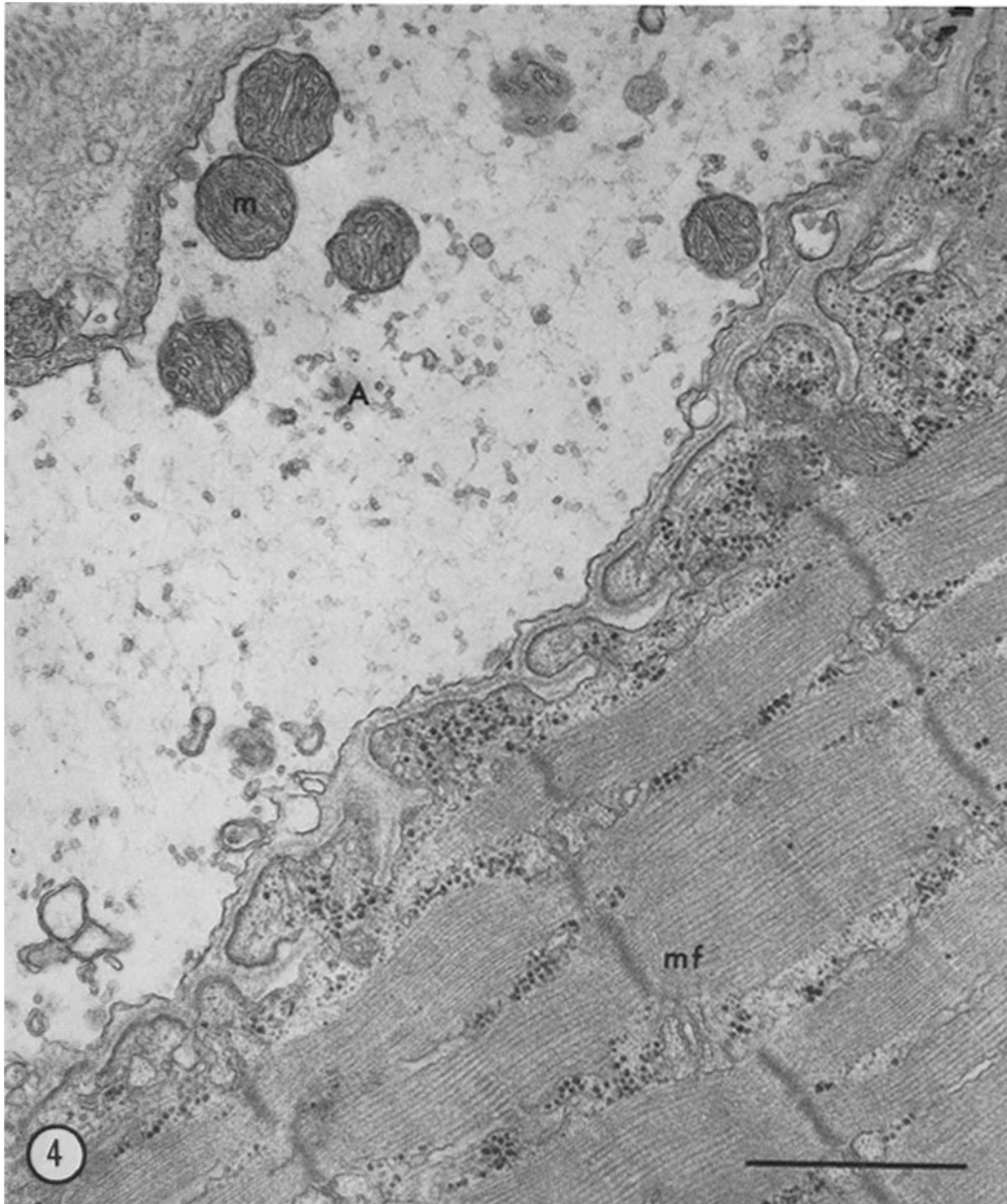


FIGURE 4 Electron micrograph showing a portion of neuromuscular junction from a preparation that had been stimulated for a total time of 8 hr. The axonal ending (*A*) appears to be swollen and contains mitochondria (*m*), neurofilaments, and elements of smooth endoplasmic reticulum. Few structures resembling synaptic vesicles are evident. (*mf*, myofibrils).  $1\ \mu$ ;  $\times 30,000$ .

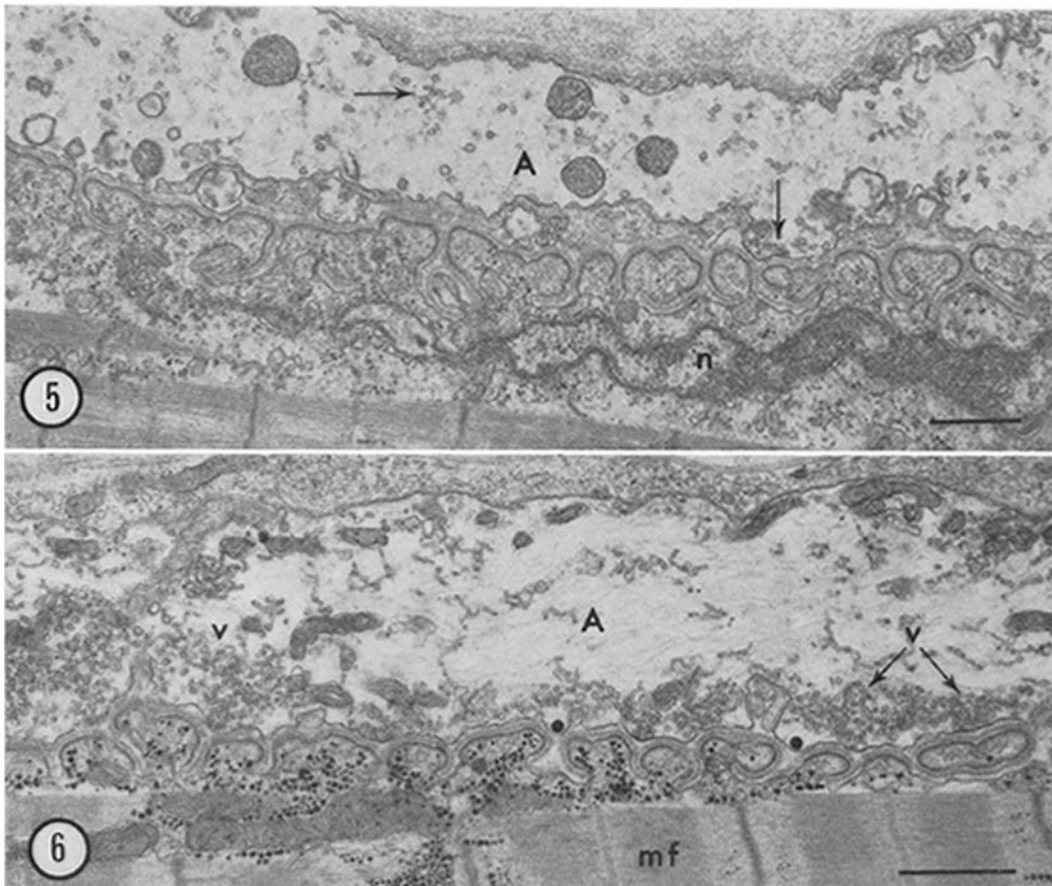


FIGURE 5 Low power electron micrograph of a portion of an end plate from a preparation that had been stimulated for 7 hr at 2/sec. The axonal ending (*A*) appears to be almost completely depleted. Only a few vesicles remain (arrows). (*n*, nucleus of muscle fiber).  $1 \mu$ ;  $\times 12,500$ .

FIGURE 6 Electron micrograph showing a portion of a neuromuscular junction from a preparation that had been stimulated continuously for 4 hr. The general organization of the axonal ending (*A*) appears normal and many synaptic vesicles (*v*) are present. A reduction in the number of vesicles in regions (*A*) away from the prejunctional membrane and focal depletion of peripheral vesicles along the prejunctional membrane (●) are evident. (*mf*, myofibrils).  $1 \mu$ ;  $\times 15,000$ .

and 10), to be possibly reutilized for the storage and subsequent release of transmitter (13).

It is difficult to explain why we succeeded in producing an extensive loss of vesicles by tetanic stimulation of the nerve whereas previous workers did not. The loss of vesicles we observed did not proceed linearly in time and was poorly correlated with the secretion of transmitter. The final massive loss of vesicles illustrated in Figs. 4 and 5 occurred relatively abruptly during the last few hours of prolonged experiments. It appears to represent a

terminal stage in the secretion process and it may have been due to the collapse of the unknown mechanisms responsible for the replacement or recycling of vesicle membrane. It is likely that the onset of this collapse depends in a complex manner on many parameters, especially the frequency, duration, and pattern of stimulation

We wish to thank Dr. George E. Palade for allowing us to use the facilities of his laboratory and for his constant encouragement. We also wish to thank

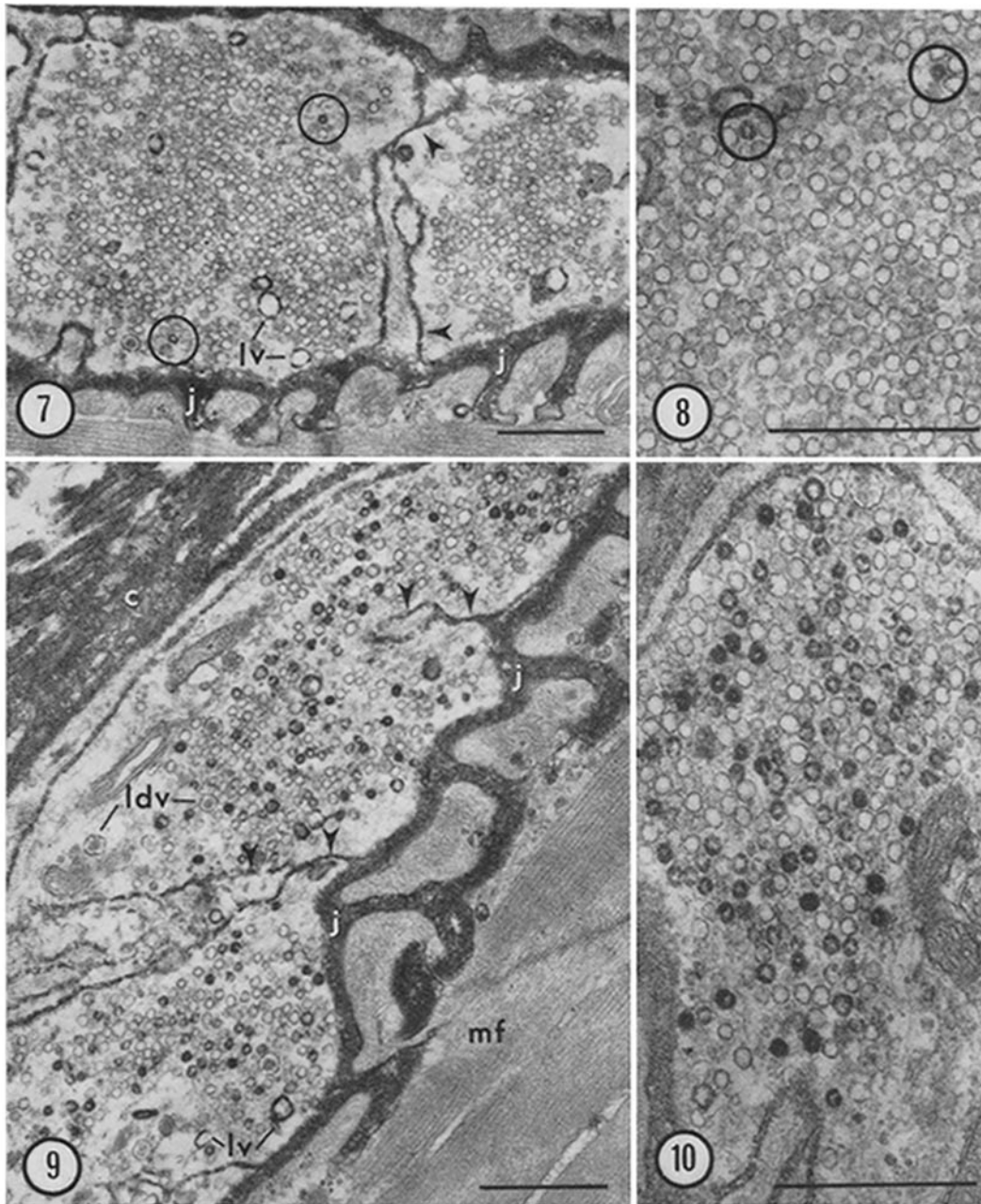


FIGURE 7 Electron micrograph of a portion of an end plate from an unstimulated preparation bathed for 2.5 hr in curare ( $3 \times 10^{-6}$  g/ml) plus horseradish peroxidase. Junctional cleft (*j*) and extracellular space delimited by infoldings of the axolemma (arrowheads) contain rich deposits of reaction product. A few vesicles (circles) also contain reaction product. Some large vesicular structures (*lv*) with a peripheral deposit of reaction product are also present.  $0.5 \mu$ ;  $\times 29,000$ .

FIGURE 8 Electron micrograph at high magnification of a portion of another end plate from the same preparation as in Fig. 7. Deposits of reaction product are present in only two synaptic vesicles (circles).  $0.5 \mu$ ;  $\times 59,000$ .

FIGURE 9 Electron micrograph of a portion of an end plate in a preparation stimulated continuously for 3 hr and rested for 0.5 hr (curare,  $3 \times 10^{-6}$  g/ml). Horseradish peroxidase was present during the last 2.5 hr of the experiment. This muscle was taken from the same frog as that used for Figs. 7 and 8. Many vesicles contain reaction product. Large vesicular structures (*lv*) with a peripheral deposit of reaction product are present as in the controls. (Arrowheads, infolding of axolemma; *ldv*, large dense core vesicle; *j*, junctional cleft; *e*, collagen fibrils; *mf*, myofibrils)  $0.5 \mu$ ;  $\times 35,000$ .

FIGURE 10 Electron micrograph at high magnification of a portion of another end plate from the same preparation as in Fig. 9. Many vesicles (about 45%) contain reaction product.  $0.5 \mu$ ;  $\times 58,000$ .



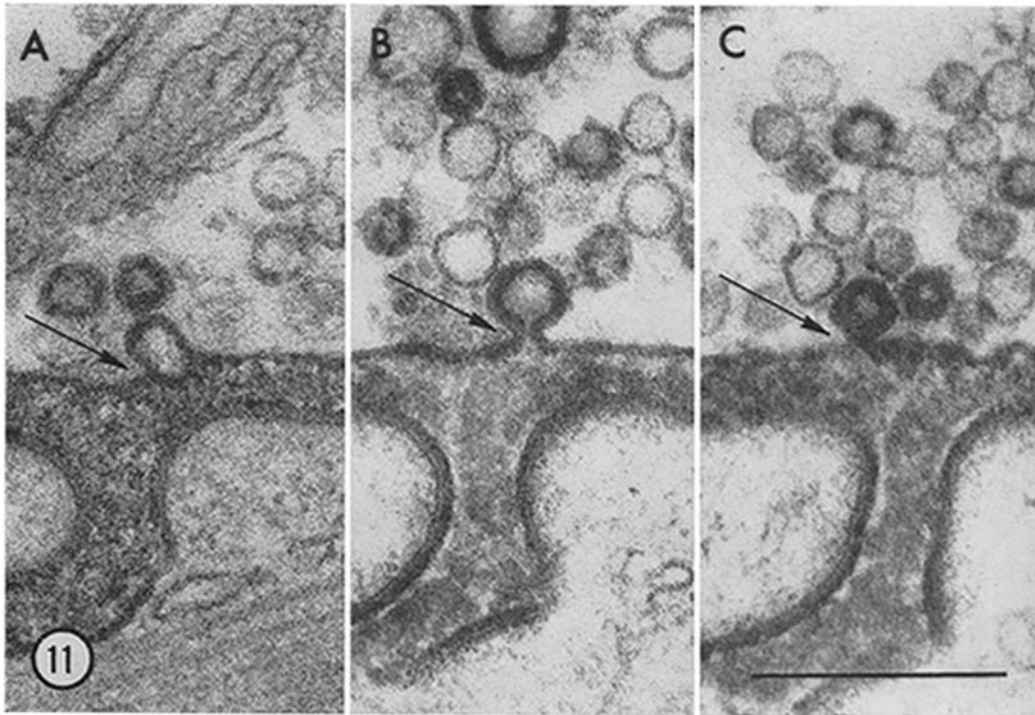


FIGURE 11 High power electron micrographs of portions of three different neuromuscular junctions at the level of the "active zone". The preparations were stimulated for 2 hr at 2/sec in curare ( $3 \times 10^{-6}$  g/ml) plus horseradish peroxidase. Junctional clefts contain rich deposits of peroxidase reaction products. The figures show three degrees of association between peroxidase-labeled vesicles and the prejunctional membrane (arrows). In A the membrane of the vesicle is completely fused with the prejunctional membrane; in B the continuity of the two membranes is maintained through a short stalk; and in C the vesicle appears to be in the process of losing contact with the axolemma.  $0.25 \mu$ ;  $\times 140,000$ .

Doctors N. Frontali, A. Grasso, and S. Bettini of the Istituto Superiore di Sanità, Rome, Italy for supplying us with the spiders

The work was done during the tenure of a fellowship awarded to Dr. Bruno Ceccarelli by the Muscular Dystrophy Associations of America, Inc.

Received for publication 22 December 1971, and in revised form 27 March 1972.

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