

TURNOVER OF TRANSMITTER AND SYNAPTIC VESICLES AT THE FROG NEUROMUSCULAR JUNCTION

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ABSTRACT

Curarized cutaneous pectoris nerve-muscle preparations from frogs were stimulated at 10/s or at 2/s for periods ranging from 20 min to 4 h. End plate potential were recorded intracellularly and used to estimate the quantity of transmitter secreted during the period of stimulation. At the ends of the periods of stimulation the preparations were either fixed for electron microscopy or treated with black widow spider venom to determine the quantities of transmitter remaining in the terminal. Horseradish peroxidase or dextran was added to the bathing solution and used as a tracer to detect the formation of vesicles from the axolemma. During 4 h of stimulation at 2/s many new vesicles were formed from the axolemma and the quantity of transmitter secreted was several times greater than the quantity in the initial store. After this period of stimulation, the terminals were severely depleted of transmitter, but not of vesicles, and their general morphological organization was normal. During 20 min of stimulation at 10/s the nerve terminals swelled and were severely depleted both of vesicles and of transmitter. During a subsequent hour of rest the changes in morphology were largely reversed, many new vesicles were formed from the axolemma and the stores of transmitter were partially replenished. These results suggest (*a*) that synaptic vesicles fuse with, and re-form from, the membrane of the nerve terminal during and after stimulation and (*b*), that the re-formed vesicles can store and release transmitter.

INTRODUCTION

From the time that del Castillo and Katz established the quantal nature of the secretion of acetylcholine from the neuromuscular junction (1), the hypothesis has been advanced that the quanta of transmitter are stored in, and released from, the synaptic vesicles present in the nerve terminal. If a synaptic vesicle contains a quantum of transmitter and must fuse with the axolemma to release it into the cleft, then an increase in the rate of secretion might eventually lead to a detectable reduction in the number of synaptic vesicles in a terminal. To obtain evidence for this hypothesis many workers have attempted to deplete

neuromuscular junctions of their synaptic vesicles by inducing a sustained rapid secretion of neurotransmitter from the nerve terminals.

In most of the early work dramatic changes in the total number of vesicles were not detected (2-4); changes were observed only in restricted regions of the axoplasm immediately adjacent to the prejunctional membrane (3, 4). Recently, however, several groups using various patterns of stimulation of the nerve have produced extensive depletions of vesicles at frog and crayfish neuromuscular junctions and in rat sympathetic ganglia (5-9). In addition, several groups have found that

after stimulation of the nerve, vesicles are formed from the axolemma of the nerve terminal (5, 6, 10). These findings suggest that during stimulation synaptic vesicles fuse with, and re-form from, the membrane of the nerve terminal (6). However, it is not known whether most of the newly formed vesicles are digested in the lysosome system (10) or whether they ultimately mature into functional synaptic vesicles capable of storing and releasing transmitter, and it is not clear whether the new vesicles arise directly from the axolemma (6) or whether they arise indirectly as the last step in a series of transformations involving intraterminal membrane compartments (5).

We have attempted to measure the rates of secretion and depletion of transmitter and to observe the depletion and re-formation of synaptic vesicles at frog neuromuscular junctions under a variety of conditions of nerve stimulation in the hope that such information would allow us to determine the turnover of the store of transmitter for comparison with changes in the number of vesicles, and thereby provide some insight into these problems. Three patterns of stimulation were used: (a) prolonged (>6 h), intermittent stimulation at 2/s; (b) continuous stimulation for 2–4.5 h at 2/s; and (c) brief (20 min) stimulation at 10/s.

The rate of secretion of transmitter was estimated from the frequency of stimulation and the amplitude of the end plate potential (e.p.p.) measured in a standard concentration of curare. The depletion of the stores of transmitter was estimated from the discharge of miniature end plate potentials (m.e.p.p.s) induced by black widow spider venom (BWSV) applied to the terminals at various times during and after the period of stimulation (11). The depletion and re-formation of vesicles was studied with the electron microscope in stimulated preparations bathed in solutions that contained either horseradish peroxidase or dextran as tracer. Some of these results have been previously published in abstract form (12).

MATERIALS AND METHODS

Material

Cutaneous pectoris nerve-muscle preparations from small frogs, *Rana pipiens*, were used. The muscle and nerve were dissected out and mounted at room temperature (20°–22°C) in Lucite chambers described previously (13). This preparation is almost ideal for work correlating changes in the electrophysiological properties of neuromuscular junctions

with changes in their ultrastructure. The muscle is very thin (about four fibers thick) and end plate regions are easy to find in the live tissue under the dissecting microscope. Furthermore, whole blocks of the fixed and embedded tissue can be mounted under a light microscope and the endplate regions readily identified and oriented appropriately for thin sectioning.

Solutions

The standard Ringer's solution contained 116 mM NaCl, 2.1 mM KCl, 1.8 mM CaCl₂, and 3 mM sodium phosphate buffer adjusted to give a pH between 6.9 and 7.2. When the ionic composition of this solution was modified the concentration of NaCl was changed to keep the tonicity constant. To change solutions the chamber was flushed at least three times using 10–20 ml of fresh solution in each flush.

Stock solutions (200 µg/ml) of *d*-tubocurarine chloride (Mann Research Labs., Inc., New York) were prepared fresh each day and diluted as required.

Horseradish peroxidase (Sigma type VI; Sigma Chemical Co., St. Louis, Mo.) was used as an extracellular tracer at a concentration of 0.4%. These solutions were prepared in small volumes and were applied with a pipette after the muscle chamber had been drained by gentle suction. The peroxidase solutions were applied to the preparations for 0.5 h before stimulation was begun and they had no obvious effects on the e.p.p. or on the ability of the terminal to support a tetanus.

Dextran, mol wt 10,000 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden), was also used as an extracellular tracer (14, 15). We prepared 10% solutions of dialyzed dextran in Ringer's with a tonicity 90% of normal. Since dextran diffuses relatively rapidly, this solution was applied to the preparations only a few minutes before stimulation was begun in the manner described for peroxidase. The 10% dextran had no obvious effect on the e.p.p. or on the ability of the terminal to withstand a long bout of stimulation. The m.e.p.p. frequency rose to about 10/s during 2 h of soaking in dextran-Ringer, and all of our experiments with dextran were shorter than this.¹

¹ It was necessary to use these high concentrations of the polysaccharide in order to see dextran particles in the electron microscope. When 20% dextran in Ringer's was applied to the preparation, the m.e.p.p. frequency rapidly increased several fold and then rose steadily with time and, after an hour, approached values of 100/s. Smaller changes in the m.e.p.p. frequency occurred at a dextran concentration of 10%. These effects were reduced when the dextran was dialyzed overnight against distilled water, and they were reduced further when the dialyzed dextran was made up in hypotonic Ringer's solution.

Preparation of BWSV

BWSV 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 μ l of the crude homogenate to the 3–4 ml of solution in the bath.

Fixation

Muscles were fixed in several different ways. In the experiments without extracellular tracers the fixing solutions contained 2% OsO₄ in 0.13 M phosphate buffer (pH 7.4) (16). The solution in the recording chamber was replaced by cold fixative and the chamber covered and stored in the refrigerator. After about 10 min the chamber was removed and under the dissecting microscope small bits of muscle containing suspected end plate regions were cut out and placed in fresh fixative at 4°C for a total fixation time of about 1 h.

The peroxidase-treated preparations were fixed for 1 h at room temperature with a solution that contained 1% glutaraldehyde and 2% sucrose in 0.1 M cacodylate buffer (pH 7.4) and then the end plate regions were cut out. The small bits of muscle were treated according to the procedure of Graham and Karnovsky to demonstrate sites of peroxidase activity (17).

The dextran-treated preparations were fixed and stained in one step with a cold solution that contained 0.7% glutaraldehyde, 0.7% formaldehyde (freshly prepared from paraformaldehyde), 1.3% OsO₄, 0.05 M arsenate buffer (pH 7.4), and lead citrate at a concentration one-sixth that of a saturated solution (15). Fixation occurred rapidly so that after only a few minutes the end plate regions were cut from the muscle and transferred to fresh fixative for a total fixation time of 15 min.

All specimens were flat embedded in Epon 812, cut with a diamond knife, and examined with a Hitachi IIB electron microscope. The OsO₄-fixed specimens were doubly stained with uranyl acetate and lead citrate, the peroxidase-treated specimens were either unstained or doubly stained, and the dextran-treated specimens were either unstained or lightly stained with lead citrate only.

Electrical Recording

End plate regions were impaled with glass micropipettes (resistance 10–30 M Ω , tip potential less than 10 mV) filled with 3 M KCl. Conventional recording apparatus was used and the e.p.p.s and m.e.p.p.s were recorded on film. The e.p.p.s were not corrected for nonlinear summation or for changes in the resting potential (18). Data were analyzed only if the resting

potential changed by less than 10 mV during an experiment. The nerves were stimulated with supra-maximal shocks 0.1 ms in duration.

General Procedure

Both muscles were dissected from a frog and mounted in separate recording chambers. In the long term experiments in which we tried to achieve complete exhaustion e.p.p.s were monitored, but we did not attempt to record from only a single junction in each preparation. These experiments lasted over 9 h and the stimulation was interrupted every few hours by rest periods 0.5 h in duration (6). As the e.p.p.s declined the curare concentration was gradually reduced from its initial concentration of 3 μ g/ml, and the intermittent stimulation was continued until the rested preparation gave only a feeble twitch in Ringer without curare. When this state had been reached the surface fibers of the muscle were explored with a micropipette to find a junction on which the effect of the BWSV could be tested. In some cases we impaled several fibers in Ringer's solution at the beginning of the experiment and then again at the end after the curare had been removed. This was easy to do near the medial and lateral edges of the muscle where the small nerve branches coursed perpendicular to the muscle fibers. In fresh preparations active junctions were almost always found near the intersections of the muscle fibers and the nerve branches, and one could impale several edge fibers to establish the presence of active junctions and record again from these same junctions after the preparations had been exhausted. The muscles always contracted vigorously when they were directly stimulated at the end of these experiments.

In the short term experiments the preparations were stimulated continuously at either 2/s or 10/s for periods up to 4 h. In these experiments we recorded from only a single neuromuscular junction in each preparation so that we could estimate the total output of transmitter from single nerve terminals. The following procedure was used. An end plate region was impaled in Ringer's and 10–20 m.e.p.p.s were recorded. Then curare (3 μ g/ml) was applied to prevent twitching and 20 min later, the time required for the effect of curare to reach a steady state, stimulation was begun. Records of the e.p.p.s were taken at the beginning of the stimulation and at convenient intervals thereafter. About 10 e.p.p.s were measured on each record to determine the average amplitude. At the end of the period of stimulation one muscle was fixed immediately for electron microscopy while the curare was washed off the other and BWSV applied 10–20 min later.

Estimation of the Number of Quanta Secreted During Stimulation

During repetitive stimulation of the nerve the rate of secretion of transmitter from a nerve terminal is determined by the number of quanta of transmitter released by each nerve impulse (i.e., the quantal content of the e.p.p.) and the frequency of stimulation, f . When the amplitude of the e.p.p. is small, then the quantal content, m , can be determined from the mean amplitudes of the e.p.p.s and m.e.p.p.s according to the formula (18)²:

$$\frac{\text{mean amplitude of e.p.p.}}{\text{mean amplitude of m.e.p.p.}}$$

Thus, when the e.p.p. is small, the rate of secretion, S , in quanta per second, is given by:

$$S = f \times m = \frac{f \times (\text{mean amplitude of e.p.p.})}{(\text{mean amplitude m.e.p.p.})}$$

At the neuromuscular junction curare acts by reducing the depolarization produced by the application of a given amount of transmitter. In blocking concentrations of curare the amount of transmitter normally released by a nerve impulse is insufficient to depolarize the muscle fiber to the threshold level. Under this condition the e.p.p.s are small and easy to measure but the amplitudes of the m.e.p.p.s cannot be determined since they have been reduced so much that they cannot be distinguished from noise. However, if one could determine the amount by which a blocking concentration of curare inhibited the response of the end plate to transmitter, then the rate of secretion of transmitter could be determined from the equation:

$$S = \frac{f \times (\text{mean amplitude of e.p.p.})_{\text{curare}}}{I_c \times (\text{mean amplitude of m.e.p.p.})_{\text{Ringer}}}$$

where I_c is the inhibition of the response of the end plate to transmitter produced by the blocking concentration of curare.

Estimation of the Number of M.E.P.P.S

Discharged by BWSV

The m.e.p.p. frequency was measured at regular intervals after BWSV had been applied, and the total number of m.e.p.p.s discharged was determined from the area under the frequency-time curve. When

² When the amplitude of the e.p.p. is large, this formula underestimates the true quantal content because of nonlinear summation of the m.e.p.p.s.

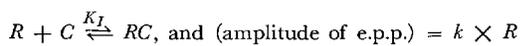
the m.e.p.p. frequency was low (<200/s), the m.e.p.p.s were discrete and their frequency could be reliably determined by counting them on moving film. When the m.e.p.p. frequency was high (>200/s), single sweeps of the oscilloscope beam were photographed using sweep speeds of 5–10 ms/cm. At the highest rates of m.e.p.p. discharge the fluctuation of the oscilloscope beam often could not be resolved into its component m.e.p.p.s even using single sweeps; the beam often underwent abrupt vertical deflections equivalent to the amplitude of several m.e.p.p.s. When this occurred the number of m.e.p.p.s was determined as the ratio of the amplitude of the deflection to the mean amplitude of the m.e.p.p.s.

RESULTS

Electrophysiology

CURARE INHIBITION OF THE E.P.P.: The results of an experiment in which curare was applied first in increasing and then in decreasing concentrations is shown in Fig. 1. The effect of curare on the e.p.p. was independent of the order in which the solutions were applied and this indicates that for these exposure times the effect of curare was reversible and steady states had been achieved.

The effect of curare, C , on the amplitude of the e.p.p. is consistent with a simple model in which curare is assumed to bind reversibly to a receptor, R , and the amplitude of the e.p.p. is assumed to be proportional to the amount of free receptor. Thus:



where K_I = dissociation constant for the binding of curare to the receptor, and k = a constant.

If the curare inhibition of the e.p.p., I_c , is defined as

$$I_c = \frac{(\text{amplitude of e.p.p.})_{\text{curare}}}{(\text{amplitude of e.p.p.})_{\text{no curare}}},$$

then the model predicts that

$$\frac{1}{I_c} = \frac{[C]}{K_I} + 1 \quad (1)$$

where $[C]$ is the concentration of curare in the bathing solution.

The inset in Fig. 1 shows the data of Fig. 1 plotted according to Eq. 1. The data fall along a straight line as required by the equation and the dissociation constant for the binding of curare to the receptor is 0.34 $\mu\text{g/ml}$ (0.49×10^{-6} M).

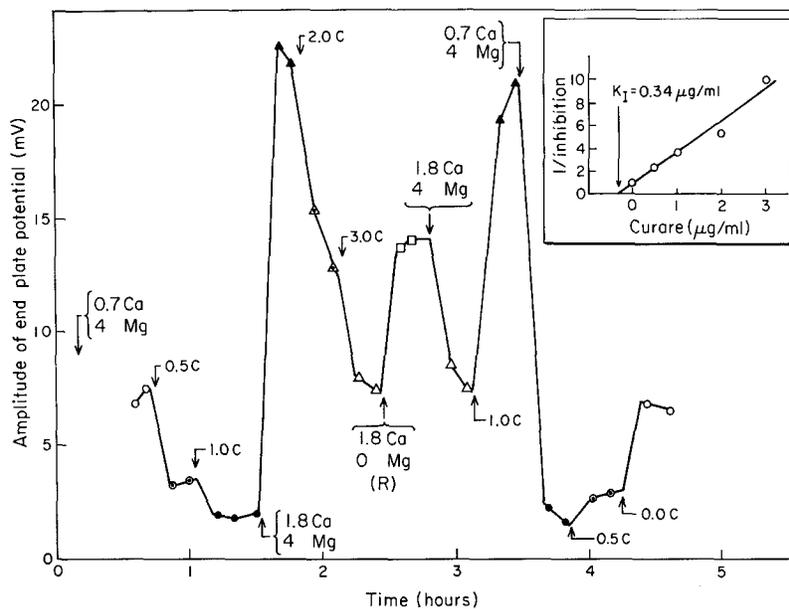


FIGURE 1 Curare inhibition of e.p.p. A junction was impaled in Ringer's solution and the amplitudes of about 20 m.e.p.p.s were recorded. A solution with 0.7 mM Ca and 4 mM Mg was run in and after a steady state had been reached the mean e.p.p. amplitude determined from 20 responses. Curare was added first at 0.5 $\mu\text{g/ml}$ (0.5 C), then at 1.0 $\mu\text{g/ml}$ (1.0 C), and the preparation was bathed for about 20 min in each solution. In the latter concentration of curare the amplitudes of e.p.p.s were usually quite small and to increase them the Ca concentration was raised to the Ringer's level (1.8 mM) while the curare concentration was held at 1.0 $\mu\text{g/ml}$ and the Mg concentration was held at 4 mM (1.8 Ca, 4 Mg). Then the curare level was increased first to 2.0 $\mu\text{g/ml}$ (2.0 C), then to 3.0 $\mu\text{g/ml}$ (3.0 C), and finally, the Mg was removed (1.8 Ca, 0 Mg). The preparation was then bathing in Ringer's solution (R) with curare at a concentration of 3 $\mu\text{g/ml}$. The solutions were then applied again in the inverse order. The m.e.p.p. amplitude in Ringer's was 0.56 mV and the resting potential varied between 90 and 95 mV. The quantal content of the e.p.p. in Ringer's was calculated to be 242. *Inset.* Data obtained at fixed level of Mg (4 mM) plotted according to Eq. 1 of the text.

Fig. 2 shows the results obtained from five experiments. The value of K_I was determined from the slope of the regression line fit to the data. When all the data were included in the analysis the value of K_I was $0.20 \pm 0.03 \mu\text{g/ml}$ ($\pm\text{SE}$). When the two highest points in Fig. 2, which deviate from the expected straight lines, were excluded from the analysis, the value of K_I was $0.28 \pm 0.02 \mu\text{g/ml}$. The latter value agrees well with the value of 0.30 $\mu\text{g/ml}$ ($0.44 \times 10^{-6} \text{ M}$) determined from the curare inhibition of the depolarization produced by carbachol or acetylcholine applied in the bath (19).

In all our stimulation experiments we used curare at a concentration of 3 $\mu\text{g/ml}$ since this was the minimum concentration that consistently blocked the muscle twitch. The inhibition of the e.p.p. at this concentration of curare, obtained by

averaging the five values in Fig. 2, was 0.073. This is equal to the degree of inhibition calculated from Eq. 1 using the average of the two values of K_I mentioned above. Therefore the formula we used to compute the rate of release of transmitter was:

$$S = \frac{f \times (\text{amplitude of e.p.p.})_{\text{curare}}}{0.073 \times (\text{amplitude of m.e.p.p.})_{\text{Ringer}}}$$

THE EFFECT OF BWSV: When BWSV is applied to unstimulated nerve terminals it provokes an enormous increase in the frequency of m.e.p.p.s that gradually subsides after 30–60 min (11). This is illustrated in Fig. 3 *a*. Terminals that have been exposed to BWSV for several hours are nearly completely depleted of synaptic vesicles and they appear to be depleted of transmitter (20). Because BWSV depletes the terminals rapidly and irre-

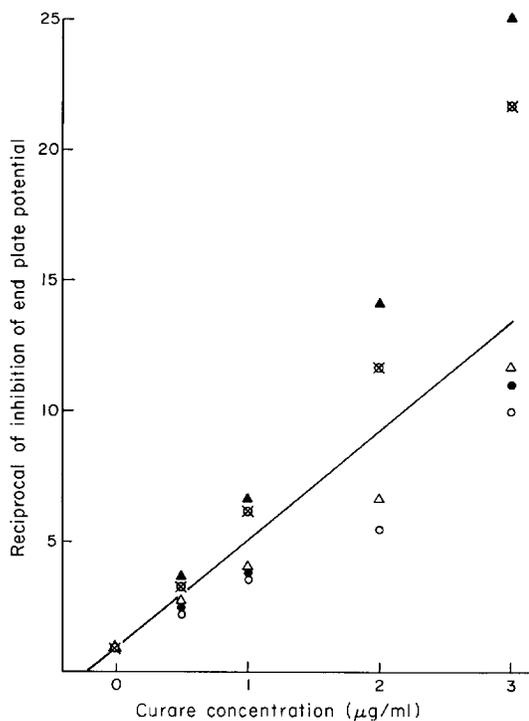


FIGURE 2 Curare inhibition of e.p.p. Data from five experiments similar to Fig. 1, each represented by a different symbol. In three experiments the curare was applied first in increasing and then in decreasing concentrations; in one, only in increasing concentrations, and in the other only in decreasing concentrations. Line drawn by eye through average values. At a concentration of 3 $\mu\text{g/ml}$ the average of the reciprocal of the curare inhibition is 14 and the average inhibition, therefore, is 0.073.

versibly it seemed to us that we could use the BWSV-induced discharge of m.e.p.p.s to determine the store of quanta present in a given nerve terminal at any given time.

BWSV was applied to 11 unstimulated preparations. Some of these preparations had been mounted *in vitro* only a few hours while others had been mounted for 7 or more hours and exposed to curare. The characteristics of the m.e.p.p. discharge were the same in both types of preparation. The results are summarized in Table I. The peak m.e.p.p. frequencies ranged from 400 to 1,100/s and the total number of m.e.p.p.s discharged ranged from 2.9×10^5 to 7.4×10^5 . These results are similar to those obtained previously with sartorius muscle (11) and they indicate that the store of transmitter in an unstimulated nerve

terminal in cutaneous pectoris muscles is 4.7×10^5 quanta on the average.

Fig. 3 *b* shows the discharge of m.e.p.p.s evoked after applying BWSV to a preparation that had been stimulated previously for 4 h at 2/s. The m.e.p.p. discharge was much attenuated: the maximum frequency was quite low (100/s), the duration of the discharge was brief (<10 min), and the total number of m.e.p.p.s counted was small (3×10^4). It is clear that stimulation had seriously depleted the stores of transmitter in this terminal. We wish to examine how the degree of depletion varies with the frequency and duration of stimulation, how the extent of depletion compares with the amount of transmitter secreted during stimulation, and whether the depletion of transmitter is correlated with changes in the ultrastructure of the terminal.

DEPLETION BY PROLONGED INTERMITTENT STIMULATION AT 2/s: We reported previously that prolonged intermittent stimulation at 2/s eventually depleted most of the nerve terminals of almost all of their stores of transmitter and their population of vesicles (6). We present here a brief statistical summary of these results. 19 preparations were stimulated intermittently for 6–9 h, and 18 ultimately gave only a feeble twitch in Ringer. BWSV was applied to 14 of these depleted preparations and six were fixed for electron microscopy without having been exposed to BWSV. (Two of the depleted muscles were divided longitudinally and the medial portion was fixed while BWSV was applied to the lateral portion.) The results obtained from the BWSV-treated preparations are summarized in Table II. All of the junctions were severely depleted of transmitter; nine gave virtually no response to BWSV and the most vigorous discharge recorded was only 20% of the normal value. The size of the BWSV-induced discharge of m.e.p.p.s was correlated with the physiological state of the terminals, i.e., those terminals still able to produce e.p.p.s gave the greatest discharge.

The data in Table II probably underestimate the proportion of depleted junctions, for we applied BWSV only after we had impaled active junctions that exhibited either e.p.p.s or m.e.p.p.s. In marked contrast to the situation in unstimulated preparations it was extremely difficult to find active junctions in depleted preparations, and we believe that many junctions gave no electrical signs of their presence. In six preparations a total of 26 fibers were impaled both before and after

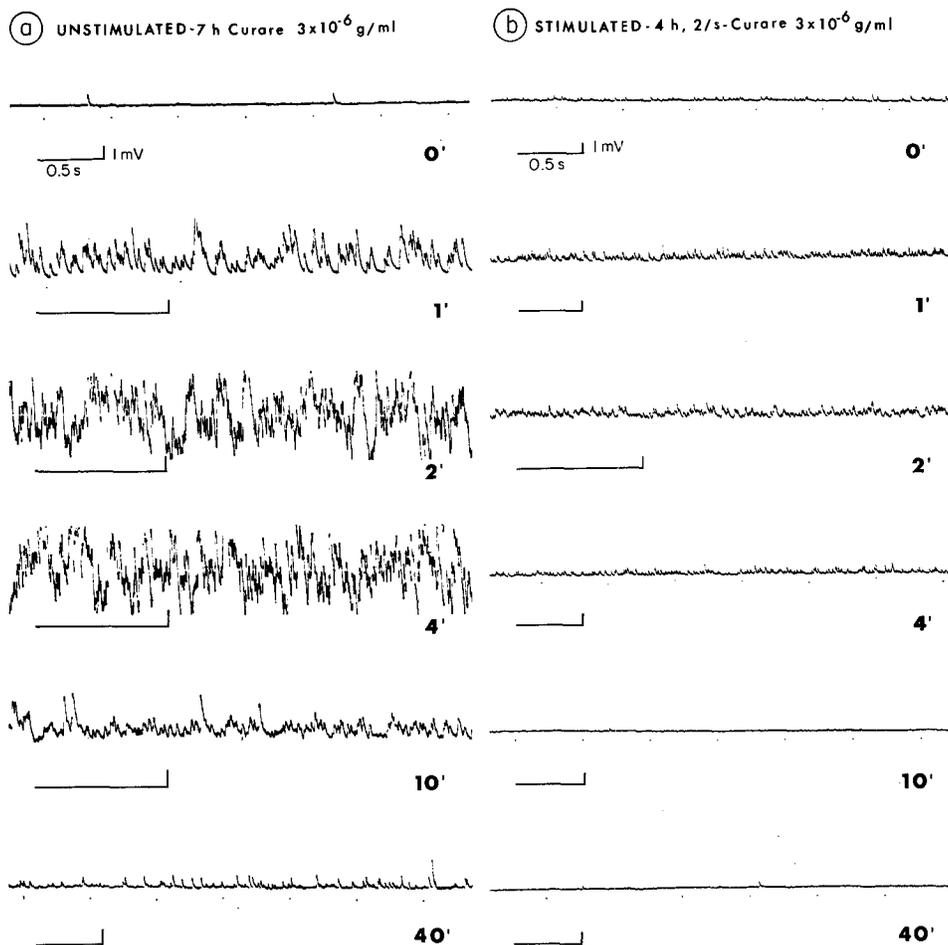


FIGURE 3 Effect of BWSV on m.e.p.p. frequency. Each column shows records on moving film obtained at various times after BWSV was applied. The time, in minutes, at which each record was taken is indicated on the right. Time and voltage calibrations: 0.5 s and 1 mV. In (a) the preparation was soaked for 7 h in Ringer's with curare ($3 \mu\text{g}/\text{ml}$), washed for 45 min with solution containing 0.5 mM Ca and 4 mM Mg, and then 50 λ of BWSV were applied. The maximum m.e.p.p. frequency in this preparation, achieved 2 min after BWSV was applied, was about 540/s, and the total number of m.e.p.p.s discharged was about 3.3×10^5 . The membrane potential was 88 mV when BWSV was first applied, fell to 65 mV at the height of the discharge, and rose to 86 mV after 40 min. In (b) the preparation was stimulated for 4 h at 2/s in curare and then the curare was washed out with Ringer's solution. After 8 min the stimulation was stopped and 13 min later 100 λ of BWSV were applied. The m.e.p.p. frequency was about 15/s when BWSV was applied. It rose to a peak value of about 90/s at 2 min and fell to less than 1/s before 10 min. The total number of m.e.p.p.s discharged was about 3×10^4 . The membrane potential was 95 mV and did not change during the discharge.

prolonged intermittent stimulation and the results are shown in Table III. None of these junctions produced e.p.p.s at the end of the experiments and we recorded m.e.p.p.s from only seven. These results indicate that the stimulation almost completely abolished all signs of electrical activity at most junctions, and they indicate that the results

shown in Table II are biased in favor of junctions that had not been completely depleted of transmitter and were still capable of some release. Hence we are confident that the program of intermittent stimulation depleted about 80% of the junctions of over 90% of their initial stores of transmitter and that the few junctions still able to re-

TABLE I
Effect of Venom on M.E.P.P. Frequency in Unstimulated Preparations

	Maximum frequency (s ⁻¹)	Minutes during which m.e.p.p. frequency exceeded			Quanta released (thousands)
		200/s	100/s	50/s	
Mean	660	11	20	33	470
Range	400-1,100	6-18	10-30	21-50	290-740

Results obtained from 11 preparations. 25 λ of venom were added to two preparations, 50 λ to seven and 100 λ to two. The venom acted faster when added in larger doses, but there were no other obvious effects of the larger doses. In one experiment the bathing solution was Ringer's. In the other experiments the bathing solution contained 4 mM Mg and either 0.5 to 0.7 mM Ca.

TABLE II
Effect of Venom on Nerve Terminals Depleted by Prolonged Intermittent Stimulation at 2/S

No. of junctions	Duration of stimulation (h)	Before venom*		Venom discharge†		
		Quantal content e.p.p.	M.e.p.p. frequency (s ⁻¹)	Maximum m.e.p.p. frequency (s ⁻¹)	Duration‡	Quanta released (thousands)
9	6-8.5	0	<1	<1	<1	<1
2	7-9	0	4-10	44-140	5	8-27
3	6.5-8	15-20	1-10	134-200	5-16	34-73

Numbers indicate spread of the data.

*E.p.p.s and m.e.p.p.s measured in Ringer's.

†50-100 λ venom applied in Ringer's.

‡Minutes during which m.e.p.p. frequency exceeded 10/s.

spond contained less than 20% of their initial stores.³

The amplitudes of the m.e.p.p.s recorded at the end of the experiments were only about one-third the initial amplitudes. We do not know whether the reduction in the size of the m.e.p.p.s was caused by a reduction in the amount of transmitter secreted in a quantum or whether it was caused by a reduction in the sensitivity of the postsynaptic membrane to transmitter. The decrease in the resting potential of the muscle fibers was too small to account for the decrease in the m.e.p.p. amplitude.

We examined in the electron microscope longitudinal sections of many neuromuscular junctions from each of the six stimulated preparations that

³ These figures were computed from Table II which shows that 11 of 14 terminals (79%) contained fewer than 27,000 quanta (less than 10% of the 470,000 quanta in unstimulated terminals) while three of 14 terminals (21%) contained less than 73,000 quanta (16% of the average initial store).

had been fixed, but not treated with BWSV. All the nerve terminals were severely depleted of vesicles as previously reported (6).

SECRETION OF TRANSMITTER DURING CONTINUOUS STIMULATION AT 2/S: Our previous work had shown that when preparations were stimulated continuously for 3-4 h at 2/s, the transmitter stores were seriously depleted, but the populations of vesicles were not (6). We wanted to know the number of quanta of transmitter secreted during such experiments to see how this quantity compared to the initial stores estimated from the number of m.e.p.p.s discharged by BWSV applied to unstimulated preparations.

We stimulated 20 preparations at 2/s for from 2 to 4.5 h. At the ends of the periods of stimulation BWSV was applied to 16 of these preparations and four were fixed and prepared for electron microscopy. In 18 cases we recorded from single junctions throughout the period of stimulation and in two cases active junctions were impaled just before

TABLE III
Effect of Prolonged Intermittent Stimulation at 2/S on M.E.P.P. Frequency and Amplitude

	δ no.	Before stimulation	δ no.	After stimulation	Ratio after/before
M.e.p.p. frequency (s^{-1})	26	1.2 (0.1-5.0)	6*	0.14 (0.05-0.4)	
M.e.p.p. amplitude (mV)	26	0.64 (0.28-2.0)	7*	0.18 (0.09-0.32)	0.33§ (0.16-0.55)
Resting potential (mV)	21†	88 (75-98)	21†	83 (71-96)	0.94 (0.91-1.05)

Mean values with ranges in parentheses.

δ = number of end plates studied.

* 19 junctions gave no m.e.p.p.s (frequency $<0.1/s$) and one, which was excluded from the average, had a m.e.p.p. frequency of 10/s.

† We failed to note the resting potentials of the muscle fibers in one experiment.

§Ratio calculated using only the seven junctions giving m.e.p.p.s at the end.

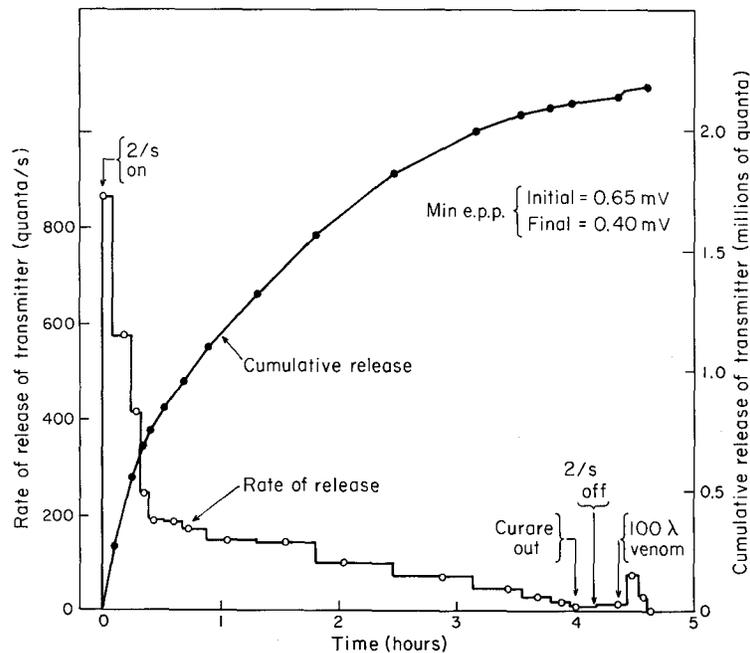


FIGURE 4 Time-course of secretion of transmitter during continuous stimulation at 2/s. Ordinates: left, rate of release (○) in quanta per second; right, number of quanta released (●). Curare, 3 μ g/ml. After stimulation was begun, the rate of release declined rapidly to a plateau level about 20% of the initial rate, remained relatively constant until about 2 h, and then gradually declined until after 4 h it was virtually zero. The curare was then washed out while the stimulation was continued. 8 min later some fibers began to twitch and the stimulation was stopped. No e.p.p.s were recorded in the impaled fiber at this time. BWSV was added 13 min later. 7 min after BWSV was applied the m.e.p.p. frequency had risen to its peak level of 87/s and it had declined to less than 1/s after 8 more min. About 3.7×10^4 m.e.p.p.s were released by BWSV. The membrane potential was constant at 95 mV.

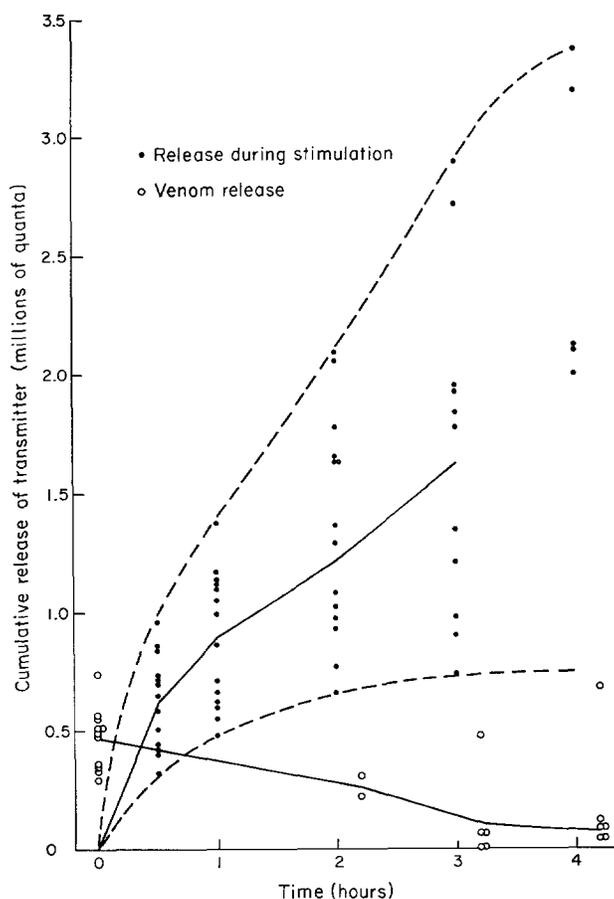


FIGURE 5 Secretion of transmitter as a function of time during continuous stimulation at 2/s. Filled symbols, quanta secreted during stimulation. The dashed lines connecting successive filled symbols indicate results obtained from single nerve terminals. Open symbols, number of m.e.p.p.s discharged after BWSV was applied after various periods of stimulation. Each open symbol represents the result obtained from a single nerve terminal. The venom could not be applied immediately at the end of the period of stimulation because the muscles began to twitch as the curare was removed. Further explanation in text. Continuous line curves drawn through average values.

BWSV was applied. Fig. 4 shows a representative example of the time-course of secretion of transmitter from a single neuromuscular junction, and the results obtained from all our experiments are summarized in Fig. 5 and Table IV.

After 2 h of stimulation all the terminals still produced e.p.p.s and the quantity of transmitter secreted was about double that in the initial store. However, we could not determine the degree of depletion of the stores of transmitter at this time because many muscle fibers began to twitch as the curare was removed. Stimulation had to be stopped to avoid dislodging the micropipettes and the washout of curare was continued until m.e.p.p.s

were clearly visible, usually about 20 min later. When BWSV was applied after the period of washing in Ringer's, the muscles often fibrillated violently and jerked the micropipettes from the fibers. To avoid fibrillation the curare was washed out with a solution containing 0.5 mM Ca and 4 mM Mg. The results in Table IV and Fig. 5 show that the depletion measured after 20 min of rest was not marked. On the other hand, after 3–4.5 h of stimulation fewer fibers began to twitch as the curare was removed. The twitching developed more slowly and was less vigorous than after 2 h of stimulation. The duration of the interval between the cessation of stimulation and the addition of

TABLE IV
Depletion of Transmitter by Continuous Stimulation at 2/S

Duration of stimulation	No. †	Quantal content e.p.p.			M.e.p.p.			Venom discharge			
		Initial	Final	Quanta released (million)	Amplitude (mV)		Frequency (s ⁻¹)		Maximum frequency (s ⁻¹)	Duration* (min)	Quanta released (thousands)
					Initial	Final	Initial	Final			
2	7	260 (60-532)	38 (7-74)	1.0 (0.4-1.8)	0.47 (0.28-0.89)	0.20 (0.16-0.25)	0.6 (0.2-1.4)	1.3 (0.4-3)	620 (570-670)	28 (11-44)	270 (230-310)
3-4.5	6	260 (138-527)	0.7 (0-4)	1.4 (0.7-2.1)	0.48 (0.29-0.65)	0.33 (0.28-0.40)	1.5 (0.5-2.9)	6.9 (1-15)	70 (1-130)	8 (0-15)	27 (0-55)
	5	348 (222-462)	15 (10-23)	2.1 (0.9-3.4)	0.43 (0.33-0.59)	0.32 (0.20-0.41)	1.6 (0.7-3)	5.3 (0.2-10)	460 (400-520)	7 (5-10)	97 (85-113)
	2	356 (137-575)	44 (41-46)	2.3 (1.4-3.2)	0.43 (0.32-0.55)	0.21 (0.14-0.27)	0.7 (0.2-2)	0.2 (0.1-0.3)	440 (290-590)	71 (47-95)	580 (480-680)

Mean values with ranges indicated in parentheses.

† Number of junctions.

* Minutes during which the m.e.p.p. frequency exceeded 10/s.

TABLE V
Depletion of Transmitter by Stimulation at 10/S for 15-20 Min

No.*	Quantal content e.p.p.		Rest period	M.e.p.p. amplitude (mV)			M.e.p.p. frequency (s ⁻¹)			Quanta released (thousands)	
	Initial	Final		Initial	After stimulation	After rest	Initial	After stimulation	After rest	During stimulation	By venom
8	317 (111-765)	9 (3-19)	0	0.54 (0.18-0.80)	0.23 (0.13-0.30)	—	1.1 (0.4-2.9)	18 (1-54)	—	580 (160-1240)	90 (25-140)
4	332 (137-628)	16 (0-37)	1.6 (1.2-2)	0.53 (0.29-0.86)	0.36 (0.19-0.60)	0.52 (0.25-0.71)	1.3 (0.3-2.8)	38 (20-54)	1.3 (0.4-2.3)	610 (300-990)	280 (180-360)

Mean values with ranges indicated in parentheses.

* Number of junctions.

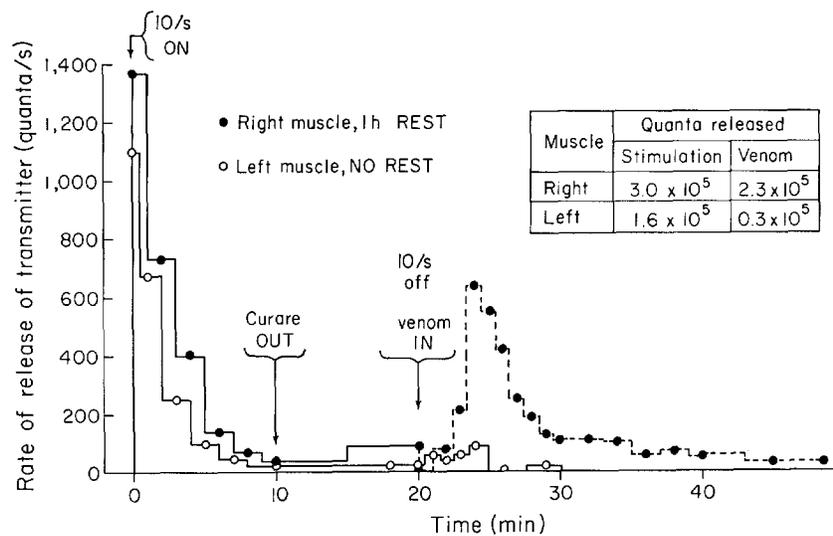


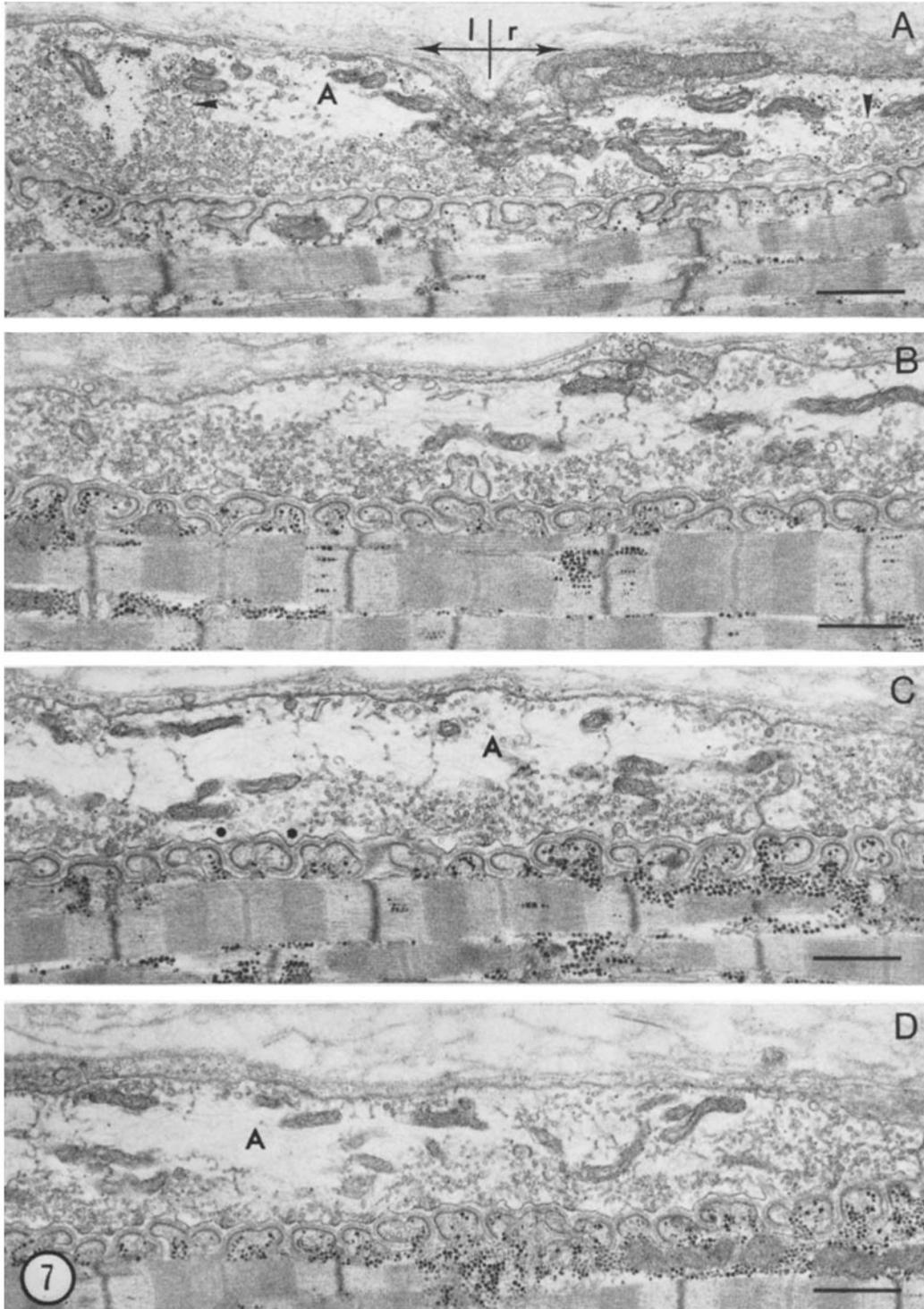
FIGURE 6 Time course of secretion of transmitter during stimulation at 10/s. Two muscles were dissected from a frog and an end plate region impaled in each muscle. Stimulation was begun in curare ($3 \mu\text{g}/\text{ml}$) and after 10 min the curare was washed off both muscles while the stimulation was continued. 10 min later the stimulation of the right muscle was stopped and 100λ of BWSV were applied to the left muscle. At this time the rate of secretion of transmitter was calculated from the directly observed m.e.p.p.s and e.p.p.s. 3 min later the e.p.p. in the left muscle failed and the stimulation of this muscle was also stopped. After the right muscle had rested for an hour in Ringer's, the bath was flushed for 10 min with a solution that contained 0.5 mM Ca and 4 mM Mg and then 50λ of BWSV were applied. The BWSV-induced discharge of m.e.p.p.s from the right muscle, which began at 90 min, was displaced to the left to coincide with the beginning of the discharge from the left muscle. In the left (unrested) muscle the m.e.p.p. amplitude and frequency were 0.7 mV and 0.5/s initially and 0.24 mV and 1/s after 20 min of stimulation. The resting potential was 92–96 mV and did not change appreciably when BWSV was applied. In the right (rested) muscle the m.e.p.p. amplitude and frequency were 0.86 mV and 0.3/s initially, 0.30 mV and 35/s after 20 min of stimulation, and 0.7 mV and 2/s after 1 h of rest. The membrane potential was 79–84 mV during the periods of stimulation and rest. It declined to about 50 mV at the peak of the BWSV-induced discharge of m.e.p.p.s and rose again to 64 mV after the discharge had subsided.

BWSV ranged from 4 to 25 min and the average duration was 13 min. BWSV was applied in Ringer's solution and no fibrillation occurred.

After 3–4.5 h of stimulation, from 1 to 3 million quanta had been secreted and there was great variation in the state of individual terminals. In

most of them (11 of 13) the e.p.p.s had failed completely or had become very small, and these terminals had been depleted of over 75% of their transmitter. In two terminals the c.p.p.s were still as large as they had been after 2 h of stimulation and these terminals had not been depleted at all.

FIGURE 7 Electron micrographs of portions of end plates from several different preparations. (A) Control preparation soaked 4 h in Ringer's with curare ($3 \mu\text{g}/\text{ml}$) but not stimulated. The axonal ending (A) contains mitochondria, neurofilaments, elements of smooth endoplasmic reticulum, and synaptic vesicles. Some large vesicular structures are also evident (arrowheads). This low power micrograph of a longitudinal section clearly shows the nonuniform distribution of vesicles along the terminal. The portion of the terminal to the left (l) contains many synaptic vesicles while the portion to the right (r) contains relatively few. (B–D) Micrographs of three different terminals taken from different preparations that had been stimulated continuously for 4 h at 2/s in Ringer's with curare. The general organization of the axonal endings (A) appears normal and many synaptic vesicles are present. Some reduction in the numbers of vesicles in regions (A) away from the prejunctional membrane and focal depletion along the prejunctional membrane (●) may have occurred. Scale marker, $1 \mu\text{m}$. $\times 13,000$.



Since the size of the residual store of transmitter was correlated with the final amplitude of the e.p.p. and with the total quantity of transmitter secreted (Table IV), these results suggest that the variations in the size of the residual store were due to differences in the capacities of individual terminals to synthesize transmitter during the period of stimulation. In the absence of exogenous choline, a typical terminal seems able to synthesize a quantity of transmitter roughly equivalent to two to three times its initial store.

The stimulation caused modest increases in the m.e.p.p. frequency, and the m.e.p.p. amplitude declined by 25–50%. The changes in m.e.p.p. amplitude were not correlated with the changes in e.p.p. amplitude or with the degree of depletion of the stores of transmitter.

SECRETION OF TRANSMITTER DURING CONTINUOUS STIMULATION AT 10/s: The results of a typical experiment at 10/s are shown in Fig. 6. The stimulation reduced the size of the BWSV-induced discharge of m.e.p.p.s and the discharge recovered toward normal levels during a subsequent period of rest. BWSV was applied to six preparations immediately at the end of the period of stimulation, and it was applied to four others after 1–2 h of rest. In four experiments no curare was used and the junctional regions were impaled after 10 min of stimulation when the muscles had ceased twitching. Essentially the same results were obtained with as without curare and the combined results are summarized in Table V.

During 15–20 min of stimulation at 10/s the amplitude of the e.p.p.s declined by 95%, the m.e.p.p. frequency rose, the BWSV-induced discharge of m.e.p.p.s was reduced by about 80%, and the terminals secreted a quantity of transmitter roughly equal to the store in unstimulated terminals. During the subsequent period of rest the store of transmitter and the frequency of the m.e.p.p.s recovered toward normal levels. The m.e.p.p. amplitude fell during the period of stimulation and recovered during the period of rest, but these changes may have been due to a slow reversal of the effect of curare on the sensitivity of the end plate to transmitter.

ANALYSIS OF ERRORS: Our estimates of the number of quanta of transmitter secreted during stimulation are based on five assumptions: (a) that curare does not reduce the amount of transmitter released by a nerve impulse but acts only postsynaptically to depress the sensitivity of the recep-

tors, (b) that during stimulation the decline in the amplitude of the e.p.p. is due entirely to a decline in the number of quanta of transmitter released by a nerve impulse, (c) that the number of molecules of transmitter contained in a quantum remains constant, (d) that the m.e.p.p. frequency does not increase during the stimulation, and (e) that the effects of nonlinear summation can be ignored.

If curare depresses the release of transmitter by a nerve impulse, then we will have overestimated the amount of transmitter released during the period of stimulation. Low concentrations of curare applied in solutions with high concentrations of Mg do not affect transmitter release (21). However, it has been suggested that high concentrations of curare applied in solutions with normal ionic concentrations do depress transmitter release in rat diaphragm (22). In frog muscle, however, even high concentrations of curare do not affect release (23). The good agreement between our estimate of the dissociation constant for the curare-receptor complex, which is based on measurements of the depression of the amplitude of the e.p.p., and Jenkinson's estimate of this dissociation constant (19), which is based on the depression of the depolarizing action of bath applied acetylcholine, also indicates that curare does not interfere with transmitter release. Hence it seems that we are justified in assuming that the effects of curare are entirely postsynaptic.

If during stimulation some of the decline in the amplitude of the e.p.p. were due to a decrease in the quantity of transmitter in a quantum or to a decrease in the sensitivity of the end plate to transmitter, then our estimates of the number of quanta released will have been too small. We observed that the m.e.p.p. amplitude fell during stimulation, and this indicates that one of these two effects must have occurred. Our ignoring of increases in m.e.p.p. frequency and our failure to correct for nonlinear summation also cause our estimates of transmitter release to be too small. The error introduced by these two simplifications is small (less than 10%) since the m.e.p.p. frequency seldom rose above 10/s (Tables I–IV) and the corrections for nonlinear summation were significant only during the first few minutes of stimulation when the amplitude of the e.p.p. exceeded 10 mV (24). The combined effect of all these sources of error is to cause our estimates of transmitter release to be too small.

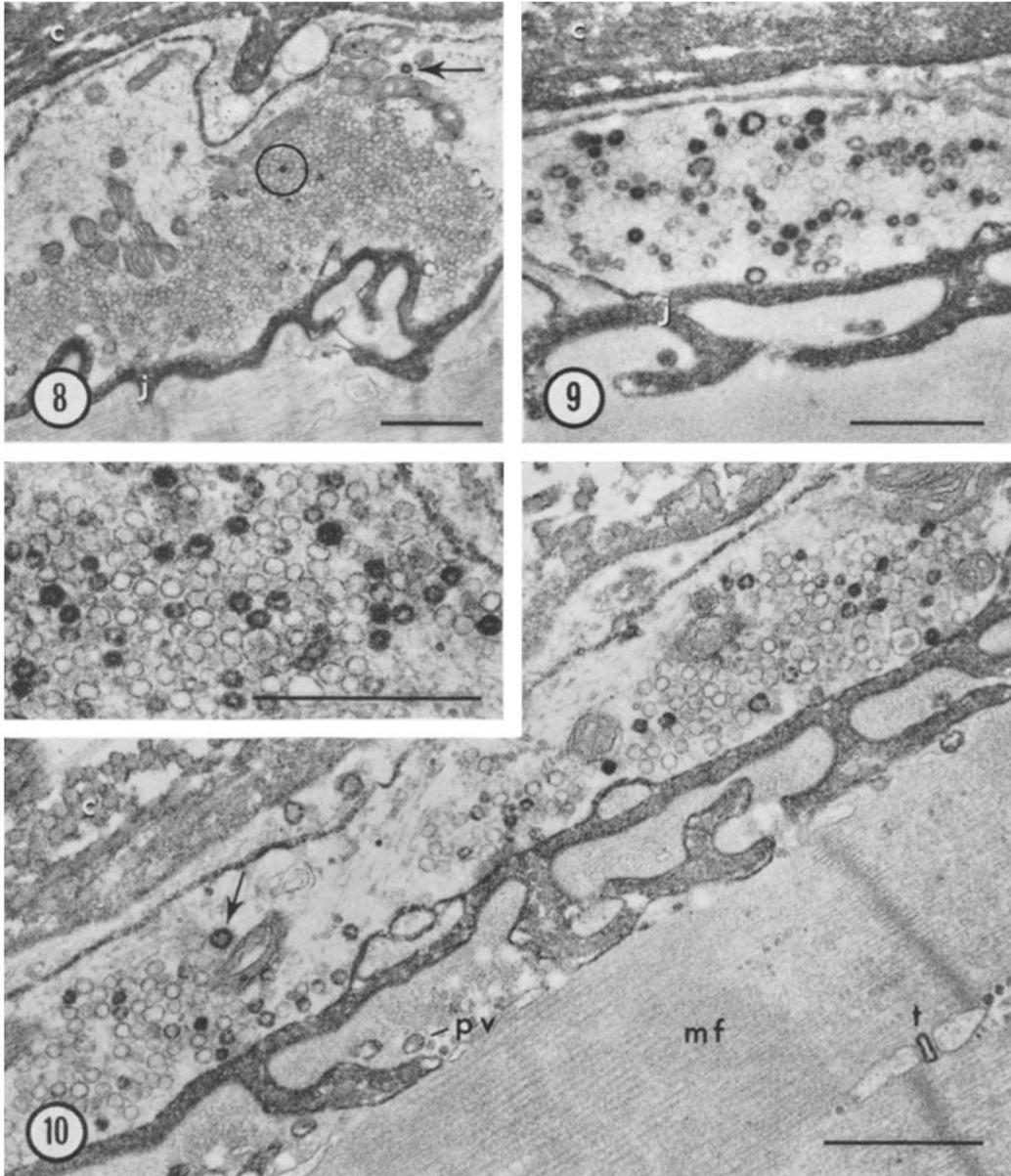


FIGURE 8 Electron micrograph of a portion of an end plate from an unstimulated preparation bathed for 2.5 h in curare ($3 \mu\text{g/ml}$) plus horseradish peroxidase. Junctional cleft (*j*) and extracellular space between the Schwann cell membrane and the axolemma contain rich deposits of reaction product. A few synaptic vesicles (circle) and a coated vesicle (arrow) containing reaction product are present. (*c*, collagen fibrils). Scale markers, $1 \mu\text{m}$. $\times 14,000$.

FIGURE 9 Electron micrograph of a portion of an end plate from a preparation stimulated continuously for 2 h at 2/s in the presence of horseradish peroxidase and fixed immediately without rest (curare, $3 \mu\text{g/ml}$). Horseradish peroxidase was applied 0.5 h before stimulation was begun. Many vesicles scattered throughout the axoplasm contain reaction product. (*j*, junctional cleft; *c*, collagen fibrils). Unstained section. Scale marker, $0.5 \mu\text{m}$. $\times 37,000$.

FIGURE 10 Electron micrograph of an end plate from another preparation stimulated for 2 h at 2/s in the presence of horseradish peroxidase and fixed immediately without rest (curare, $3 \mu\text{g/ml}$). Peroxidase was added 0.5 h before stimulation was begun. The proportion of labeled vesicles is smaller than in Fig. 9. The T system (*t*) contains a peripheral deposit of reaction product. (arrow, coated vesicle; *c*, collagen fibrils; *pv*, pinocytotic vesicle in the muscle fiber; *mf*, myofibrils.) *Inset*: electron micrograph of a portion of another end plate from the same preparation. A higher proportion of vesicles are labeled in this field. Scale marker, $0.5 \mu\text{m}$. $\times 44,000$. *Inset*: Scale marker, $0.5 \mu\text{m}$. $\times 63,000$.

It is difficult to assess the error in our estimate of the number of m.e.p.p.s discharged by BWSV applied to unstimulated terminals. When the m.e.p.p. frequency exceeded 200/s the individual m.e.p.p.s were not discrete and the estimates of m.e.p.p. frequency were not very accurate (see Methods). Improved methods for measuring m.e.p.p. frequency must be developed before the estimate of the number of quanta in the initial store can be confirmed. Our estimates of the stores of transmitter remaining in depleted terminals are not subject to such errors since the m.e.p.p. frequency usually did not rise to very high levels when BWSV was applied.

Ultrastructure

EFFECTS OF STIMULATION AT 2/s: Four preparations were fixed immediately at the ends of the periods of stimulation and then prepared for electron microscopy. The cutaneous pectoris muscle is so thin that complete fixation occurs in a few minutes. Therefore these muscles had, at most, only a few minutes for recovery and they should have been depleted by at least as much as those to which BWSV was subsequently applied. Micrographs of a control terminal and of terminals that had been stimulated for 4 h at 2/s are shown in Fig. 7. All the terminals examined appeared normal and there was no obvious change in the numbers of vesicles or in the surface area of the terminal even though the electrophysiological results indicated that most terminals were severely depleted of transmitter. It is likely that most of the vesicles seen in Fig. 7 B-D contained little or no transmitter. This corroborates and extends our earlier findings indicating that terminals can be severely depleted of transmitter before a large decrease in the number of vesicles occurs (6).

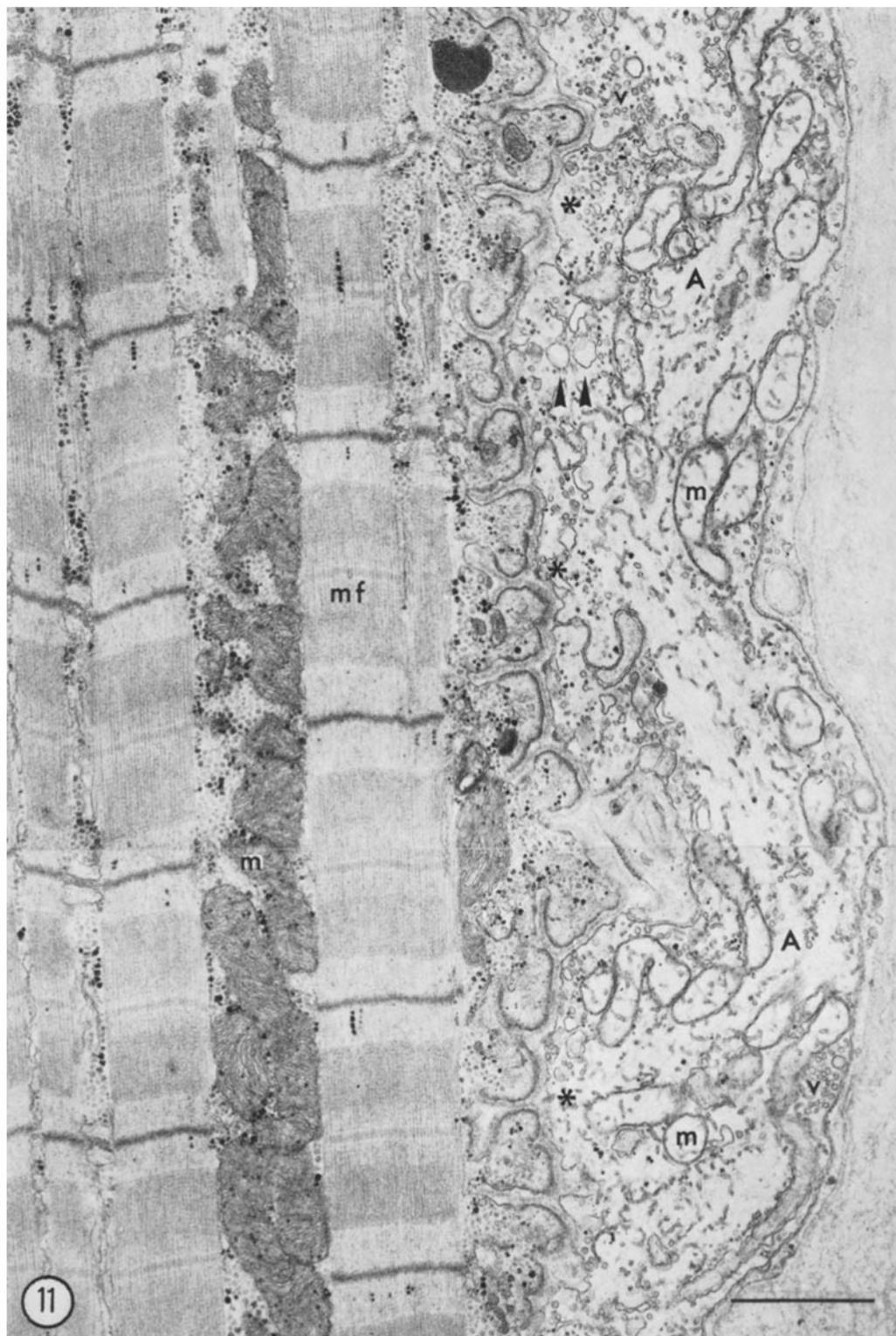
In our previous report we suggested that the vesicle population was maintained constant during stimulation at 2/s by the re-formation of vesicles directly from the nerve terminal membrane. We

concluded this because when the preparations were stimulated in the presence of horseradish peroxidase the terminals contained many vesicles labeled with the enzyme (6). In our earlier experiments the preparations had been stimulated for 2 h at 2/s and then allowed to rest for 0.5 h. We have repeated these experiments, but omitted the period of rest, in order to determine if the peroxidase is taken up during the period of stimulation. Figs. 8-10 show the results of these experiments. Although there was considerable variation in the labeling of individual terminals, both the numbers of vesicles in the terminals and the fraction of vesicles that were labeled were similar to what we observed in our previous experiments that included a period of rest (6). It seems that at low frequencies of stimulation most of the vesicles are re-formed during the period of stimulation so that during the subsequent period of rest neither the total number nor the proportion of labeled vesicles changes appreciably.

EFFECTS OF STIMULATION AT 10/s: Two pairs of muscles were stimulated for 20 min at 10/s. One muscle of each pair was fixed immediately at the end of the period of stimulation and the other muscles were fixed after 1 h of rest. Micrographs of stimulated, unrested terminals are shown in Figs. 11 and 12. All of the terminals we examined in the unrested preparations were swollen and the presynaptic membrane frequently showed many deep invaginations that originated from the regions of the axolemma between adjacent "active zones" (25). There was a clear reduction in the number of synaptic vesicles especially from the regions of the axoplasm close to the presynaptic membrane. In addition, the mitochondria in many of the terminals were swollen (Fig. 11).

Fig. 13 is a typical electron micrograph of a terminal from a preparation that had been stimulated and then rested for an hour. There are pronounced differences between these rested terminals and the unrested terminals shown in Figs. 11 and

FIGURE 11 Electron micrograph of a portion of an end plate from a preparation that had been stimulated at 10/s for 20 min and fixed immediately without rest (curare, 3 μ g/ml). The axonal ending (A) appears to be swollen (the degree of swelling can be roughly judged by comparing the diameter of the nerve terminal with the length of a sarcomere). (Compare Fig. 11 with Fig. 7.) The mitochondria (m) of the axonal ending are clearly swollen and although some clusters remain (v), there is an obvious reduction in the number of synaptic vesicles especially along the presynaptic membrane (*). A few large vesicular structures (arrowheads) are evident. Scale marker, 1 μ m. $\times 22,000$.



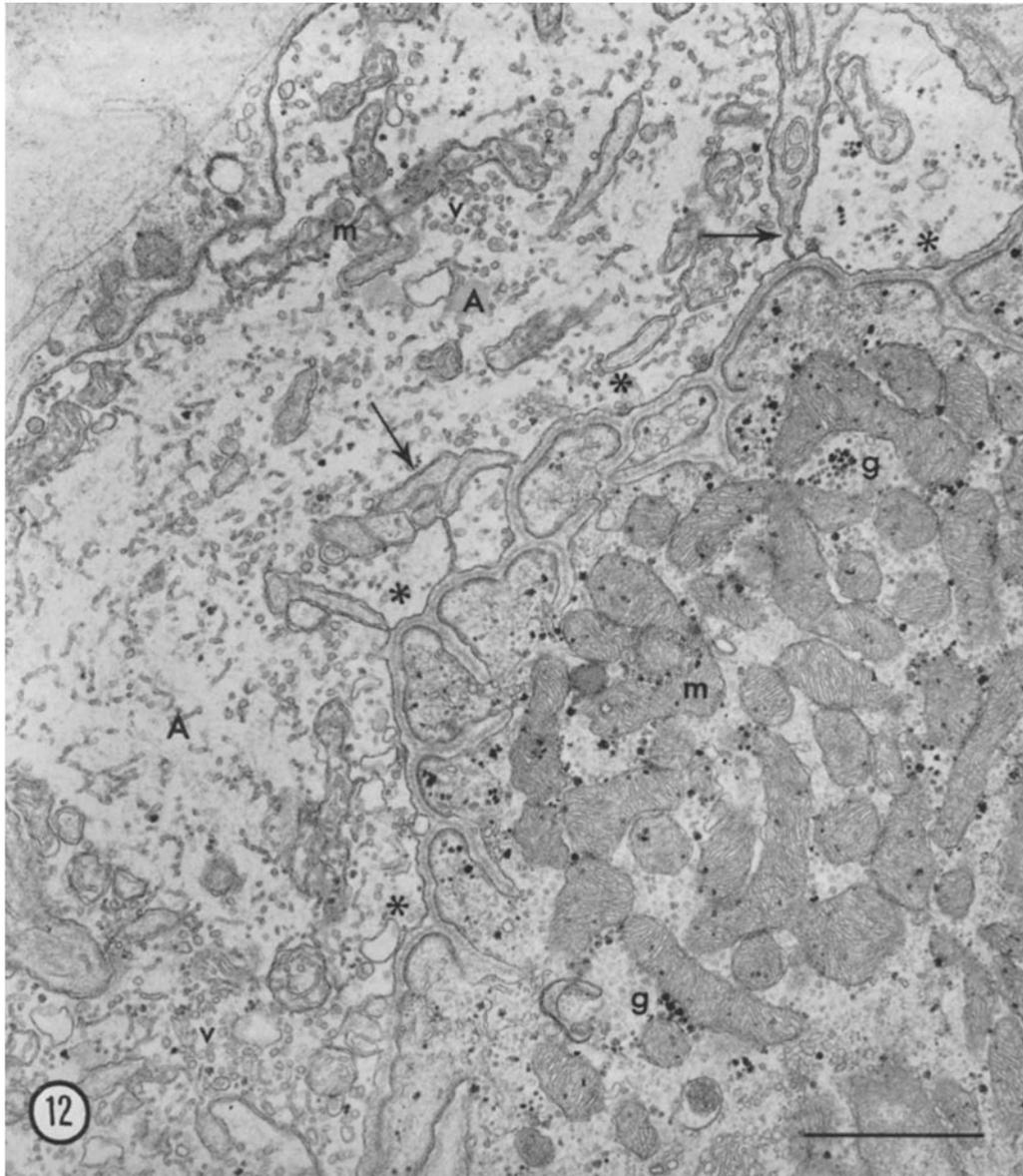


FIGURE 12 Electron micrograph of a portion of an end plate from a preparation stimulated for 20 min at 10/s and fixed immediately without rest (curare, 3 $\mu\text{g}/\text{ml}$). The axonal ending (A) is swollen and contains mitochondria (m), neurofilaments, and elements of smooth endoplasmic reticulum. There is a clear reduction in the number of synaptic vesicles, particularly in regions near the junctional membrane opposite the postjunctional folds (*). Some vesicles remain, often in clusters (v). Elaborate infoldings (arrows) of the axolemma that often envelop projections of the glial cells are present. (g, glycogen). Scale marker, 1 μm . $\times 24,000$.

12. The rested terminals are nearly normal in appearance. Although some invaginations of the axolemma remain, neither the terminals nor their mitochondria are swollen, and the number of vesi-

cles has increased toward normal levels. The vesicles occur mainly in the peripheral regions of the axoplasm that lie near the prejunctional membrane, and in these regions they tend to occur in

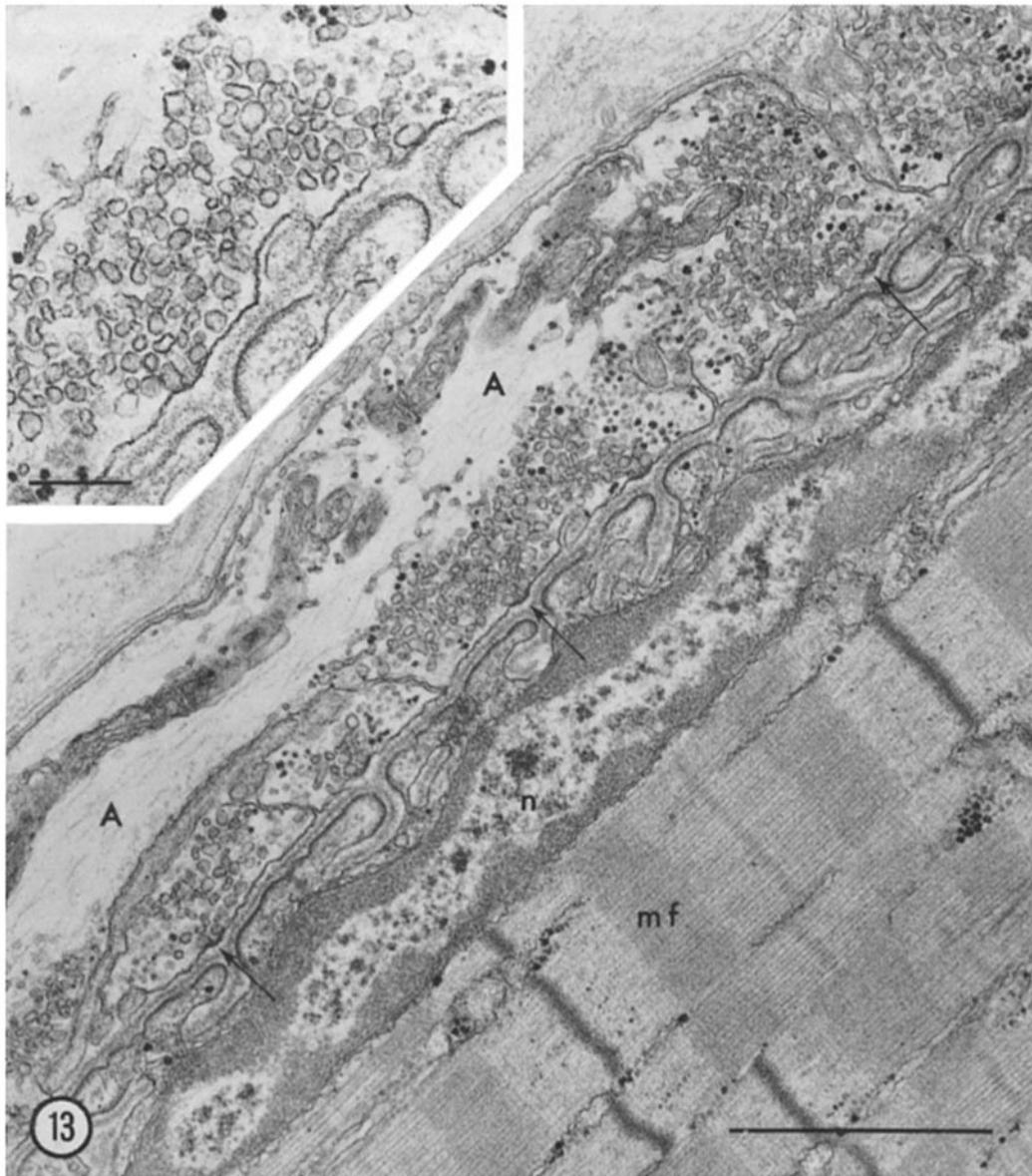


FIGURE 13 Electron micrograph of a portion of an end plate from a preparation stimulated for 20 min at 10/s and then rested for an hour before being fixed (curare, 3 $\mu\text{g}/\text{ml}$). This muscle, and the one used for Fig. 12, were paired muscles from the same frog. The general organization of the axonal ending (*A*) is nearly normal and many synaptic vesicles are present. The vesicles occur mainly in the regions near the prejunctional membrane and they tend to occur in groups concentrated in the areas opposite the postjunctional folds (arrows). A reduction in the number of vesicles in regions of the axoplasm away from the prejunctional membrane is evident and some invaginations of the axolemma can also be seen. (*n*, nucleus of muscle fiber; *mf*, myofibrils). *Inset*: higher magnification of a portion of the same end plate a few sections removed. Many synaptic vesicles are concentrated in groups near the active zones. Scale marker, 1 μm . $\times 35,000$. *Inset*: Scale marker, 0.25 μm . $\times 56,000$.

groups that are concentrated in the areas opposite the postjunctional folds.

The effects on the ultrastructure of relatively brief stimulation at 10/s are strikingly different from the effects of several hours of stimulation at 2/s. At 2/s, no obvious changes in the ultrastructure occurred before 4 h (Fig. 7 B-D) even though the electrophysiological results indicated that the store of transmitter was severely depleted and that the number of quanta secreted was several times greater than the number in the initial store. On the other hand, at 10/s many vesicles were lost within 20 min and the nerve terminals swelled. These changes in ultrastructure were reversed during a subsequent period of rest, and the increase in the number of vesicles was accompanied by an increase in the number of quanta in the transmitter store.

UPTAKE OF DEXTRAN: Since our previous work with peroxidase indicated that many vesicles were formed directly from the nerve terminal membrane during stimulation at 2/s, it was important to determine whether those vesicles that formed during a rest period after stimulation at 10/s were also derived from the axolemma. For these relatively short experiments we chose dextran 10,000 as a tracer because it has a small molecular weight and should diffuse rapidly into the junctional cleft and because it can be visualized directly in the electron microscope without first carrying out a histochemical reaction. When dextran-treated tissue is both fixed and stained in one step, individual dextran molecules, or aggregates of several molecules, appear as small, irregularly shaped, dense particles (14, 15). In our micrographs small particles of the size of individual dextran molecules ($\sim 30 \text{ \AA}$ in diameter)⁴ could not be reliably distinguished from background, but particles larger than 60 \AA in diameter, which represent aggregates of several dextran molecules, were easily seen.

In all of our dextran-treated preparations, particles were found in many intracellular compartments and in those secluded extracellular compartments that equilibrate slowly with the bath. Thus, dextran particles were found in pinocytotic vesicles near the surface of the muscle fibers, in pinocytotic vesicles in the Schwann cells, in the T system and in the confined extracellular spaces between the membranes of the Schwann cells and nerve terminals (Figs. 14 and 17). The presence of

particles in these compartments proves that dextran must also have penetrated the junctional clefts and the extracellular space around the muscle fibers. Few particles (Figs. 14-18) were found in these relatively accessible extracellular spaces, probably because most of the dextran diffused out during dehydration. It may also be that these spaces contained many individual dextran molecules that were difficult to visualize, but that few aggregates were present because the degree of aggregation was less than in the small membrane-bounded compartments. Except for these open extracellular compartments, the distribution of dextran was similar to that of peroxidase, and this indicates that dextran is an adequate tracer for our purposes.

Experimental preparations were stimulated in dextran solutions for 20 min at 10/s and then rested for an hour before being fixed. The control preparations were soaked for 1.5 h in the dextran solution but were not stimulated. Electron micrographs of terminals from control and experimental preparations are shown in Figs. 14-18.

The major difference between the stimulated and the control preparations was in the number of dextran-loaded vesicles within the nerve terminals. In the control terminals few vesicles contained dextran, and these were scattered randomly throughout the axoplasm. In the stimulated and rested terminals about 50% of the vesicles contained dextran, and these were mainly concentrated in the regions of the axoplasm opposite the postjunctional folds. This estimate of the fraction of labeled vesicles in the experimental terminals was corrected for background particles of the same size. It probably underestimates the true extent of the labeling since many vesicles that seemed to contain no dextran particles may in fact have contained such particles in regions of the vesicles outside of the plane of section. These results obtained with dextran fully corroborate the results obtained previously with peroxidase in showing that vesicles may be formed directly from the nerve terminal membrane in the regions opposite the postjunctional folds.

DISCUSSION

The analysis of errors indicates that we may have underestimated the secretion of transmitter from our preparations and that our estimate of the store of transmitter in unstimulated terminals is not accurate. However, one indication of the validity

⁴ Simionescu, N., M. Simionescu, and G. E. Palade. Personal communication.

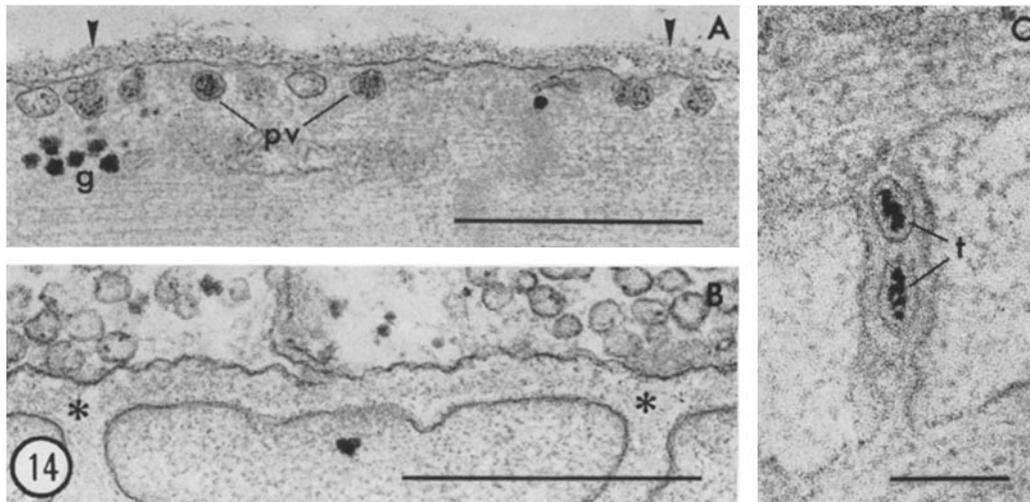


FIGURE 14 Electron micrographs of portions of muscle fibers and a neuromuscular junction from preparations that had been bathed in dextran solutions for about 1 h and 20 min. (A) Portion of the surface of a muscle fiber in a region far removed from the junction. Many pinocytotic vesicles (*pv*) labeled with dextran lie along the surface of the fiber and many dextran particles seem to be trapped in the basement membrane (arrowheads). (B) High power micrograph of a portion of a junctional cleft (*) from a preparation that had been stimulated for 20 min at 10/s and then rested for an hour. It is difficult to see dextran in the cleft but many dextran-labeled synaptic vesicles are present near the active zones. (C) High power electron micrograph of a portion of a muscle fiber showing dextran particles in the T system (*t*). Compare with Fig. 10. (*g*, glycogen). (A) Scale marker, $0.5 \mu\text{m}$. $\times 66,000$. (B) Scale marker, $0.5 \mu\text{m}$. $\times 80,000$. (C) Scale marker, $0.1 \mu\text{m}$. $\times 160,000$.

of our results is their internal consistency. Thus, during stimulation the rate of secretion of transmitter fell rapidly from a high initial level to an intermediate plateau level and then remained relatively constant for long periods of time. During the plateau period, each terminal may have been in a steady state with the rate of release of transmitter being balanced by an equal rate of synthesis. At 10/s the average rate of secretion during the plateau period (due to e.p.p.s and m.e.p.p.s) was about 140 quanta/s (Table V; Fig. 6). At 2/s the average rate of secretion during the plateau period (30–60 min) was about 135 quanta/s. These results suggest that during stimulation transmitter is synthesized at a rate of about 140 quanta/s and that this rate is relatively independent of the frequency of stimulation (26). The depletion of the store of transmitter should be determined by the difference between the amount of transmitter synthesized and the amount secreted. During 20 min of stimulation at 10/s about 6×10^5 quanta were secreted and the contents of the stores were reduced to 0.9×10^5 quanta (Table V). If transmitter had been synthesized at a rate of 140

quanta/s throughout this period of stimulation, then the total number of quanta synthesized would be 1.7×10^5 . If we use these figures to estimate the size of the initial store, we get: $(6.0 + 0.9 - 1.7) \times 10^5 = 5.2 \times 10^5$ quanta. This agrees quite well with our estimate of the size of the initial store in unstimulated terminals.

If the rate of synthesis remained constant during 2 h of stimulation at 2/s, then the number of quanta synthesized would be about 10^6 , which is equal to the number secreted in this same interval (Table IV). This result implies that the stores should not have been depleted during 2 h of stimulation at 2/s, and we found that such terminals, when tested after 20 min rest, were not markedly depleted (Table IV; Fig. 5).

On balance, then, the internal consistency of our results indicates that we have not seriously underestimated the size of the initial store of transmitter, and our previous considerations suggest that we may have underestimated the number of quanta of transmitter secreted during the period of stimulation. Hence it seems valid to conclude that during prolonged stimulation at 2/s the number of quanta

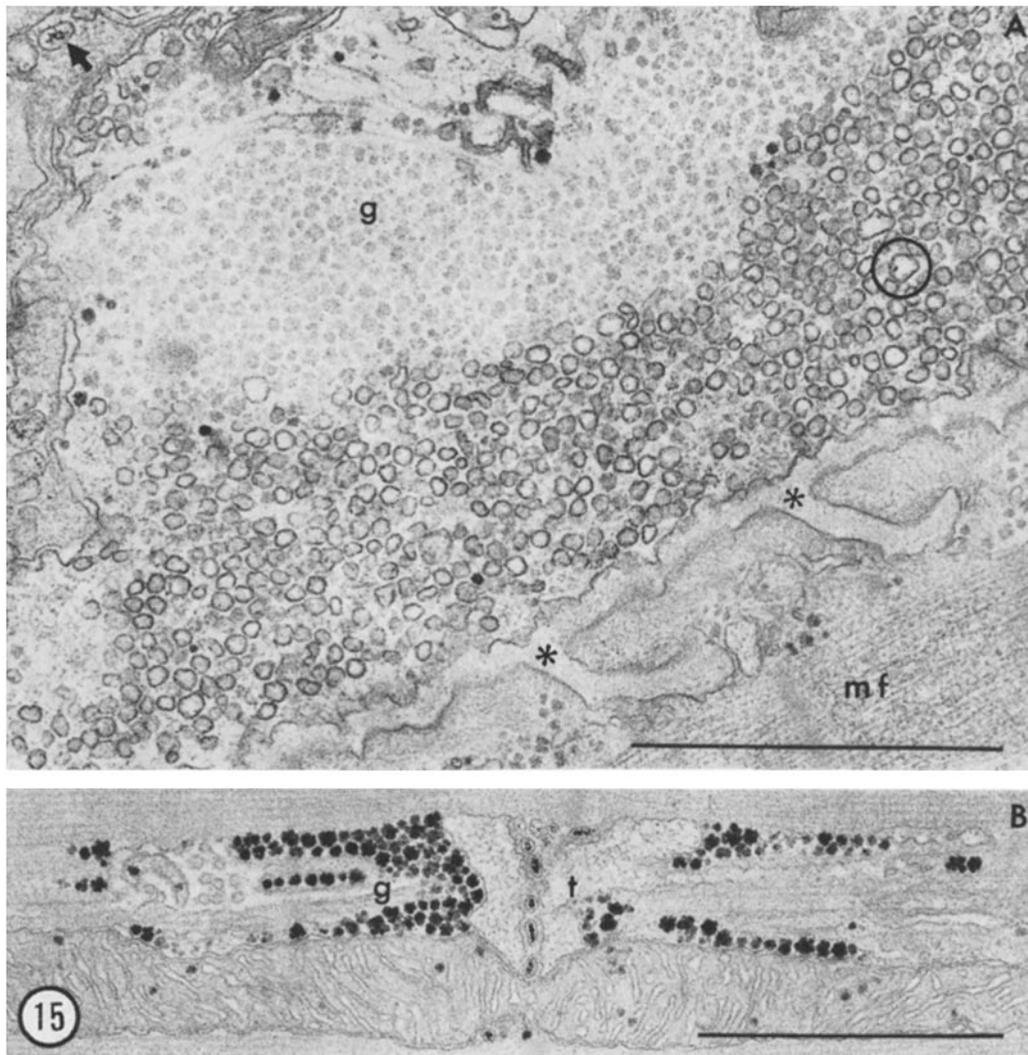


FIGURE 15 Electron micrographs of portions of a neuromuscular junction and muscle fiber that had been bathed in a dextran solution for about 1 h and 20 min but not stimulated. (A) Micrograph of a portion of the neuromuscular junction. Many synaptic vesicles are present in the terminal but only a few are clearly labeled with dextran (circle). In the upper left a pinocytotic vesicle labeled with dextran is present in the Schwann cell process surrounding the terminal (arrow). (B) Micrograph of another portion of the same muscle fiber shown in (A). Many dextran particles are evident in the T system (*t*). (*g*, glycogen; *mf*, myofibrils; *, junctional cleft). (A) Scale marker, 1 μ m. \times 50,000; (B) Scale marker, 1 μ m. \times 41,000.

secreted exceeds by several fold the number in the initial store. If a vesicle must fuse with the axolemma to release a quantum of transmitter, then this result implies either that the original vesicles are used repeatedly or that the fused vesicles are replaced by new ones capable of storing and releasing transmitter (6, 27).

The electron microscope observations provide strong evidence that vesicles fuse with, and are formed from, the nerve terminal membrane. The experiments with dextran and peroxidase give direct evidence that vesicles are formed from the axolemma during and after stimulation. The axolemma may not be the only source from which

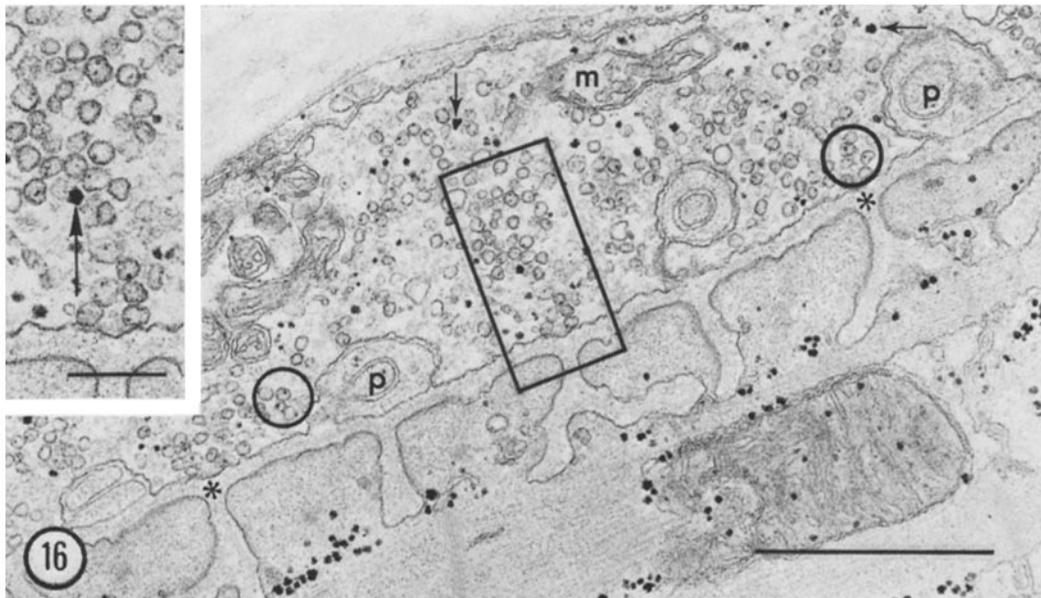


FIGURE 16 Electron micrograph of a portion of an end plate from a preparation that had been stimulated for 20 min at 10/s and then rested for an hour in a solution with dextran (curare, 3 $\mu\text{g}/\text{ml}$). Even at the relatively low magnification of this micrograph the small dense particles, representing aggregates of dextran molecules, are easily seen (circle) in most of the synaptic vesicles in the field. *Inset*: higher magnification of the portion of the terminal delineated by the rectangle. Almost all the vesicles in this field contain dextran particles. (arrow, glycogen; *m*, mitochondria; *, junctional cleft; *p*, projection of glial cell). Scale marker, 1 μm . $\times 32,000$. *Inset*: scale marker, 0.25 μm . $\times 56,000$.

new vesicles are derived since some vesicles may be formed from the smooth endoplasmic reticulum within the terminal and some may be transported into the terminal from the axon. However, the experiments with the tracers show that at least 50% of the vesicles in our stimulated preparations were derived from the axolemma.

Evidence for the fusion of vesicles with the axolemma comes from the experiments at 10/s in which we observed that during stimulation the number of vesicles decreased while the surface area of the terminal increased. However, during stimulation at 2/s neither the number of vesicles within a terminal nor the surface area of the terminal changed appreciably. The fact that the total number of vesicles remained relatively constant while many new vesicles were formed from the axolemma implies that many other vesicles had been lost, possibly by fusion with the axolemma. These results suggest that at both frequencies of stimulation vesicles were fusing with, and re-forming from, the membrane of the nerve terminal. Whether or not noticeable changes occurred either

in the surface area of a terminal or in the number of vesicles depends upon how many vesicles had been incorporated into the axolemma before the rates of fusion and re-formation became equal. During stimulation at 10/s the initial rate of fusion (about 3,000 vesicles/s) far exceeded the resting rate of re-formation. The number of vesicles fell and the surface of the terminal expanded before a new balance was achieved at a reduced rate of fusion (caused in part by the drop in the number of vesicles) and an accelerated rate of re-formation. During stimulation at 2/s the initial rate of fusion was less (about 600 vesicles/s) and the decline in the number of vesicles should also have been less before a new balance was achieved between the rates of fusion and re-formation. Once a balance was achieved both the vesicle population and the surface area of the nerve terminal remained relatively constant for long periods of time. According to this interpretation some reduction in the number of vesicles and some increase in surface area of a terminal should have occurred during stimulation at 2/s. Indeed, some of our micro-

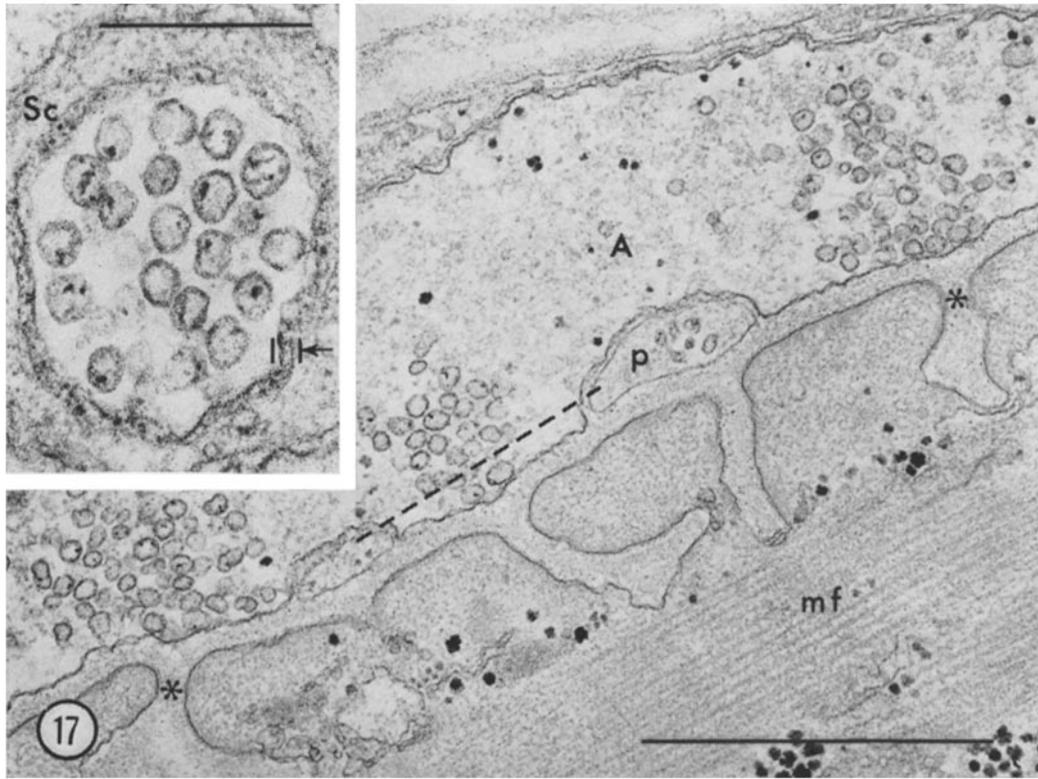


FIGURE 17 Electron micrograph of a portion of an end plate from the same preparation as Fig. 16. Clusters of vesicles are seen in the regions of the axoplasm (*A*) opposite the postjunctional folds (*). Many vesicles contain dextran. The process (*p*) probably represents a projection of a Schwann cell containing pinocytotic vesicles some of which are labeled with dextran. *Inset*: high magnification of a portion of axoplasm surrounded by a ring of Schwann cell cytoplasm (*Sc*). The arrow marks the intervening extracellular space which contains dextran particles. This section probably passes through a plane similar to that indicated by the dashed lines in the main part of the figure. (*mf*, myofibrils). Scale marker, 1 μm . $\times 48,000$. *Inset*: scale marker, 0.25 μm . $\times 110,000$.

graphs suggest that a small loss of vesicles did occur during 4 h of stimulation at 2/s, but the non-uniform distribution of vesicles within nerve terminals almost precludes our obtaining convincing quantitative data which demonstrate small changes in vesicle population (Fig. 7).

Some authors have suggested that the labeled vesicles found in nerve terminals are not functional synaptic vesicles and are fated to be digested in the lysosome system (28). Other authors have suggested that the labeled vesicles are synaptic vesicles formed, not from the axolemma directly, but from extensive intraterminal membrane compartments that represent depots of membrane material transported from the axolemma by coated vesicles (5). Our results do not support either of

these interpretations, for even in those preparations that had been stimulated for several hours our micrographs failed to show increases in the numbers of autophagic vacuoles, multivesicular bodies, lysosomes, coated vesicles, or intraterminal membrane compartments. For this reason we favor the alternative interpretation that the labeled vesicles are true synaptic vesicles that formed directly from the axolemma. We have not demonstrated directly that the newly formed vesicles store and release transmitter. That they do so, we infer from our electrophysiological results which indicate (*a*) that during stimulation at 2/s the number of quanta of transmitter secreted by a terminal can be several times larger than the number of quanta initially present, and (*b*) that after stimulation at 10/s the

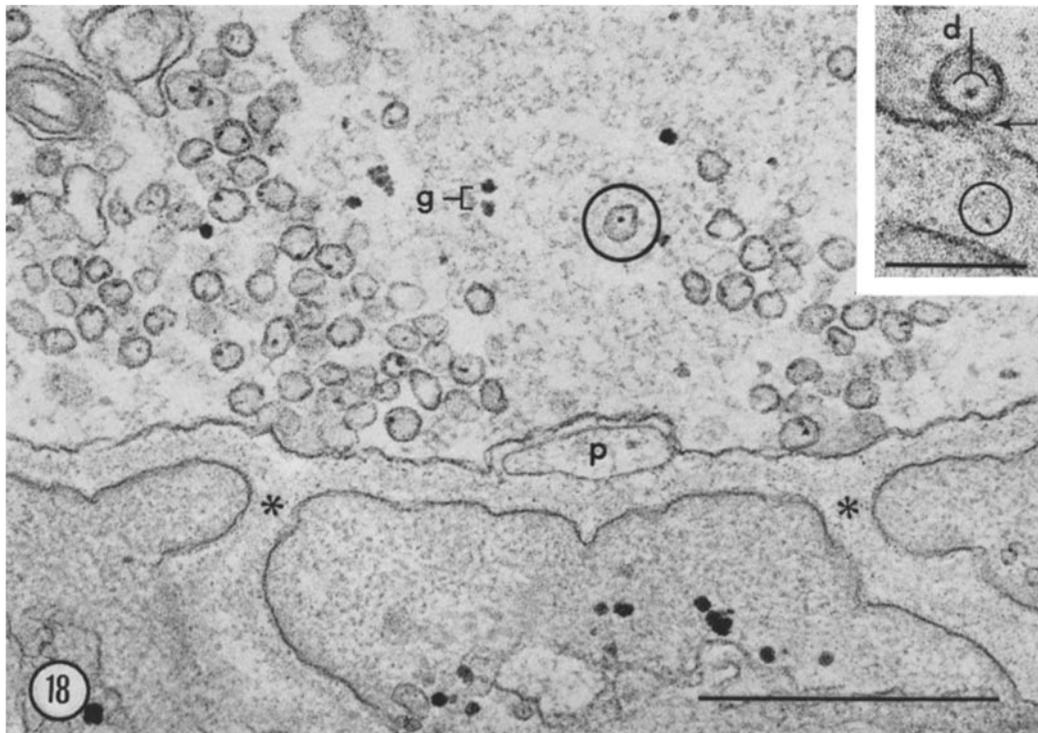


FIGURE 18 High power electron micrograph of a region of an end plate from a preparation that had been stimulated at 10/s for 20 min and then rested for 1 h in a solution of dextran. Most of the vesicles are labeled with dextran (circle) and they occur in clusters near adjacent active zones. *Inset*: high power electron micrograph of a dextran-labeled vesicle (*d*) in contact with the axolemma (arrow). Some small dense particles (circle), presumably dextran, are seen in the junctional cleft. (*g*, glycogen; *p*, projection of a Schwann cell; *, junctional cleft). Scale marker, 0.5 μm . $\times 80,000$. *Inset*: scale marker, 0.1 μm . $\times 190,000$.

increase in the number of vesicles is accompanied by an increase in the number of m.e.p.p.s discharged by BWSV.

The picture that emerges from these studies is that during stimulation there is rapid and extensive recycling of membrane between synaptic vesicles and the axolemma. The rate at which new vesicles are formed must be at least as great as the rate at which new quanta of transmitter are synthesized, and we have estimated this latter rate to be 140 quanta/s. The formation of vesicles can continue for several hours since the population of vesicles begins to fall only after more than 4 h of stimulation at 2/s when about 2×10^6 quanta of transmitter have been secreted. If we assume the vesicles to be spheres with an average diameter of 400 \AA , these results imply that membrane can be recycled at a rate of about $\pi \times (400)^2 \times 140 \times 10^{-8} = 0.7 \mu\text{m}^2/\text{s}$, and that the total area of membrane

that can be recycled is $\pi \times (400)^2 \times 2 \times 10^6 \times 10^{-8} = 10^4 \mu\text{m}^2$, or about five times the surface area of a typical terminal in the cutaneous pectoris muscle (diameter about 2 μm ; length about 300 μm) (29).

One important question that we cannot answer is how rapidly newly formed vesicles are able to accumulate transmitter. If there were no changes in the sensitivity of the postsynaptic membrane, then the time-course of the change in m.e.p.p. amplitude would give an indication of how rapidly vesicles were filled. We always found that the m.e.p.p. amplitudes fell during stimulation, but the interpretation of this finding is ambiguous since we have not established whether there were changes in the sensitivity of the postsynaptic membrane. The postsynaptic sensitivity of the end plate does not change during brief stimulation (30), but this may not be true for the very

long stimulation we have used. In addition, we cannot be sure that the effect of curare on the postsynaptic membrane was completely reversible after it had been applied for several hours.

Although we do not know how rapidly individual vesicles may fill with transmitter, the terminal as a whole can synthesize quanta at a rate of about 140/s for many hours even in the absence of an exogenous supply of choline. It appears, however, that the terminals lose their ability to synthesize or to store transmitter before they lose their ability to form new vesicles. The loss of the ability to synthesize transmitter may simply reflect the exhaustion of the supply of endogenous choline or it may indicate that the vesicles formed in the latter stages of the experiments are defective in their ability to store transmitter.⁵

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 Drs. N. Simionescu and M. Simionescu for giving us the details of their procedures for dextran before they were published.

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 31 October 1972, and in revised form 12 January 1973.

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⁵ Since the nerve terminals were separated from their perikarya throughout these experiments, their biosynthetic activities may have been severely curtailed. This may be important for the synthesis vs. recycling of synaptic vesicle membranes (or membrane components).