

EVIDENCE FOR RECYCLING OF SYNAPTIC VESICLE MEMBRANE DURING TRANSMITTER RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

J. E. HEUSER and T. S. REESE

From the Laboratory of Neuropathology and Neuroanatomical Sciences, National Institutes of Neurological Diseases and Stroke, Bethesda, Maryland 20014. Dr. Heuser's present address is the Department of Biophysics, University College, London, England.

ABSTRACT

When the nerves of isolated frog sartorius muscles were stimulated at 10 Hz, synaptic vesicles in the motor nerve terminals became transiently depleted. This depletion apparently resulted from a redistribution rather than disappearance of synaptic vesicle membrane, since the total amount of membrane comprising these nerve terminals remained constant during stimulation. At 1 min of stimulation, the 30% depletion in synaptic vesicle membrane was nearly balanced by an increase in plasma membrane, suggesting that vesicle membrane rapidly moved to the surface as it might if vesicles released their content of transmitter by exocytosis. After 15 min of stimulation, the 60% depletion of synaptic vesicle membrane was largely balanced by the appearance of numerous irregular membrane-walled cisternae inside the terminals, suggesting that vesicle membrane was retrieved from the surface as cisternae. When muscles were rested after 15 min of stimulation, cisternae disappeared and synaptic vesicles reappeared, suggesting that cisternae divided to form new synaptic vesicles so that the original vesicle membrane was now recycled into new synaptic vesicles. When muscles were soaked in horseradish peroxidase (HRP), this tracer first entered the cisternae which formed during stimulation and then entered a large proportion of the synaptic vesicles which reappeared during rest, strengthening the idea that synaptic vesicle membrane added to the surface was retrieved as cisternae which subsequently divided to form new vesicles. When muscles containing HRP in synaptic vesicles were washed to remove extracellular HRP and restimulated, HRP disappeared from vesicles without appearing in the new cisternae formed during the second stimulation, confirming that a one-way recycling of synaptic membrane, from the surface through cisternae to new vesicles, was occurring. Coated vesicles apparently represented the actual mechanism for retrieval of synaptic vesicle membrane from the plasma membrane, because during nerve stimulation they proliferated at regions of the nerve terminals covered by Schwann processes, took up peroxidase, and appeared in various stages of coalescence with cisternae. In contrast, synaptic vesicles did not appear to return directly from the surface to form cisternae, and cisternae themselves never appeared directly connected to the surface. Thus, during stimulation the intracellular compartments of this synapse change shape and take up extracellular protein in a manner which indicates that synaptic vesicle membrane added to the surface during exocytosis is retrieved by coated vesicles and recycled into new synaptic vesicles by way of intermediate cisternae.

INTRODUCTION

Synaptic vesicles are generally thought to package neurotransmitters and release them from nerve terminals in multimolecular "quanta" (46). During this process, synaptic vesicles may "recycle" locally (18) in a manner which allows each vesicle to package and release quanta repeatedly (8). This is the simplest interpretation of the observation that the number of synaptic vesicles contained in a frog motor nerve terminal when it is isolated from its cell body is far fewer than the number of quanta it can subsequently release (34), and that these vesicles do not disappear when it releases quanta at moderate rates (12). Disappointingly few morphological changes that might reflect such vesicle recycling have been found to accompany evoked transmitter release at this synapse (6, 7, 12), but several interesting changes have recently been found to occur when transmitter release is driven to irreversible exhaustion: synaptic vesicles finally disappear and the plasma membrane enlarges (12, 13) or irregular membrane-walled compartments appear in place of vesicles (34, 49). It is of interest to know whether these morphological changes represent abnormal displacements of vesicle membrane or whether they represent accumulations of vesicle membrane at different points along a normal path of vesicle recycling.

The present experiments attempt to answer this question by reexamining the effects of nerve stimulation on the morphology of the frog motor nerve terminal. They establish that synaptic vesicle depletion and membrane redistribution appear during brief periods of nerve stimulation and disappear during rest. Additional experiments follow the movement of a protein tracer through the intracellular compartments formed by this membrane redistribution, including its entry into synaptic vesicles as others have found (12, 36), to illustrate how the morphological changes could represent transient accumulations of synaptic vesicle membrane along a cyclical path that normally allows vesicles to package and release quanta repeatedly. These experiments have been presented in preliminary form elsewhere (31, 32, 35).

MATERIALS AND METHODS

Sartorius nerve-muscle preparations were dissected from small *Rana pipiens*, mounted on Plexiglas frames at rest length, and maintained in an appropriate Ringer solution (see below). Nerves were stimulated with 5 V, 0.1 ms square pulses and the nerve action

potential was monitored at all times. Transmitter release was monitored either by recording averaged end plate potentials (e.p.p.'s) with a pair of wires on the muscle (22) or by recording individual muscle fiber e.p.p.'s with 3 M KCl-filled micropipettes (47). After stimulation the muscles together with their frames were rapidly removed from the recording chamber and immersed in fixative. Then the muscle was further dissected into small cubes containing fine intramuscular nerve branches, where most synapses are concentrated (Fig. 1) (67). For histochemical localization of horseradish peroxidase (HRP) (24), the cubes had to be chopped (77) into 70- μ m thick sections parallel to the long axis of the muscle, to assist penetration of the incubation medium. After immersion in OsO_4 and uranyl acetate block treatment, all tissues were dehydrated in ethanol for several hours, passed briefly through propylene oxide, and embedded in English Araldite (Ciba Ltd., Duxford, Cambridge, England). Silver-gray thin sections were stained with uranyl acetate and lead citrate and examined in an AEI-6B electron microscope. The several variations or additions to these basic methods are described as their results are presented.

Solutions

NORMAL RINGER: 116 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 3 mM glucose.

MG-RINGER: 113 mM NaCl, 2 mM KCl, 6 mM MgCl_2 , 3 mM glucose.

HRP-RINGER: Normal Ringer with 10 mg/ml HRP (Worthington Biochemical Corp., Freehold, N. J., or Sigma type VI, Sigma Chemical Co., St. Louis, Mo.) dialyzed 16 h at 4°C against $\times 1,000$ normal Ringer.

ALDEHYDE FIXATIVE: 2% formaldehyde (from paraformaldehyde) and 3% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) in 90 mM cacodylate buffer, pH 7.2, containing 20 mM CaCl_2 ; fix for 16 h at 4°C.

OSMIUM FIXATIVE: 2% OsO_4 in 30 mM barbital buffer, pH 7.4, containing 20 mM CaCl_2 and 50 mM NaCl; fix 4 h at 1°–4°C (Figs. 2–19, 22).

HRP INCUBATION: 0.05% diaminobenzidine and 0.01% H_2O_2 in 90 mM cacodylate buffer, pH 7.2, containing 20 mM CaCl_2 ; incubate and stir 1 h at 1°–2°C. (Tissue washed in several changes of cacodylate buffer plus calcium for 2 h before and 12 h after incubation (Figs. 20, 23–35).

URANYL BLOCK TREATMENT: 1% uranyl acetate in 50 mM acetate buffer, pH 5.2; soak 4–16 h at 1°–4°C. (Tissue rinsed in 100 mM acetate buffer, pH 5.2, before and after block stain.)

URANYL GRID STAIN: 10% uranyl acetate in 1:1 methanol:water, for 15 min at 22°C.

LEAD GRID STAIN: 0.4% lead citrate in 0.15 N NaOH, for 1.5 min at 22°C.

RESULTS

Resting Appearance

Nine muscles were fixed in osmium after a 2–4 h soak in normal Ringer. Neuromuscular junctions in these control muscles were considered to be resting before fixation. At a frog neuromuscular junction or “end plate” the motor axon lost its myelin sheath and divided to form a few cylindrical terminal branches which extended along the length of the muscle fiber for 100–300 μm (Figs. 1–3). These terminal branches remained enclosed in thin Schwann processes except at the immediate interface with the muscle, where adjacent nerve and muscle plasma membranes were apposed to form wide synaptic clefts (70). Within each terminal were a large number of synaptic vesicles of rather uniform size and shape located near the presynaptic surface, and many small, dense, elongated mitochondria located in its central core (Figs. 2–3). Also present were a number of narrow, tortuous, membrane-walled tubes that were found all along the motor axon and extended into the core of the terminal to a variable extent (Fig. 2, 3, 16–19) (6, 7, 70). Connections between this axonal endoplasmic reticulum and synaptic vesicles were not observed.

Each terminal branch was differentiated along its length by repeating constellations of morphological features conveniently termed “synaptic units.” Each unit centered upon a 100 nm wide band of dense material lining the plasma membrane (Fig. 3). This dense band was oriented perpendicularly to the axis of the terminal and always located just above a vertical fold in the muscle plasma membrane (Figs. 3–6). Above the dense band synaptic vesicles appeared to cluster, and immediately around it vesicles came closest to the plasma membrane (Fig. 10). This has been observed before and has led to the suggestion that the dense band is the site of transmitter release from synaptic vesicles (7, 14, 25, 63). At greater distances from the dense band, vesicles did not come as close to the plasma membrane. Each synaptic unit was bounded by Schwann processes which extended intermittently between the nerve and muscle to enclose the terminal and separate one

unit from the next (Fig. 3). Where the terminal was contacted by Schwann processes, its plasma membrane was coated with a filamentous material that often assumed the discrete polygonal appearance of a “basket” (Figs. 3, 13) (44). The morphological differentiation of resting terminals into repeating synaptic units was accentuated by stimulation, which introduced characteristic changes in particular portions of each unit.

Appearance after 15 Min of Stimulation

Eight muscles were curarized (10^{-5} g/ml or 0.02 mM), stimulated at 10 Hz for 15 min at 10°C, and immediately plunged into osmium fixative. During this stimulation the average e.p.p. amplitude monitored with external electrodes progressively fatigued to plateau at 5–10% of initial amplitude. Intracellular recordings insured that this fatigue was due to a reduction in the amount of transmitter released from all end plates, not to a complete cessation of release from certain end plates which would have occurred if they had failed to conduct the nerve impulse at 10/s (20, 51).

Consistent with the uniform involvement of all end plates in this fatigue, structural changes appeared in all 160 stimulated terminals that were examined (Figs. 4, 6, 11, 16). These terminals contained fewer synaptic vesicles than normal, but did not appear empty because the vesicles had been replaced by large irregularly shaped membrane compartments hereafter called “cisternae” (62), and by coated vesicles (Figs. 4, 6, 15). The cisternae most often assumed either a broad, flattened form (arrow, Fig. 16) or an irregular, rounded form (Figs. 4, 11), but they assumed intermediate forms often enough that it was impossible to classify them into two distinct types. Furthermore, frequent continuities between flattened and rounded cisternae were apparent in series of sections. The mitochondria in stimulated terminals were swollen and occasionally contained granular deposits in their electron-lucent inner compartments similar to the granules found in mitochondria actively accumulating calcium (30, 33, 65). Some mitochondria appeared to have moved nearer to the presynaptic surface to mingle with the new cisternae (Figs. 16, 17). Such mitochondrial swelling and relocation has previously been observed during stimulation of this (7) and other neuromuscular junctions (43).

To determine the extent of these changes, some measurements of membrane areas were made



FIGURE 1 Light micrograph of the region of the frog sartorius muscle used in these studies because it is particularly rich in neuromuscular junctions. Nerve terminals (circled) appear in cross section as discrete oval profiles lying in shallow grooves on the surface of the muscle fiber. Several profiles appearing on one fiber represent different terminal branches of one neuromuscular junction. $\times 1,200$.

upon 100 randomly cross-sectioned terminals from two of the stimulated muscles, in comparison with 100 terminals from the contralateral unstimulated muscles from the same animals. Simple counts of synaptic vesicles revealed that stimulated terminals contained less than half as many vesicles as unstimulated controls (Table I).¹ Based on a total end

¹ To determine the true number of vesicles in a section it was necessary to correct the actual counts by eliminating half of those vesicles which would also be

plate length of 500 μm (56), an average end plate had lost about 3×10^5 vesicles; it became of interest to know if it had released a similar number of quanta. This number could not be accurately

counted in either of the adjacent serial sections. Even when vesicles were counted only if they had a clearly defined membrane around at least half their perimeter, it was found that 12% of them were also counted in an adjacent section. Thus all vesicle counts were reduced by 6%.

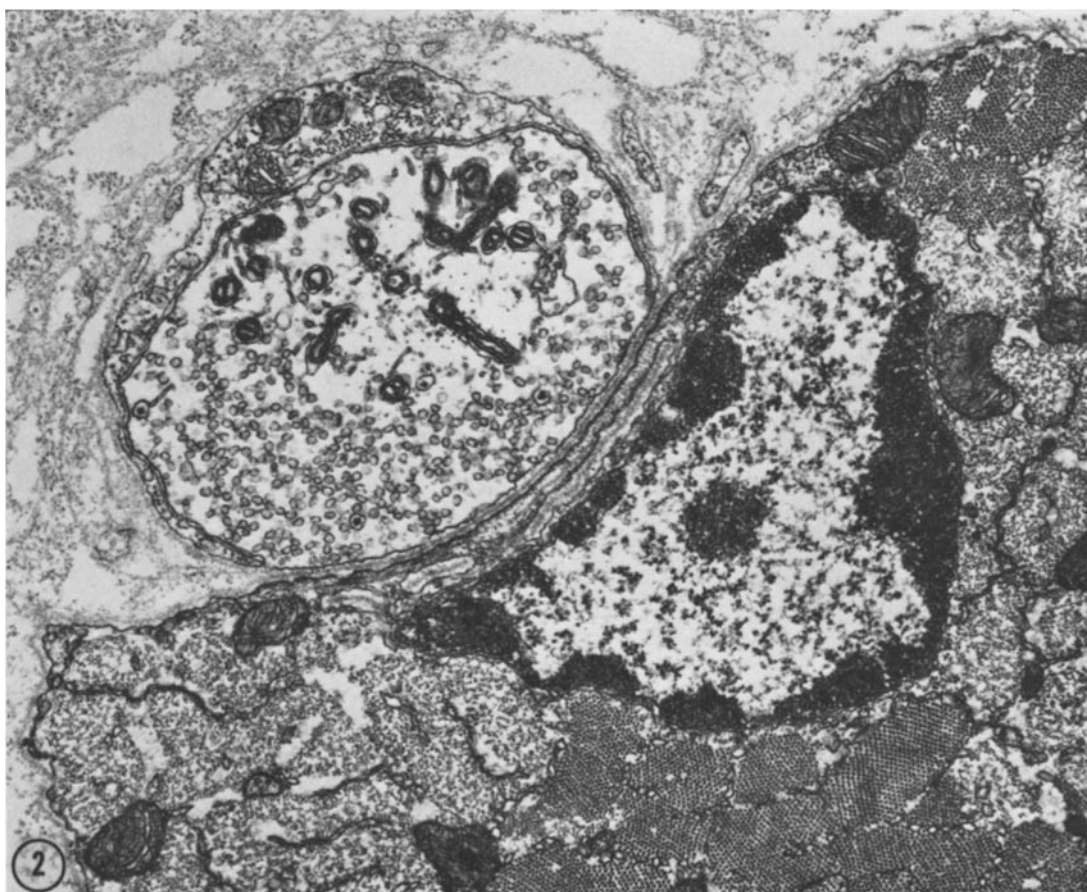


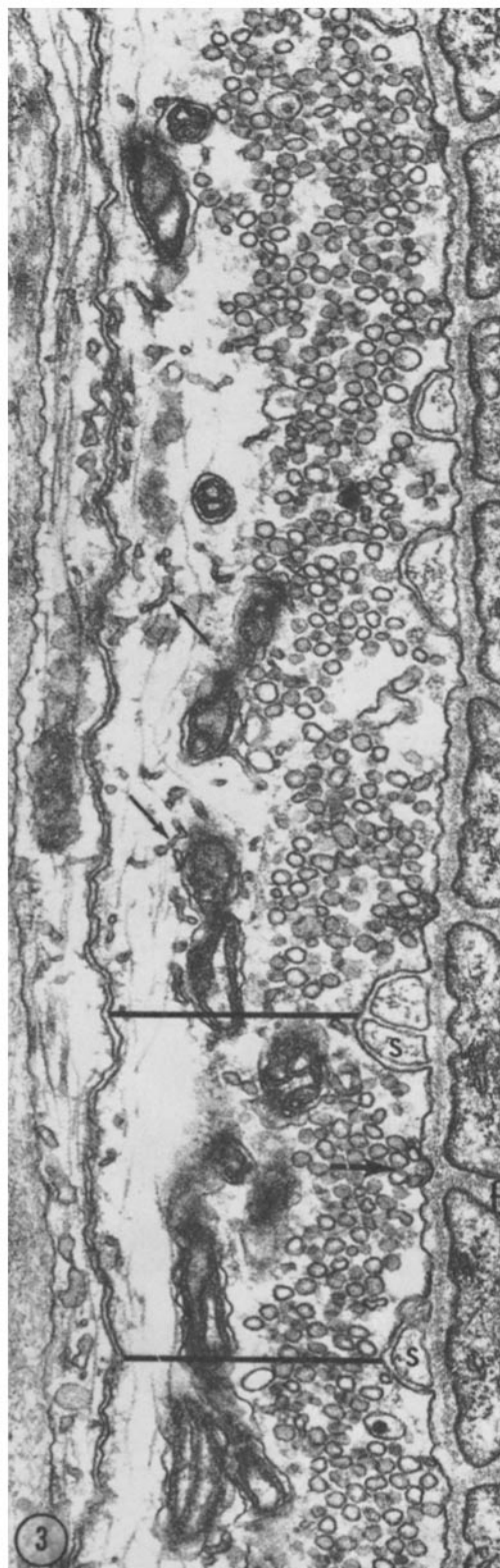
FIGURE 2 Cross section of a large unstimulated nerve terminal typical of one of the regions circled in Fig. 1. The terminal contains many synaptic vesicles distributed around a core of microfilaments, dense mitochondria, and axonal endoplasmic reticulum. It is covered by a thin Schwann sheath except at its interface with the muscle fiber. $\times 20,000$.

measured by current methods over the wide range of quantal output occurring during the stimulation (39); instead it was estimated by correlating our own and others' measurements of e.p.p. fatigue (12, 15, 78) with published values of initial quantal release rates (45, 54). These estimates ranged from 2×10^5 to 5×10^5 quanta released by 15 min of 10 Hz stimulation at 10°C , which are at least the same order of magnitude as the number of vesicles depleted, and so are consistent with the possibility that under these conditions one vesicle transiently disappeared for each quantum released.

To determine whether the missing vesicle membrane could have been totally redistributed into new membrane compartments in stimulated terminals, or whether some vesicle membrane had

completely disappeared, the amounts of membrane in each compartment were measured in the matched photographs. The methods and numerical results are presented in Table I and summarized graphically in Fig. 8. After these results were analyzed, all the terminals whose membrane distributions were well within 1 standard deviation of mean values for each compartment were collected, and those thought to be representative were traced to make the pictorial summary of the measurements in Fig. 9.

The first step in these measurements was to calculate the amount of vesicle membrane that had disappeared on the basis of measurements of vesicle sizes and numbers in resting and stimulated terminals. A Zeiss particle analyzer was used to de-

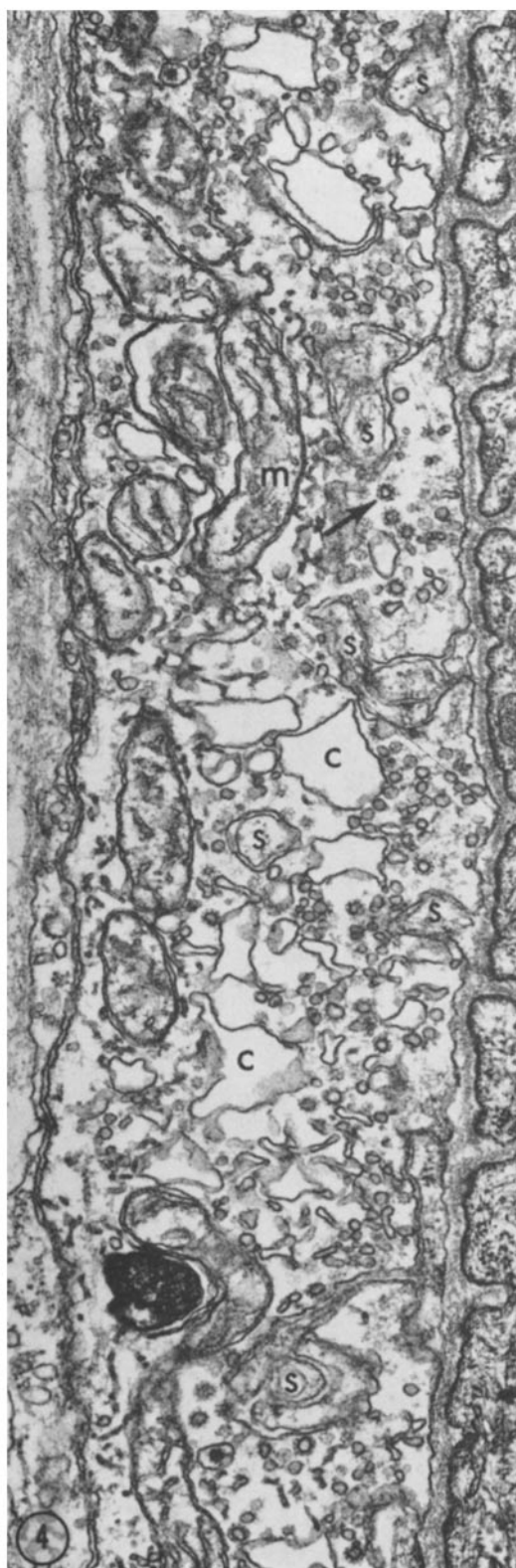


termine that the mean external diameter of synaptic vesicles in resting terminals was 52 nm ($n = 1097$) and in stimulated terminals was 54 nm ($n = 380$), which was significantly larger at the $P < 0.01$ level only. The size histograms presented in Fig. 7 illustrated that much of this increase resulted from the appearance in stimulated terminals of vesicles that were larger than normal; these perhaps should have been counted as small cisternae.

These measurements of external diameter were averaged with measurements of the vesicles' mean internal diameter (37 nm) to obtain a corrected value of 45 nm for vesicle diameter with respect to the center of its membrane. It seemed appropriate to use this corrected diameter to calculate the amount of membrane forming a vesicle; however, if the external diameter had been used instead in the calculations, Fig. 8 would have portrayed even larger vesicle membrane changes. As it was, Fig. 8 illustrates that about $3 \times 10^3 \mu\text{m}^2$ of membrane was used to form vesicles in an average resting end plate and $1.2 \times 10^8 \mu\text{m}^2$ formed vesicles in an average end plate stimulated for 15 min; thus more than half of the membrane which had initially formed vesicles was missing after stimulation.

The next step was to measure the amount of membrane appearing around cisternae by moving a calibrated map reader around the perimeter of the cisternae and calculating their surface area by multiplying the perimeter by the section thickness. The thickness was estimated to be 55 nm on the basis of silver-gray interference colors and this estimate was supported by finding that synaptic vesicles this size or larger occasionally appeared as faint smudges in both neighboring serial sections. Assuming that both control and stimulated end plates averaged 500 μm in length, we calculated that cisternal membrane increased more than sixfold during stimulation, from only $0.25 \times 10^3 \mu\text{m}^2$ in control end plates to $1.5 \times 10^8 \mu\text{m}^2$ in the stimulated end plates (Fig. 8). In addition, Fig. 8 illustrates that this increase in

FIGURE 3 Longitudinal section of an unstimulated terminal. The terminal contains a large number of synaptic vesicles on the side facing the muscle (right), and neurofilaments, dense mitochondria, and axonal endoplasmic reticulum (small arrows) on the side facing the Schwann sheath (left). Presynaptic densities (large arrow) occur opposite folds (f) in the muscle surface, and Schwann processes (s) periodically insinuate between the nerve and muscle to separate one presynaptic density from the next and define a series of "synaptic units." $\times 40,000$.



cisternal membrane was sufficient to account for over 80% of the missing synaptic vesicle membrane. Because there was no obvious alteration in the amount or distribution of the axonal endoplasmic reticulum in stimulated terminals, this membrane was not included in the above measurements.

Finally, the amount of membrane added to the surface of stimulated terminals was measured by moving the map reader around their circumference and multiplying this value by section thickness (Table I) and then by total end plate length (Fig. 8). The average amount of plasma membrane around a control end plate was $2.2 \times 10^3 \mu\text{m}^2$ and around a stimulated end plate was $2.7 \times 10^3 \mu\text{m}^2$ so the surface area of nerve terminals increased during stimulation by 20% ($P < 0.01$).² Fig. 8 illustrates that this increase in plasma membrane approximated the net decrease in internal membrane. Thus, our measurements are consistent with the conclusion that the total amount of visible membrane composing a terminal remained approximately constant during stimulation but they do not rule out a small change in total membrane. Thus, it appears that during 15 min of stimulation of this synapse, some vesicle membrane was incorporated into the plasma membrane and some into new cisternae, but none disappeared. Consistent with previous observations on permanganate-fixed frog synapses (81), no differences were found in the trilaminar structure or thickness of the membranes forming vesicles, cisternae, or

² To determine whether stimulated terminals had also increased in volume, their profiles were cut from the photographs and weighed, in order to measure their areas. Average end plate volume was $635 \mu\text{m}^3$ and did not increase after 15 min of stimulation. Thus, synaptic vesicle density fell from 840 vesicles μm^3 in normal terminals, similar to previous estimates of 1000 V μm^3 (7), to 330 V μm^3 in stimulated terminals. Since stimulated terminals had increased their surface without increasing their volume, they necessarily assumed a more irregular profile as illustrated in Fig. 9.

FIGURE 4 Longitudinal section of a terminal stimulated for 15 min. The terminal contains many cisternae (c) and coated vesicles (arrow) in place of the missing synaptic vesicles, appears less organized, and is distorted by enlarged Schwann processes (s). Coated vesicles (arrow) and filamentous material occasionally forming discrete baskets are particularly prominent around the Schwann processes. Mitochondria (m) are swollen and pale. $\times 30,000$.

plasmalemma that might preclude such membrane redistribution.

Appearance after 1 Min of Stimulation

To observe the onset of the membrane redistribution accompanying transmitter release, nine curarized muscles were stimulated at 10 Hz for only 1 min at 10°C or 20°C and immediately plunged into OsO₄ fixative. Simple inspection of longitudinal and cross sections of these muscles indicated that their nerve terminals already contained more cisternae than normal (Figs. 12 and 14). In addition, some cross sections of these nerve terminals appeared depleted of vesicles, but many cross sections did not appear depleted and it was necessary to count the vesicles in 75 randomly cross-sectioned terminals from one of these muscles to reveal that the overall number of vesicles was already reduced by more than 25% (Table I). This depletion of about 10⁶ vesicles per end plate was similar to rough estimates of the number of quanta released by 1 min of stimulation. It constituted a loss of about 1,000 μm^2 of vesicle membrane from an average end plate.

Statistical analysis of further measurements on these 75 terminals (Table I) confirmed that cisternae were twice as extensive as usual, and in addition revealed that coated vesicles were twice as numerous as usual (Fig. 15); unlike the situation found in terminals stimulated for 15 min, the 275 μm^2 increase in membrane forming these structures could not account for much of the missing vesicle membrane (Fig. 8). On the other hand, the statistical analysis also revealed that the plasma membrane of these terminals was increased nearly as much as after 15 min of stimulation (Figs. 8 and 9). This increase amounted to 425 μm^2 per end plate, which could account for much of the missing vesicle membrane.

Thus 25% of the membrane had rapidly disap-

peared from inside each end plate, while a similar amount of membrane rapidly appeared on the surface. Since the calculated amount of visible membrane in vesicles, cisternae, and plasmalemma remained nearly constant after 1 min of stimulation as well as after 15 min of stimulation (Fig. 8), the changes in relative amounts could be interpreted as a movement of intact membrane between the different configurations. An early increase in surface membrane at the expense of vesicle membrane, followed by a later increase in cisternal membrane, would be the result of vesicle membrane first joining the surface and then being retrieved as cisternae.

Cisternae in Stimulated Terminals

The morphology of stimulated terminals was considered in more detail to gather some clues as to how such a vesicle membrane might be retrieved from the surface membrane. After either 1 or 15 min of stimulation, the cisternae appeared to be distributed randomly within the areas usually occupied by synaptic vesicles (Figs. 4, 11, 12, 14, 16, 17), so they were presumed not to originate at any particular site near the surface of the terminal. Direct connections with the surface were sought in both single and short series of sections but none were found. In two experiments, terminals previously fixed in aldehyde were soaked in colloidal lanthanum chloride (1%, pH 7.4) which resulted in a coating of dark precipitate around some terminals but never labeling of cisternae. A third reason for concluding that cisternae are usually not connected with the surface membrane was produced by the experiments using HRP, discussed below, where it was found that cisternae did not release peroxidase accumulated as a result of stimulation, even when the peroxidase was washed out on the extracellular spaces around the terminal (Fig. 27).

FIGURE 5 Glancing longitudinal section of the presynaptic surface of an unstimulated terminal. The presynaptic densities are parallel bands lying in register with muscle folds (large arrows) and are bounded by rows of synaptic vesicles. Along parts of the dense bands, some synaptic vesicles are replaced by folds in the presynaptic membrane (small arrows at top). $\times 40,000$.

FIGURE 6 A similar glancing longitudinal section through the presynaptic surface of a terminal stimulated for 15 min. Large numbers of synaptic vesicles have been replaced by cisternae (c) and coated vesicles. The presynaptic surface is clearly distorted by the enlarged Schwann processes (s). The faint 20 nm periodicity outside the terminal (small arrows at right) is apparently a structural feature of the basal lamina which separates nerve and muscle at this synapse. $\times 40,000$.

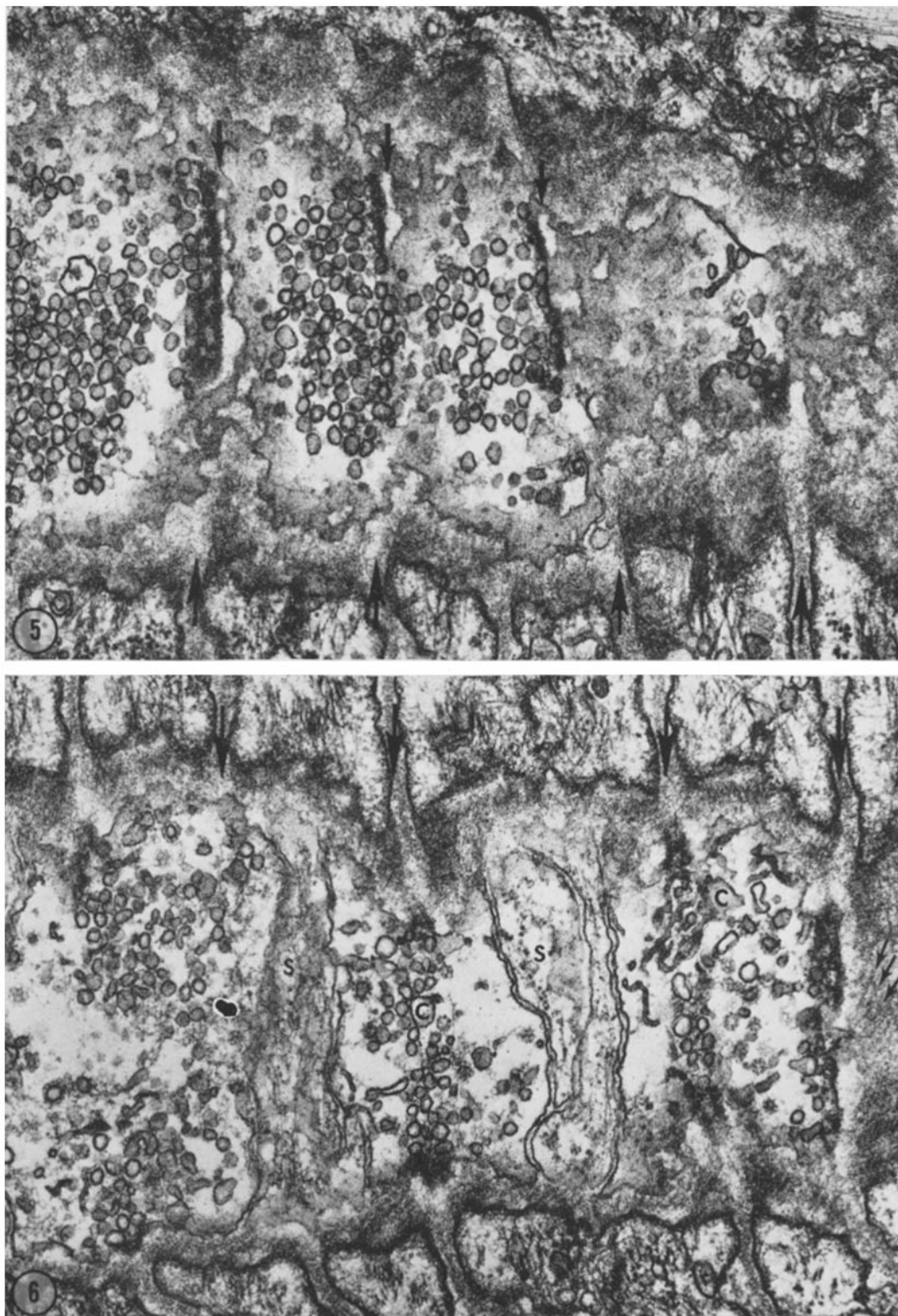


TABLE I
Effect of Stimulation on the Distribution of Membrane in Nerve Terminals

Type of membrane	Amount of membrane per cross section, in $\mu\text{m}^2 \times 10^{-3}$		
	At rest ($n = 100$)	Stimulated 1 min ($n = 75$)	Stimulated 15 min ($n = 100$)
Synaptic vesicle membrane (no. of vesicles per section)	$330 \pm 24^*$ (58.6)	$219 \pm 17^\ddagger$ (38.8)	$130 \pm 11^\ddagger$ (23.1)
Cisternal membrane	23 ± 4	$57 \pm 7^\ddagger$	$171 \pm 20^\ddagger$
Coated vesicle membrane (no. of vesicles per section)	2.5 ± 0.8 (0.31)	5.5 ± 0.8 (0.69)	8.0 ± 0.8 (1.0)
Plasma membrane	246 ± 10	$292 \pm 13^\ddagger$	$302 \pm 11^\ddagger$
Total membrane	$600 \pm 32^\S$	$567 \pm 34 \text{ ns}$	$608 \pm 30 \text{ ns}$

n , number of cross sections measured.

* Mean \pm standard error of the mean.

‡ Significantly different from at rest.

§ Standard error derived by calculating total membrane for each cross section separately.

ns , not significantly different from at rest.

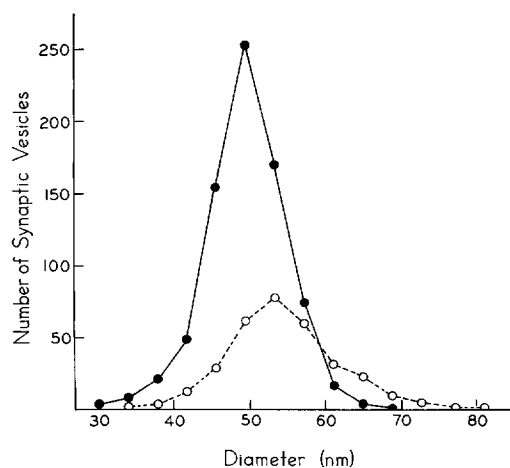


FIGURE 7 Outside diameters of synaptic vesicles from eight sections of large resting endings (closed circles) and eight sections of large endings stimulated for 15 min (open circles), both fixed in osmium. During stimulation the total number of vesicles fell to less than half the total number at rest. At the same time, the number of larger vesicles increased. To insure that the measured diameter was close to the true diameter of each vesicle, only those vesicles with sharply defined membranes around at least half of their perimeters were measured. This was effective because serially sectioned vesicles which met this criterion in two adjacent sections had the same apparent diameter in both sections.

Cisternae were often connected to one or more coated vesicles and even when not joined to a coated vesicle were usually partially covered with a filamentous material which appeared similar to that around coated vesicles. Cisternae were coated most frequently in terminals stimulated for 1 min (Figs. 12, 14, 24), but it was still common in terminals stimulated for 15 min (Figs. 4, 11). In addition, coated vesicles were often connected to the plasma membrane in regions of the nerve terminal covered by a Schwann process, especially just at the edge of the synaptic cleft (Figs. 4 and 14). In these regions, resting nerve terminals contained a filamentous material forming what appeared to be empty baskets (Fig. 3), while stimulated terminals contained increased numbers of coated vesicles and coated pits (Figs. 4, 11, 15). This association of coated vesicles with the plasma membrane and with cisternae raised the possibility that membrane was retrieved from an enlarged surface by budding of coated vesicles which subsequently coalesced to form the cisternae. In this case, the occasional appearance of two coated vesicles attached together (Figs. 22, 23) could represent the earliest stage of formation of cisternae.

Factors Affecting the Appearance after Stimulation

To determine whether stimulation alone, without transmitter release, would produce any of the

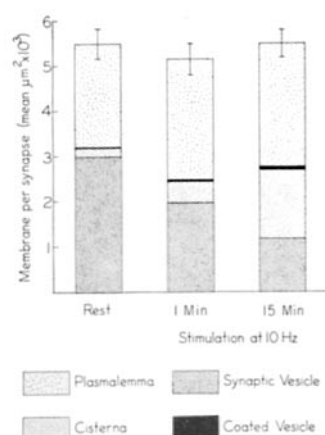


FIGURE 8 Amounts of membrane forming synaptic vesicles, coated vesicles, cisternae, and plasmalemma in an average frog neuromuscular junction at rest, 1 min of stimulation, and 15 min of stimulation. The total amount of membrane forming an average end plate did not change appreciably during stimulation, even though the relative amounts of membrane forming different components of the end plate changed dramatically. As discussed in the text, this suggests that synaptic vesicle membrane remains visible and simply becomes redistributed during stimulation. The values for average whole end plates were derived from the measurements on individual sections presented in Table I, by multiplying the values in Table I by 9,090, the number of 55-nm sections in an end plate 500 nm long. Ranges on tops of bars are standard errors of the total membrane area which was computed separately for each section of a terminal.

changes in distribution of membrane described above, three muscles were soaked in Ringer with 6 mM Mg^{++} replacing Ca^{++} before and during 15 min of 10 Hz stimulation. This treatment greatly reduced evoked transmitter release, but as others have found, did not entirely abolish it (40, 57). 50 random micrographs of terminals in these muscles showed no differences from the resting appearance, so nerve stimulation alone was not adequate to produce the changes in distribution of membrane.

To determine whether cooling to 10°C had artifactually exaggerated these changes, six other muscles were stimulated for 15 min at room temperature. All terminals examined in these muscles contained many cisternae and all contained fewer vesicles, but comparative counts have not been done to determine whether the vesicle depletion was as severe as at 10°C, or whether new vesicles were

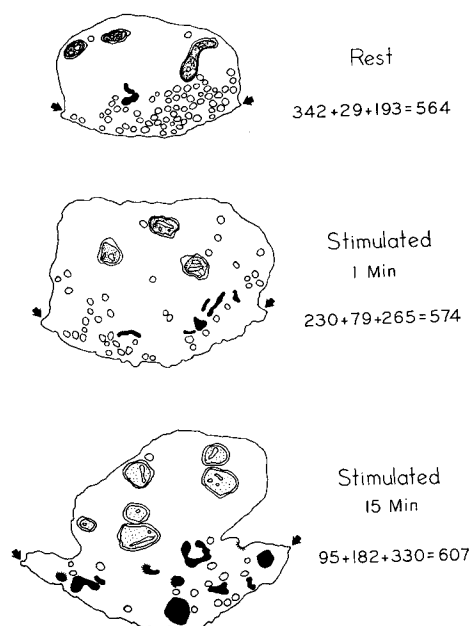


FIGURE 9 Tracings of representative nerve terminals at rest, after 1 min of stimulation, and after 15 min of stimulation. They illustrate a progressive depletion of synaptic vesicles (open circles), appearance of cisternae (filled in black), and increase in surface area. The numbers indicate the area per section of synaptic vesicle membrane + cisternal membrane + surface membrane = total membrane. An ending from each experimental group was selected in which the area of each type of membrane was close to the average value for that group given in Table I. The edge of the synaptic cleft lying below each terminal is indicated by the pairs of arrows. $\times 35,000$.

formed more rapidly during the stimulation at higher temperature.

To determine whether availability of transmitter or precursors controlled the appearance of these changes, three muscles were stimulated at 1 Hz for 1 h in 10^{-5}M hemicholinium-3 to exhaust intracellular stores of both (73), before receiving the usual 15 min stimulation at 10 Hz. In distinct contrast to what has been reported for rat motor nerve terminals (43), this treatment did not obviously exaggerate or otherwise alter the membrane changes. Also, two muscles were presoaked in the anticholinesterase drug neostigmine methyl sulfate 10^{-6} g/ml for 1 h before stimulation, to prevent breakdown of released transmitter and to deprive these endings of much of their choline

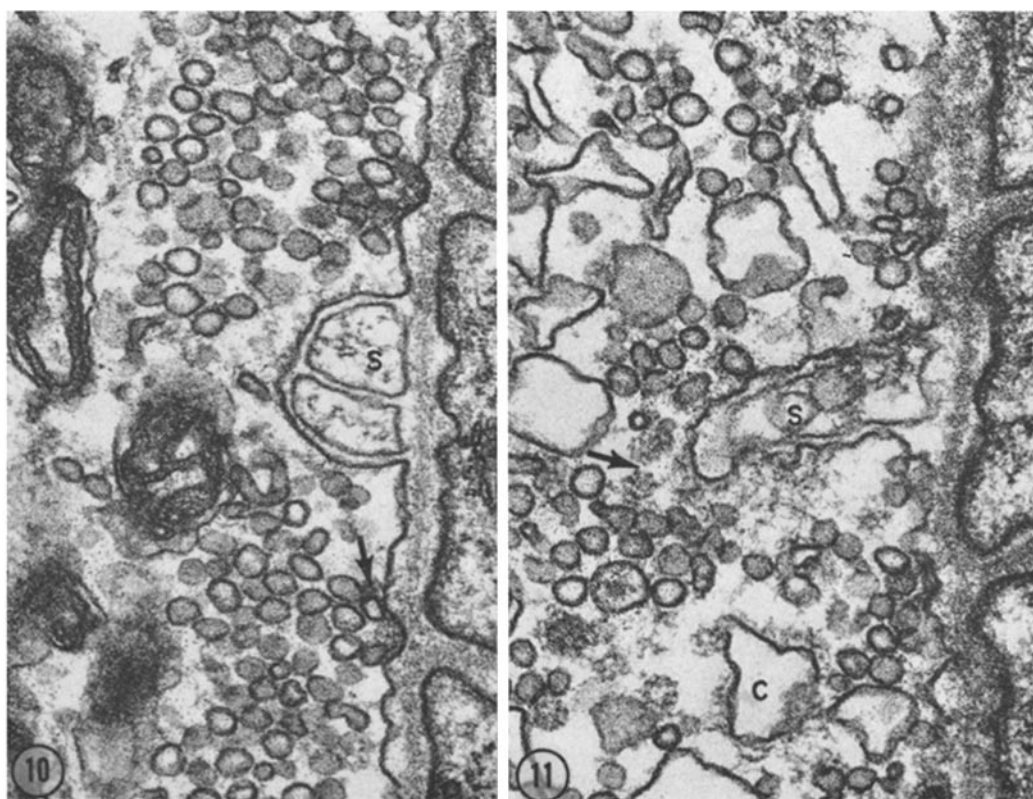


FIGURE 10 Higher magnification of the longitudinal section through an unstimulated nerve terminal shown in Fig. 3. The plasma membrane of the terminal is slightly pitted, approached by synaptic vesicles adjacent to the presynaptic densities (arrow), and underlain by a faint filamentous material where the Schwann process (*s*) intervenes. $\times 70,000$.

FIGURE 11 Higher magnification of a longitudinal section through a terminal stimulated for 15 min. The plasma membrane is more pitted and many synaptic vesicles are replaced by cisternae (*c*). Filamentous material, either faint wisps or discrete empty baskets (arrow), surrounds some cisternae and is associated with coated pits and coated vesicles in various stages of formation along the surface where the Schwann process (*s*) invaginates. $\times 70,000$.

supply (68), but again the structural changes were not obviously exaggerated. Conversely, three muscles were presoaked for 1 h in 1 mM choline in an attempt to augment intracellular supplies of transmitter substrates during release. This did not prevent the appearance of these structural changes.

Appearance during Recovery

To determine the reversibility of these structural changes, six muscles were rested at 10°C for 15, 30, or 60 min after 15 min of stimulation at 10 Hz and then immersed in osmium fixative. During this rest period the number of cisternae progress-

ively declined and the number of synaptic vesicles partially recovered (Figs. 16–19), suggesting that cisternae were dividing to form new synaptic vesicles. Nevertheless, such a conversion was not clearly visualized; only occasionally were cisternae varicose or directly adjacent to rows of vesicles (Fig. 17). However, varicose cisternae are quite common after prolonged stimulation with lanthanum ion (37). During recovery cisternae were rarely associated with coated vesicles, in contrast to earlier formative periods described above.

Monitoring averaged or individual e.p.p.'s during the rest period at 10°C revealed that most



FIGURE 12 Cross section of a large terminal stimulated for 1 min. Synaptic vesicles are not obviously depleted, mitochondria are slightly expanded, and a cisterna has formed. However, even at this early stage the cisterna lies far away from the plasma membrane and appears to have formed indirectly by the coalescence of coated vesicles (*inset*). $\times 50,000$; *inset* $\times 100,000$.

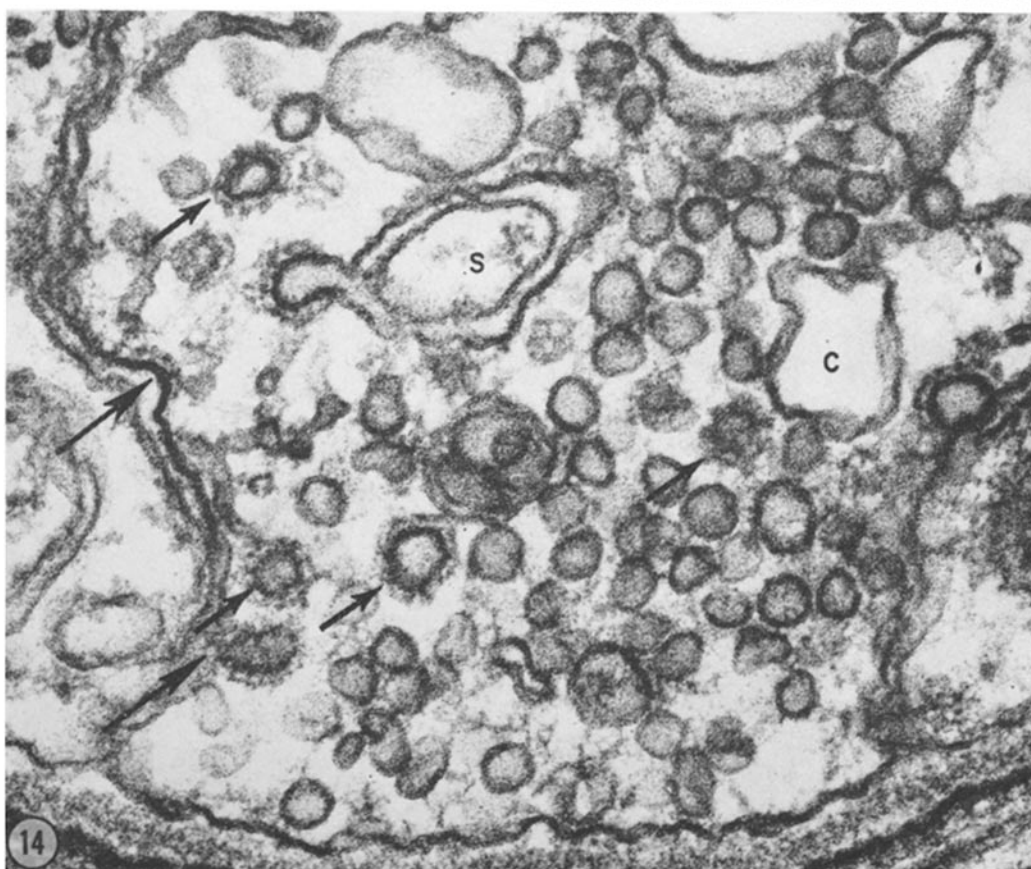
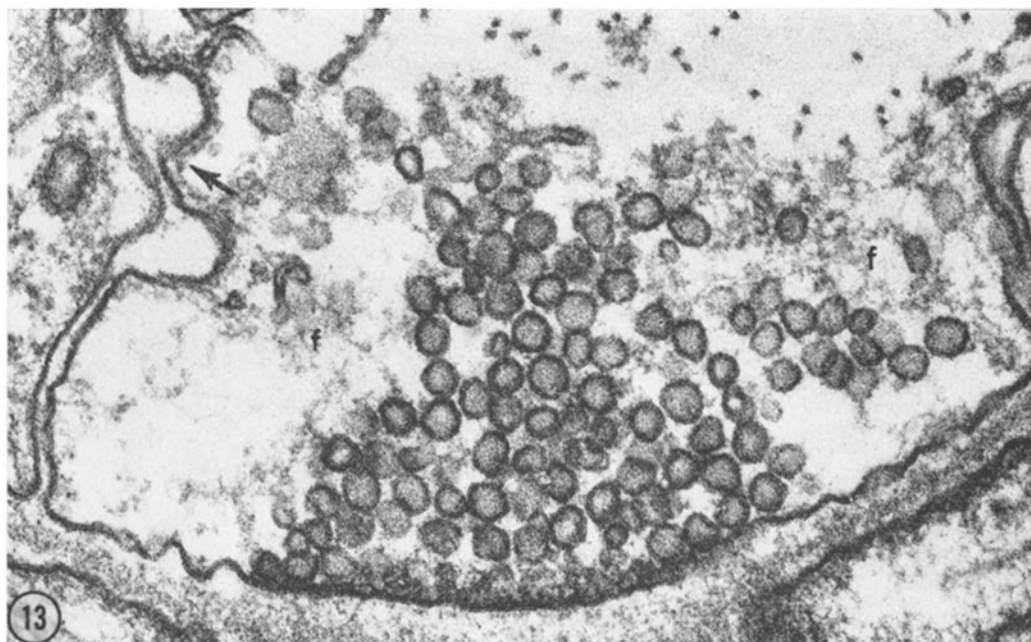


FIGURE 13 Cross section of an unstimulated terminal. A filamentous material (*f*) extends from the cluster of synaptic vesicles to the lateral edges of the terminal covered by Schwann processes, where it appears to participate in forming organized coats (arrow) on the plasma membrane. $\times 90,000$.

FIGURE 14 Cross section of a terminal stimulated for 1 min. At this time most of the filamentous material is found around coated vesicles (small arrows), which tracer uptake studies (Fig. 21) suggest form along the lateral edges of the terminal (large arrows) and coalesce to form cisternae (*c*). A Schwann process (*s*) invaginates into this terminal. $\times 110,000$.

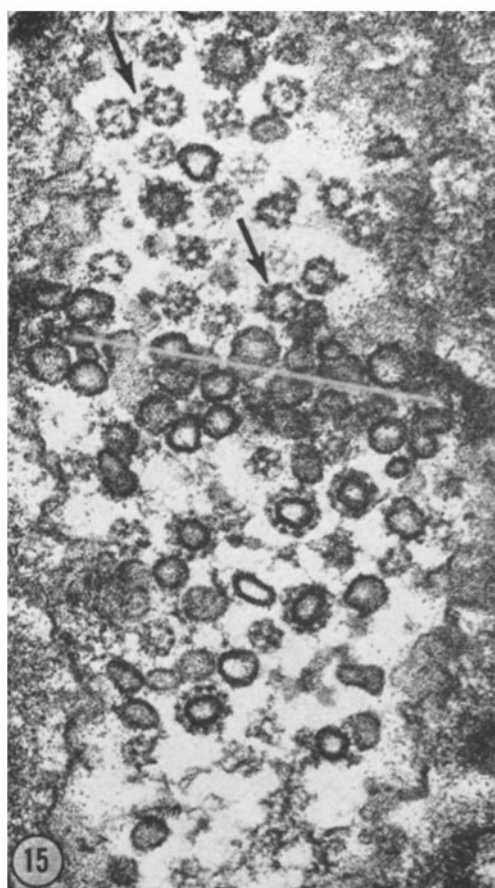


FIGURE 15 Glancing longitudinal section through a terminal stimulated for 1 min. Adjacent to the pre-synaptic dense band and its overlying cluster of synaptic vesicles (bisected by a white line) are several coated vesicles and many empty coats or baskets (arrows). $\times 80,000$.

terminals required only a few minutes to recover their ability to release full amounts of transmitter in response to infrequent test stimuli, but required an hour to recover their ability to sustain normal levels of transmitter release during tetani. Even then, some cisternae persisted and synaptic vesicles appeared more pleomorphic than usual (Fig. 18). During recovery mitochondria progressively shrank to normal size and density and returned to their normal position away from the presynaptic surface (Figs. 16–19).

Temperature had a definite effect on the rate of reversal of structural changes. Three muscles warmed to room temperature for the 1 h rest

period showed virtually complete recovery of vesicle numbers and disappearance of cisternae (Fig. 19), while four muscles cooled to 0°C for the 1 h rest period showed no apparent recovery of vesicle numbers and still contained many cisternae.

In contrast, the availability of transmitter or precursors had no apparent effect on the rate of recovery. Two muscles, which were pretreated with hemicholinium-3 until intracellular stores were presumed exhausted and then stimulated, still showed complete recovery of vesicle numbers after a 1 h rest at room temperature.

During the recovery period, narrow invaginations of the plasma membrane capped by coated vesicles and containing Schwann processes became especially prominent along the lateral edges of the terminals (Fig. 18). These invaginations began to appear during the stimulation (Figs. 4, 14) and reached their maximum extent 1 h after stimulation at 10°C . They may be distortions of the terminals which develop as their surface area increases (Figs. 8, 9) but their constant association with coated vesicles inside and Schwann processes outside the terminal (Fig. 4) may indicate an additional specific role in membrane retrieval. It is important to note here that these invaginations are not to be mistaken for cisternae connected to the surface because the invaginations always contain a Schwann process.

Uptake and Discharge of Horseradish Peroxidase

To determine the spatial and temporal connections among the internal compartments and the surface of stimulated nerve terminals, the movement of HRP through these compartments was studied in some detail. This tracer does not cross intact membranes (24), so its movement from one membrane-walled compartment to another indicates that the compartments were connected at some time during the experiment. However, HRP generally does not adhere to membranes and is free to diffuse along a concentration gradient (11), so its movement does not necessarily indicate that membrane moved from one compartment to another. Only when HRP moves in one direction through a series of compartments does it indicate that membrane is also moving along to form dynamic compartments that are sequestering and transporting it.

SOAK: HRP permeated this muscle rapidly, so a 30 min soak in HRP-Ringer completely surrounded most surface nerve terminals with the tracer (Fig. 20). In this and all subsequent tracer experiments the muscles were maintained for convenience at room temperature rather than 10°C. This soak alone resulted in very little entry of HRP into the terminals. Its occasional entry into coated vesicles (Fig. 20), which has been reported previously (82), could be interpreted in terms of the findings described below to result from a slow turnover of synaptic vesicles accompanying spontaneous transmitter release.

SOAK PLUS BRIEF STIMULATION: 12 muscles soaked 30 min in HRP-Ringer were stimulated at 10 Hz for 1 min and immediately fixed. The results were striking and consistent; in 174 randomly sectioned terminals, every new cisternae contained HRP (Fig. 21). Also, nearly all the coated vesicles proliferating near the surfaces covered by Schwann processes contained HRP (Figs. 21, 23). These coated vesicles may have carried HRP to the cisternae, since HRP-containing cisternae never appeared directly connected to the surface but often appeared connected to one or more HRP-containing coated vesicles (Fig. 24).

It was important to determine whether coated vesicles were the exclusive path of HRP uptake, or whether some ordinary synaptic vesicles were also involved. This was difficult because coats were often poorly visualized after the histochemical incubation. In the best preparations, half of the HRP-containing vesicles were clearly coated, while most of the remaining half were indistinctly coated and were located nearer to a Schwann process than to the synaptic cleft which is characteristic of coated vesicles in this synapse (Figs. 4, 14).

Of all the HRP-containing vesicles observed, only about 10% (41 out of 379) were clearly not coated and lay nearer to the synaptic cleft, where transmitter release presumably occurred, than to the Schwann process. These HRP-containing vesicles constituted a tiny minority of the total number of synaptic vesicles lying near the synaptic cleft (Fig. 21), and they could be interpreted in terms of the findings described below to be new synaptic vesicles that had already re-formed. Thus it seemed unlikely that ordinary, uncoated synaptic vesicles took up HRP directly from the pre-synaptic surface, unless they transported it so rapidly to cisternae that very few were fixed in transit.

SOAK PLUS LONG STIMULATION: 10 muscles soaked 30 min in HRP-Ringer were stimulated for 2–15 min. As the period of stimulation was lengthened, cisternae became progressively more numerous; still all contained HRP (Figs. 25, 26). This suggested that cisternae were formed exclusively from HRP-containing vesicles, since if they were formed also from empty synaptic vesicles, a proportion of them should have initially contained no HRP. At the end of 15 min of stimulation, some synaptic vesicles contained HRP (Fig. 26).

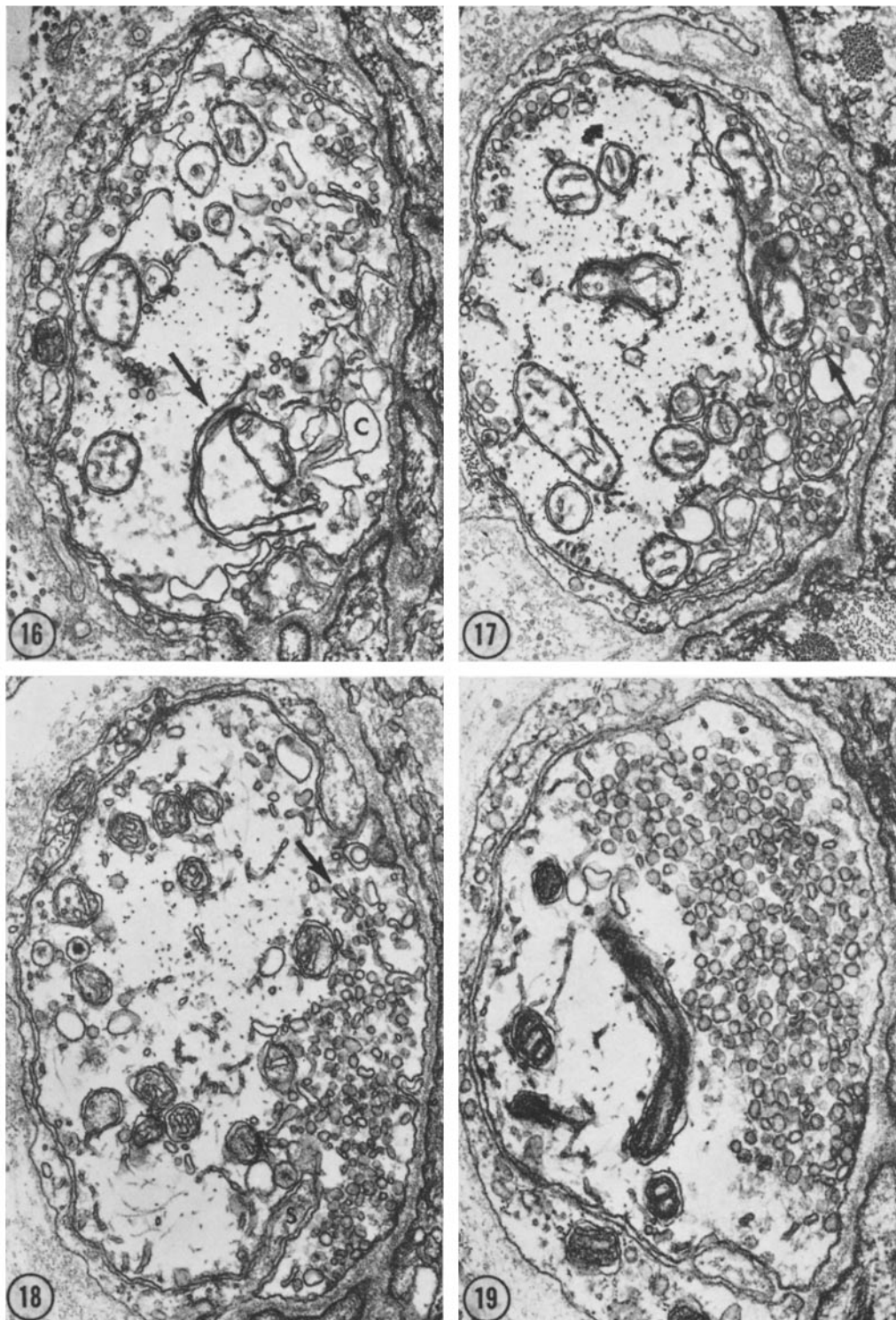
LOAD: SOAK, PLUS LONG STIMULATION, PLUS REST: 10 muscles, soaked in HRP-Ringer and stimulated for 15 min to load their cisternae with HRP, were then rested for 1 h. During the latter half of the rest period the muscles were washed in normal Ringer, which removed most of the discernible HRP from the extracellular spaces but none from cisternae inside the terminals (Fig. 27). This finding was additional evidence that the cisternae were not open directly to the surface (see the section on cisternae in stimulated

FIGURE 16 Cross section of a terminal stimulated for 15 min and fixed immediately. Cisternae, either vacuolar (c) or flattened (arrow), replace a large proportion of the synaptic vesicles. $\times 30,000$.

FIGURE 17 Cross section of a terminal stimulated for 15 min and then rested for 30 min at 10°C. The rest has resulted in partial recovery of synaptic vesicle numbers and disappearance of cisternae. Some cisternae may be dividing into vesicles (arrow). Mitochondria remain swollen. $\times 30,000$.

FIGURE 18 Cross section of a terminal stimulated for 15 min and then rested for 60 min at 10°C before fixation. Synaptic vesicle numbers have nearly recovered and cisternae (arrow) have been reduced to short lengths. Mitochondria have begun to return to normal size and density. $\times 30,000$.

FIGURE 19 Cross section of a terminal stimulated for 15 min and then rested for 60 min at room temperature to demonstrate the complete reversibility of the changes occurring during stimulation. Synaptic vesicles have recovered, cisternae have disappeared, and mitochondria have returned to normal size and density. Osmium. $\times 35,000$.



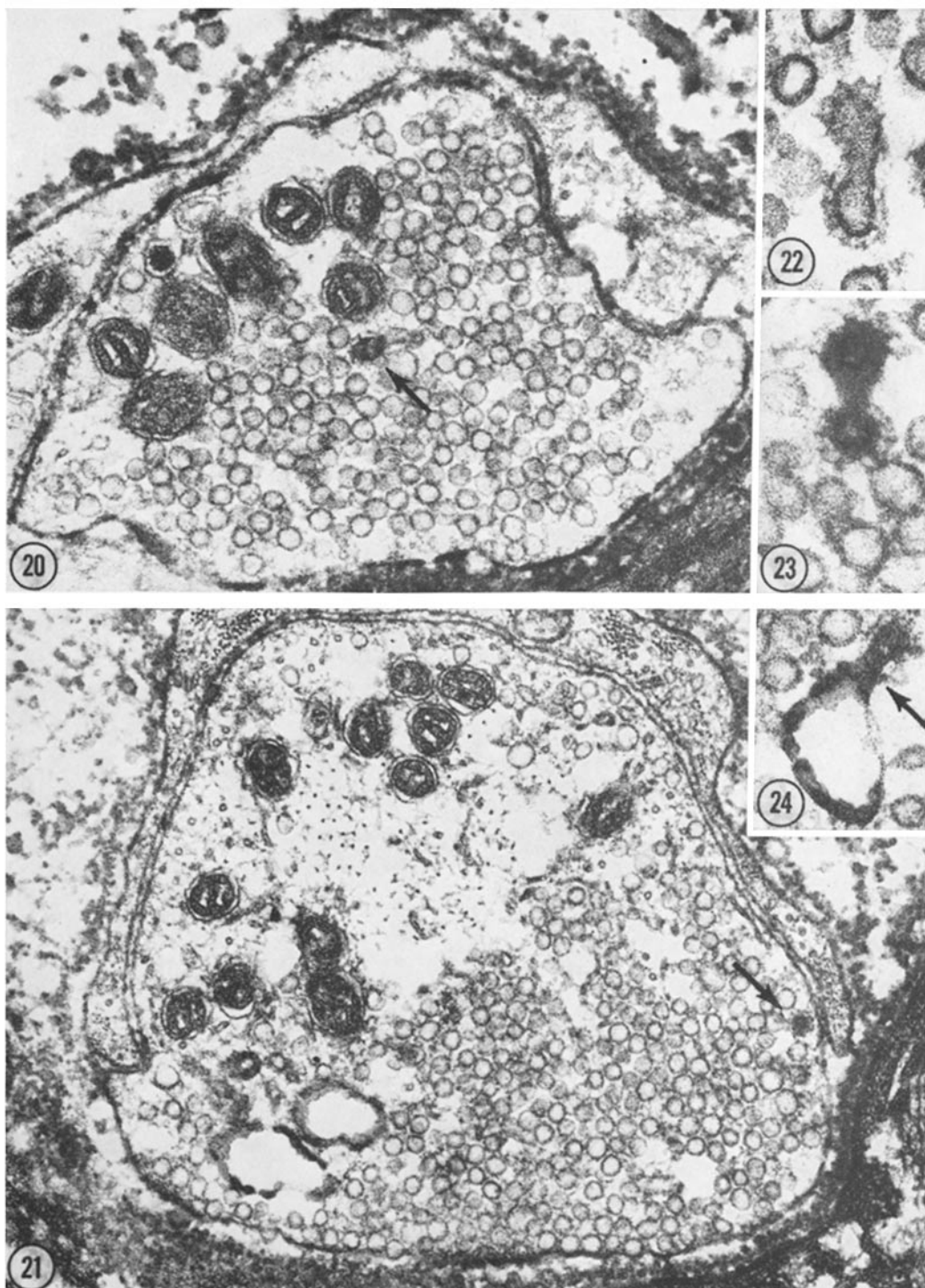


FIGURE 20 Cross section of a terminal soaked in HRP for 30 min. Tracer has surrounded the terminal and entered one coated vesicle (arrow). $\times 60,000$.

FIGURE 21 Cross section of a terminal soaked in HRP for 30 min and stimulated for 1 min. Tracer has entered the newly formed cisternae and the one coated vesicle present along the lateral edge (arrow), but but is excluded from synaptic vesicles lying near the synaptic cleft. $\times 45,000$.

FIGURES 22-24 Higher magnifications of coated vesicles which appear to be fusing with each other (Figs. 22 and 23) or with a cisterna (Fig. 24). Fig. 22 is from an experiment employing osmium fixation but Figs. 23 and 24 are from experiments where terminals were stimulated in peroxidase. Fig. 22, $\times 105,000$; Fig. 23, $\times 110,000$; Fig. 24, $\times 100,000$.

terminals). The rest period resulted in the usual decline in the number of cisternae, though some persisted at the end of the hour (Fig. 27), and it resulted in recovery of the usual number of synaptic vesicles, a large proportion of which now contained HRP (Figs. 27–30). Some images of the remaining HRP-containing cisternae were consistent with the possibility that they divided to form HRP-containing vesicles (Fig. 27), but this was not clearly visualized. In fully recovered regions, more than 50% of the synaptic vesicles contained HRP (Fig. 30), which corresponded to the number of vesicles which could have been depleted by the stimulation and re-formed from HRP-containing cisternae (Fig. 8). The loaded vesicles were evenly distributed among the synaptic vesicles free in the terminal (Figs. 28, 32) and among the vesicles in contact with the inside surface of the plasma membrane (Fig. 29).

LOAD PLUS BRIEF STIMULATION: Six muscles loaded as detailed above were again stimulated for 1 min at 10 Hz, after most of the HRP had been washed from the extracellular spaces. Averaged and individual e.p.p. monitors revealed no gross abnormality in evoked transmitter release in these muscles. As expected, terminals in these muscles contained a new population of scattered cisternae and coated vesicles, and it was of great interest that most of these compartments did not contain HRP (Fig. 33). The rare HRP-containing cisterna which was found could be interpreted as one persisting from the first stimulation as in Fig. 27. This suggested that cisternae were not formed by nonspecific coalescence of synaptic vesicles, since at this point most vesicles contained HRP and their coalescence would have resulted in the appearance of HRP in the new cisternae. It also meant that the tracer movements described so far were not simply the result of diffusion along a concentration gradient between the extracellular space and various intermittently connected internal compartments. If such two-way movements were occurring, then reversing the concentration gradient, by removing HRP from the extracellular space while it remained in internal compartments, would have resulted in movement of tracer during the second stimulation from vesicles into cisternae and coated vesicles. This did not occur. Instead, the tracer behaved as a pulse and was chased during the second stimulation by tracer-free extracellular fluid included in the newly formed compartments (Fig. 33). This strongly suggested that these new com-

partments were formed by a one-way inward movement of surface membrane, that sequestered and transported an extracellular tracer in the process, so that the same membrane was first a coated vesicle, then part of a cisterna, and finally a synaptic vesicle.

LOAD PLUS LONG STIMULATION: Three muscles were stimulated for 15 min in normal Ringer after they were loaded. Terminals from these muscles contained many cisternae, nearly all of which were devoid of HRP, and reduced numbers of synaptic vesicles (Fig. 34). In addition, the proportion of loaded to nonloaded vesicles appeared to decline.

LOAD PLUS LONG STIMULATION PLUS REST: Seven muscles that had been loaded, washed free of HRP, and stimulated a second time at 10 Hz for 15 min as detailed above, were then rested again for 1 h to allow vesicle numbers to return toward normal. Only a few synaptic vesicles still contained HRP after this treatment (Fig. 35). Paired muscles, identically loaded but simply washed in normal Ringer while the others were stimulated and rested a second time, still had a large proportion of HRP-containing vesicles. Therefore the second stimulation had caused the disappearance of HRP from vesicles.

Since the HRP had clearly not exited backwards through cisternae and coated vesicles, the question remained where it had gone. During the first 1–3 h after entry of HRP into the terminals, we found no sign of sequestration or digestion of HRP-containing vesicles (Figs. 27–30). However, in the light of reports that HRP can be carried back to the motor neuron perikaryon (50, 52), it was important to determine whether the loaded synaptic vesicles moved out of the terminals and up the axon during stimulation. In two loaded muscles the nerve was tied within 5 mm of the terminals and massive transmitter release produced by soaking the muscles in 1 mM La^{+++} . All HRP-containing vesicles disappeared from terminals in these muscles without any sign of HRP-containing vesicles accumulating in the preterminal axons below the ties. Thus on the basis of exclusion, we presume that stimulation produced release of HRP from vesicles by exocytosis. In spite of an intensive search we have failed to visualize such a process (Figs. 29–32), presumably because the few HRP molecules from inside the vesicle are immediately diluted in the relatively huge volume of the synaptic cleft.

It should be added that many of the tracer

experiments were unsuccessful and some deleterious effects of the HRP-Ringer were uncovered. All these effects appeared to be minimized by using the purest HRP available and dialyzing it against a large volume of normal Ringer before use. If the initial HRP soak was prolonged more than 1 h, many terminals failed to form new membrane compartments or take up any HRP during stimulation; instead they developed multiple accumulations of membrane along their surfaces. The terminal in Fig. 30 is typical of many that contained a mixture of normal regions and damaged regions (Fig. 31). In addition, after terminals had been stimulated to take up HRP, many continued to display persistent or intermittent bursts of high frequency miniature e.p.p.'s (Fig. 32). These terminals initially confounded the study of the release of HRP, because they would empty themselves during the normal Ringer wash before they could be stimulated a second time. Finally, during the second stimulation some terminals released only small amounts of transmitter and only partially developed the expected membrane changes, while others failed completely to develop the membrane changes or release HRP, indicating that they had stopped conducting the nerve impulse. These failures prompted the preparation of 210 muscles before it was possible to consistently reproduce the results described above.

DISCUSSION

The present study confirms that during transmitter release the membrane-walled compartments inside frog motor nerve terminals undergo morphological changes and intermittently connect with the surface (34). Furthermore, the present study has ordered the morphological changes and

uptake of extracellular tracer into a temporal sequence which suggests that during transmitter release synaptic vesicle membrane remains visible and physically moves along a definable path that permits it to be reused several times (5). The hypothetical path of vesicle membrane recycling is illustrated in Fig. 36: synaptic vesicles discharge their content of transmitter by coalescing with the plasma membrane at specific regions adjacent to the muscle; then equal amounts of membrane are retrieved by coated vesicles arising from regions of the plasma membrane adjacent to the Schwann sheath; and then, the coated vesicles lose their coats and many coalesce to form cisternae which accumulate in regions of vesicle depletion and slowly divide to form new synaptic vesicles. The existence of such membrane recycling cannot be proven until it becomes possible to directly visualize or label individual membrane components (41), but it serves as a helpful working hypothesis to relate our observations (in italics in the following paragraphs) with other similar observations on resting and stimulated synapses.

Transmitter Release via Exocytosis

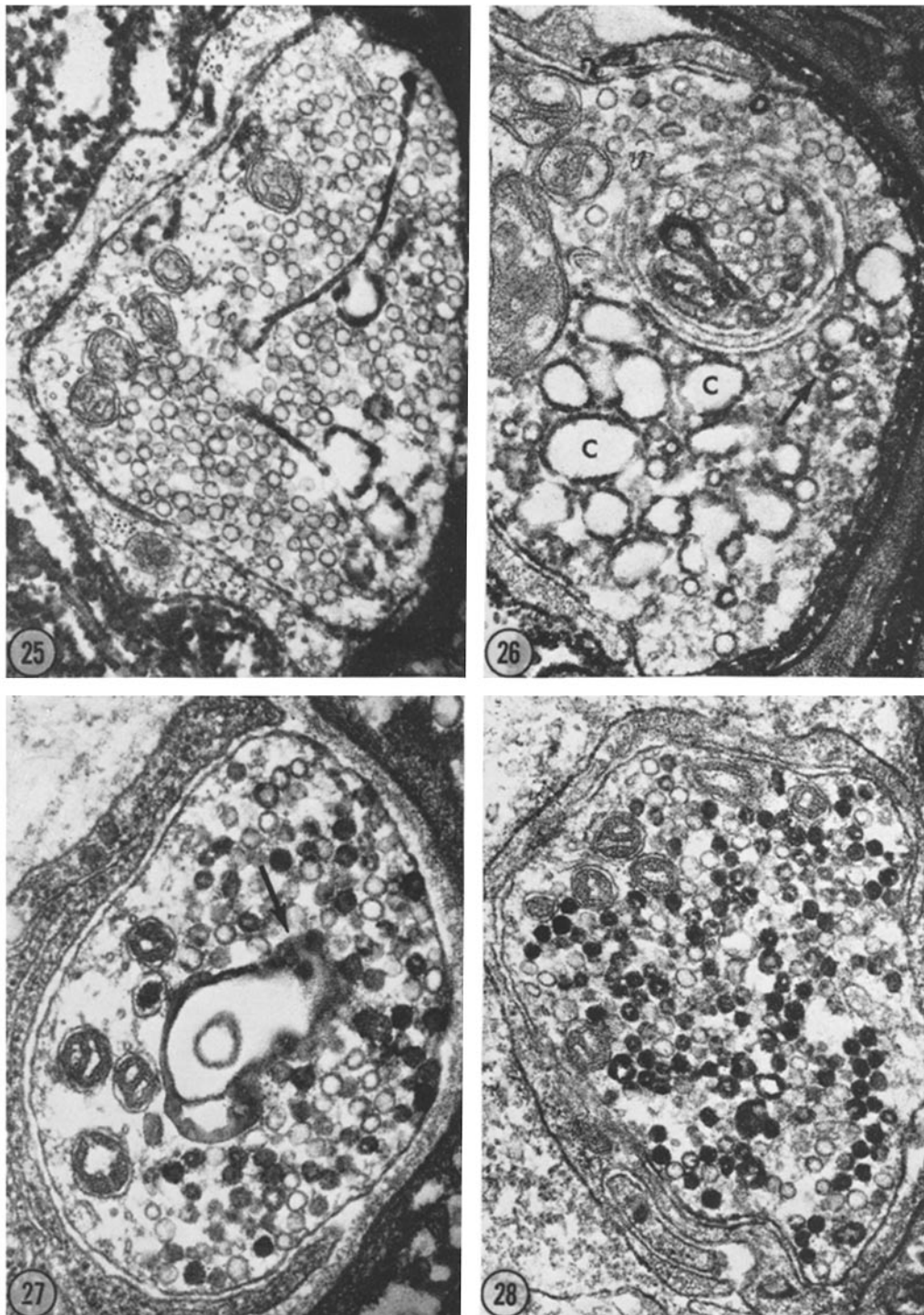
Enlargement and distortion of the terminal plasma membrane as synaptic vesicle membrane disappears would indicate that synaptic vesicle membrane is incorporated into the plasma membrane when vesicles burst onto the surface to discharge transmitter. Such expansions of the plasma membrane or reduction in vesicle numbers have been observed and similarly interpreted in other studies of synaptic changes during transmitter release (4, 12, 13, 38, 43, 66, 69). Such an expansion of the plasma membrane has also been seen in other

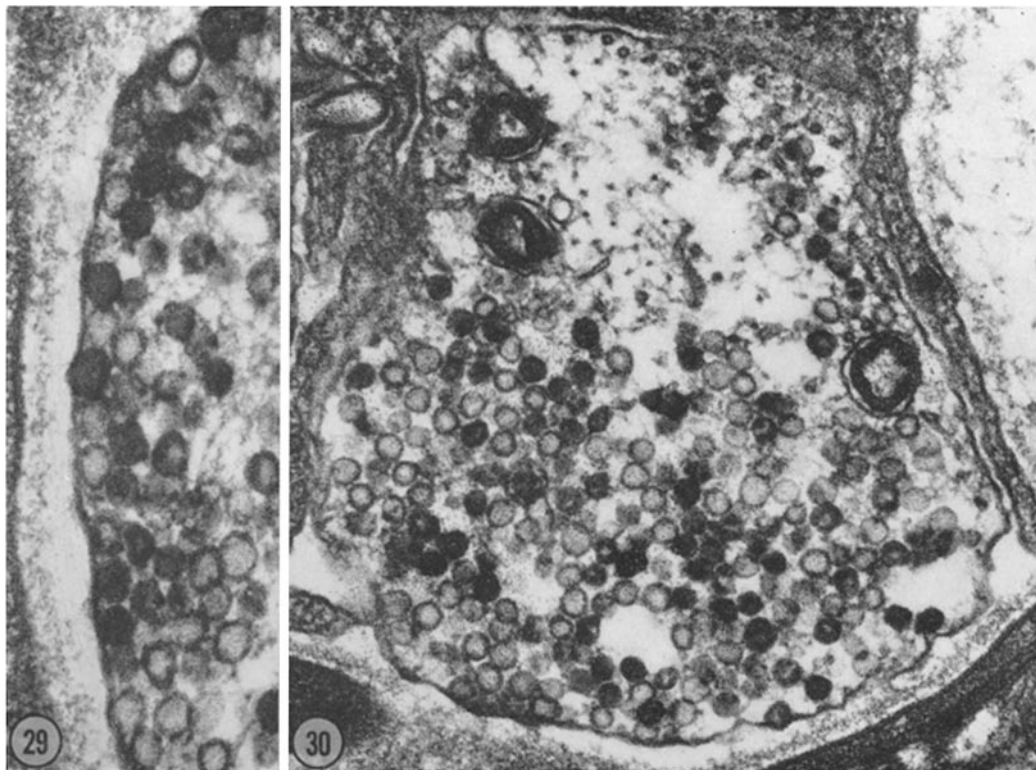
FIGURE 25 Cross section of a terminal soaked in HRP and stimulated for 2 min. Cisternae have become more numerous and all contain the tracer. However, no synaptic vesicles near the cleft (right) contain tracer which is evidence that they do not carry tracer to cisternae. $\times 40,000$.

FIGURE 26 Cross section of a terminal soaked in HRP and stimulated for 15 min. Rounded cisternae (c) with HRP coating their inner surfaces largely replace synaptic vesicles, and some synaptic vesicles (arrow) now contain HRP as well. $\times 45,000$.

FIGURE 27 Cross section of a "loaded" terminal (soaked in HRP, stimulated in HRP for 15 min, and rested for 1 h while HRP was washed from the extracellular spaces). A large number of HRP-containing synaptic vesicles have appeared, presumably by division of HRP-containing cisternae (arrow). $\times 50,000$.

FIGURE 28 Cross section of a terminal loaded twice in HRP and allowed to rest for 1 h. More than 70% of the synaptic vesicles contain HRP. $\times 45,000$.





FIGURES 29 and 30 Cross sections through normal regions of loaded terminals. Nearly 50% of the vesicles contain HRP, which corresponds to the number which should have been depleted by the stimulation and reformed from HRP-containing cisternae. Fig. 30 illustrates that the HRP-containing vesicles appear to be distributed randomly within the nerve terminal, including the area near the presynaptic surface, shown in more detail in Fig. 29 to illustrate that HRP-containing vesicles in contact with the plasma membrane do not discharge HRP, even though the tracer has been washed out of the synaptic cleft. Fig. 29, $\times 85,000$; Fig. 30, $\times 33,000$.

secretory cells, where it is possible actually to visualize that this results from exocytosis, or coalescence of vesicles with the plasma membrane to release their contents (1, 29, 41, 58, 59, 61). Furthermore, the disappearance of HRP from synaptic vesicles coincident with transmitter release would also indicate that exocytosis is occurring; that is, vesicles are opening wide enough to discharge their entire soluble contents. Such coincident release of large proteins and transmitter has been found to occur at other synapses (42, 76) and neurosecretory cells (48, 79), and is considered to be good evidence for exocytosis (75).

Membrane Recovery via Coated Vesicles

The apparent failure of HRP to enter synaptic vesicles near the synaptic cleft during the early phases of stimula-

tion would indicate that synaptic vesicles are not directly retrieved after exocytosis. The alternative that vesicles are directly recovered but fail to take up HRP in the process, has been rendered unlikely by the finding that they apparently can discharge HRP during stimulation. The uptake of HRP by coated vesicles proliferating near the Schwann coat during stimulation would seem to be sufficiently rapid and extensive to represent the major route for retrieving membrane from the surface.³ This

³ The density of empty coats or baskets along the Schwann-covered surface of resting terminals often reaches $50/\mu\text{m}^2$ (Fig. 15), from which it may be calculated that a resting end plate could contain at least 10^4 free coats in all. Consistent with this estimate is the presence of nearly 10^4 coated vesicles in terminals stimulated for 15 min (Table I and Fig. 8),

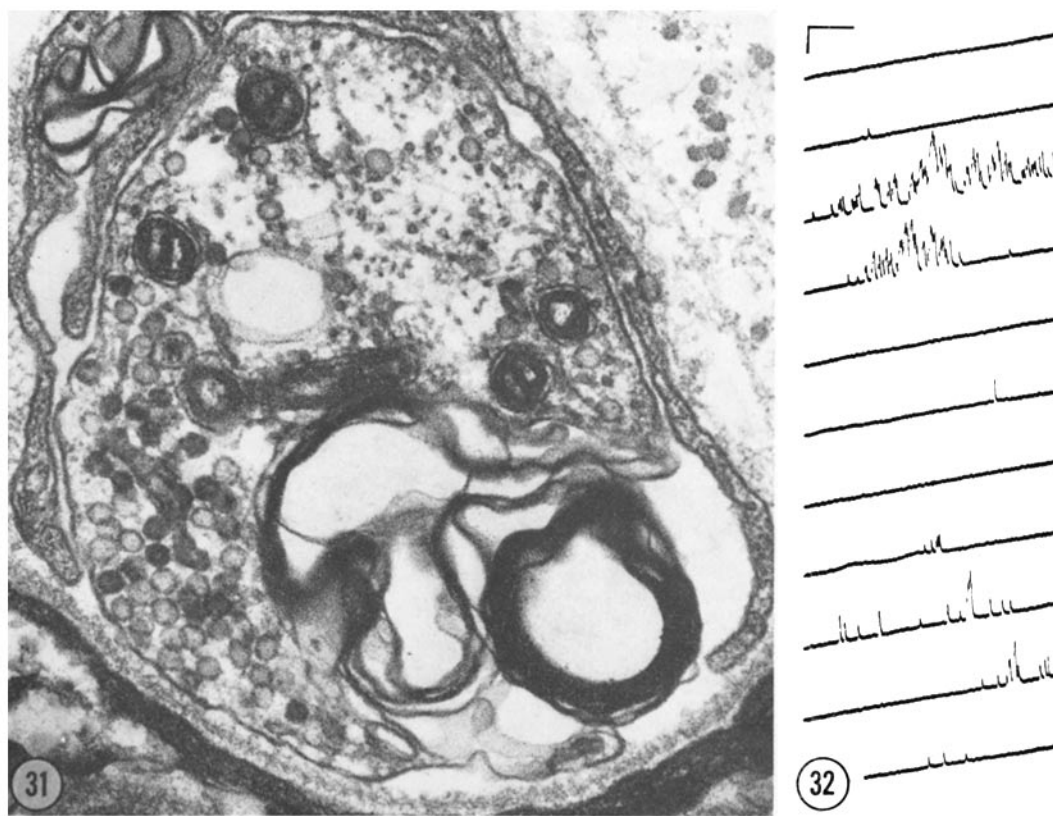


FIGURE 31 Cross section through a damaged region of the same loaded terminal as in Fig. 30. Typical of the deleterious effects of HRP, a large whorl of membrane has accumulated at the lateral surface of the terminal. $\times 55,000$.

FIGURE 32 Another deleterious effect of HRP is illustrated in the physiological record of m.e.p.p. activity in a different end plate approximately 15 min after it was loaded. Short bursts of high frequency m.e.p.p.'s recurred at variable intervals. Calibration, 1 mV, 100 ms.

pathway has been suggested, on purely morphological grounds, in the past (2, 3, 26, 27, 64, 80). The coat apparatus has been considered to be the visible aspect of a special mechanism for deforming the plasma membrane and pinching off vesicles from it (44, 72). In addition, since there may be chemical differences between the plasma membrane and vesicle membrane (21, 37),

when membrane retrieval would be operating at a high rate. Given this many coats, simple calculations suggest that during continuous stimulation membrane could be retrieved from the surface as fast as it was added by exocytosis of transmitter if each coat took around 1 min to attach to the plasma membrane, pinch off a vesicle, and detach from the vesicle to reorganize on the plasma membrane again.

the coat apparatus could maintain this chemical differentiation by gathering components of vesicle membrane and excluding components of the plasma membrane as it pinched off vesicles. The release of membrane at one locus and recovery at a distant locus would require the rapid migration of the vesicle membrane within the plasma membrane. Such rapid mixing of different membranes has recently been directly visualized (23) and a model embracing membrane fluidity proposed (74).

It was not surprising to find that coated vesicles take up HRP at this synapse, since they are specifically involved in protein absorption in many types of cells (9, 72). Furthermore, coated vesicles have previously been found to take up extracellular

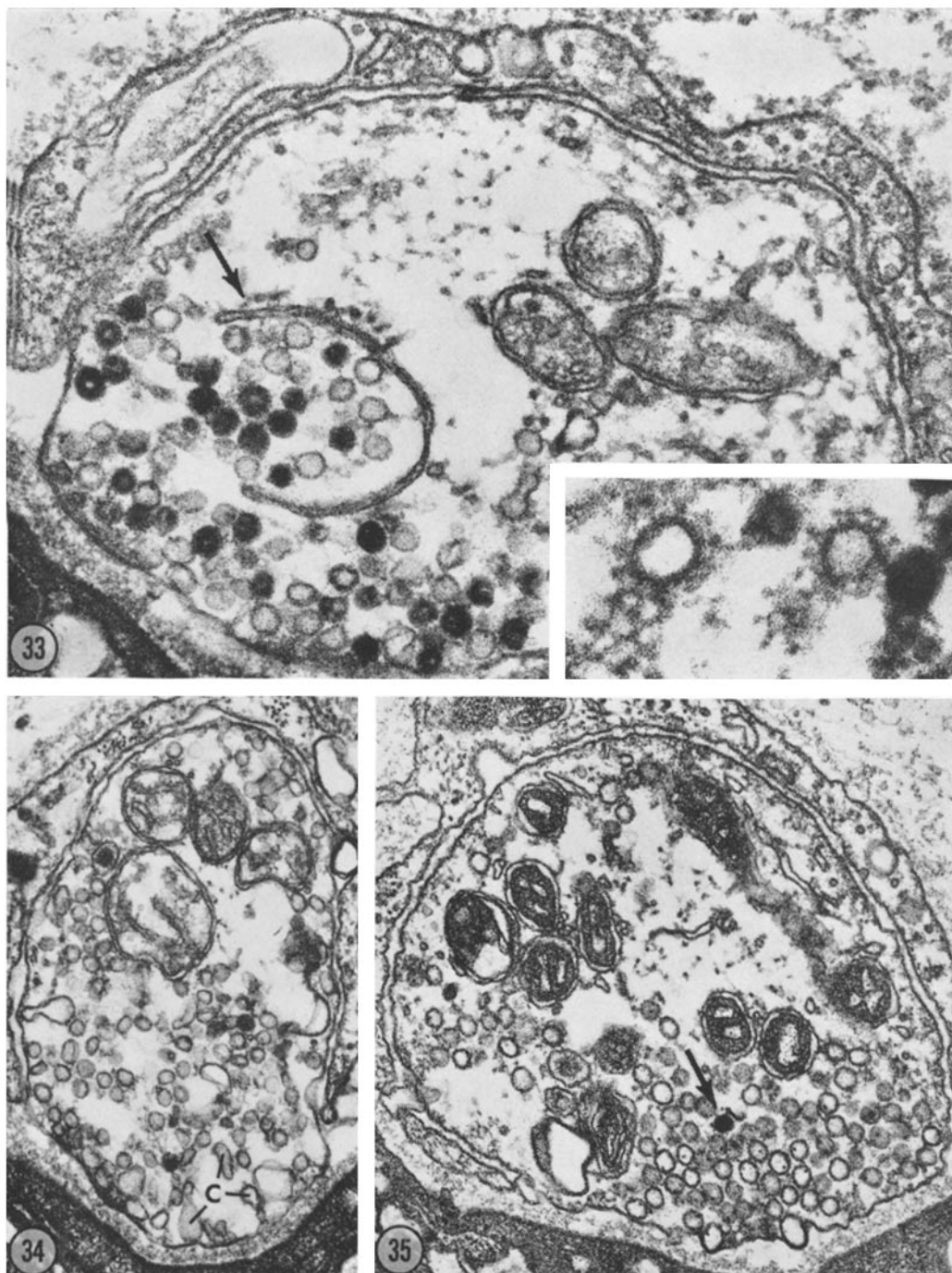


FIGURE 33 Cross section of a loaded terminal that was restimulated for 1 min. The new cisterna (arrow) and coated vesicles (*inset*) formed as a result of the second stimulation do not contain HRP, presumably because they have formed from the surface and have accumulated tracer-free extracellular fluid, rather than forming from coalescence of synaptic vesicles which now contain the tracer. $\times 70,000$; *inset*, $\times 150,000$.

FIGURE 34 Cross section of loaded terminal that was restimulated for 15 min. None of the new coated vesicles or cisternae (*c*) contain HRP, and the proportion of HRP-containing synaptic vesicles has declined. $\times 40,000$.

FIGURE 35 Cross section of a loaded terminal that was restimulated for 15 min and then allowed to rest again for 1 h. Tracer remains in only one synaptic vesicle (arrow) laying among a normal complement of empty synaptic vesicles. $\times 50,000$.

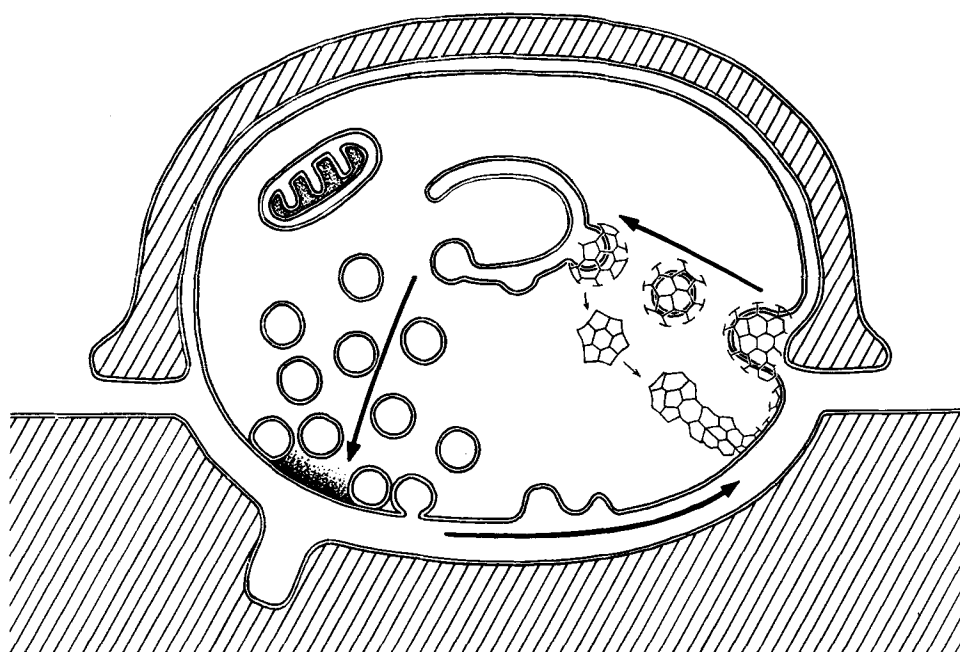


FIGURE 36 Diagrammatic summary of the path of synaptic vesicle membrane recycling proposed in this study: synaptic vesicles discharge their content of transmitter by coalescing with the plasma membrane at specific regions adjacent to the muscle; then equal amounts of membrane are retrieved by coated vesicles arising from regions of the plasma membrane adjacent to the Schwann sheath; and then, the coated vesicles lose their coats and coalesce to form cisternae which accumulate in regions of vesicle depletion and slowly divide to form new synaptic vesicles.

proteins at rat neuromuscular junctions (82), and at central nervous system synapses (10) and neurosecretory terminals (19, 60). Nevertheless, there is some question about whether coated vesicles are the primary route of HRP entry and membrane retrieval during stimulation (12). They appear to be the primary route in certain neurosecretory cells, where the retrieved membrane is apparently not used over again immediately because HRP does not enter the secretory vesicles (19, 58). But at the neuromuscular synapse HRP does enter a large proportion of synaptic vesicles after prolonged stimulation (12, 36) presumably because the retrieved membrane is immediately used over again to make new vesicles. Once a large number of vesicles contain HRP, it becomes difficult to determine whether those connected to the surface are forming or discharging (12). The present experiments attempted to avoid this complication by examining the first moments of HRP entry. *After short periods of stimulation HRP-containing vesicles were few and most of them were of the*

coated variety. Still it was not possible to be certain that coated vesicles represented the primary route of HRP entry and membrane retrieval, because *after short periods of stimulation many cisternae contained HRP as well.* If these cisternae form as direct invaginations of the plasma membrane, as others have suggested (13), then they would represent the major route of membrane retrieval. Nevertheless, in spite of an intensive search it was not possible to visualize direct connections of cisternae with the plasma membrane in the present material. It is quite possible that such connections would break during the osmium fixation used in the purely morphological part of this study (17, 71). However, it was not possible to remove HRP from cisternae by washing it from around nerve terminals and it was not possible to introduce lanthanum tracer into cisternae after aldehyde fixation. Thus it would appear that most cisternae do not arise directly from the plasma membrane by invagination.

Instead, cisternae appeared to form by coales-

cence of coated vesicles. *During the period of their formation cisternae were often connected to coated vesicles*, and several intermediate forms that could be interpreted as coalescing coated vesicles were found. The alternative interpretation of these observations, that coated vesicles form from cisternae as well as from the plasma membrane, seems less likely because *coated vesicles were less frequently connected to cisternae during periods of rest when the cisternae were disappearing*. Thus it appears, on the basis of the present evidence, that cisternae are indirectly connected to the plasma membrane by coated vesicles which represent the major route of synaptic vesicle membrane retrieval.

Conclusive determination of the exact sequence of membrane movements between cisternae and coated vesicles in these nerve terminals may be aided by additional information on the mode of formation of the empty vacuoles that appear in the apical cytoplasm of other secretory cells during plasma membrane retrieval (1, 41). These vacuoles may be equivalent to the cisternae found in stimulated synapses. Currently these vacuoles are thought to form by direct invagination of the plasma membrane (41), but improved methods for visualizing coat material may reveal that they form by coalescence of coated vesicles that indirectly link them with the surface.

Formation of New Synaptic Vesicles from Cisternae

The initial entry of HRP into cisternae before it enters synaptic vesicles, and the gradual disappearance of cisternae as vesicle numbers recover after stimulation would indicate that during intense stimulation of this isolated synapse, cisternae represent an intermediate step in the formation of new synaptic vesicles, and that the final step in the recycling of synaptic vesicle membrane is the division of cisternae into new vesicles. The existence of such an intermediate in retrieval of vesicle membrane was presciently hypothesized some years ago (2). During the early phases of low rates of transmitter release, the division of cisternae into vesicles could proceed so rapidly that few cisternae accumulate or grow to noticeable size (6, 7, 12). In fact, if division of cisternae proceeded as rapidly as formation, it would become impossible to distinguish these intermediates from normal synaptic vesicles and it would appear that new synaptic vesicles emerge directly from coated vesicles (27). On the other hand,

during the later phases of intense transmitter release, the division of cisternae into vesicles would appear to be the rate-limiting step in the entire membrane cycle, because under these conditions cisternae progressively accumulate as synaptic vesicles disappear (34, 49).

This formation of cisternae during stimulation, reflecting what may be a slow step in the recycling of synaptic vesicles, raises the possibility that quantal size remains relatively constant during fatigue of transmitter release (15, 16), because cisternae divide to form vesicles only when they become filled with transmitter. This attractive possibility may not be the case. The availability of extra transmitter substrates outside the terminal does not speed cisternal division, and the application of hemicholinium-3 to interrupt substrate availability and completely exhaust transmitter stores in the terminal does not prevent cisternal division. In fact, *after prolonged stimulation in the presence of 1 mM hemicholinium-3, frog motor nerve terminals recover a large population of synaptic vesicles* while spontaneous and evoked release remains too small to measure, indicating that they can form new synaptic vesicles from cisternae that contain no transmitter. This is consistent with findings at other synapses, where application of hemicholinium-3 during stimulation does not exaggerate synaptic vesicle depletion (28, 69). However, it should be stressed that all of our experiments on the effects of hemicholinium on vesicle membrane changes were evaluated only by visual inspection and will have to be evaluated quantitatively, since they differ from some other reports on the effects of hemicholinium (43), and since the interrelation of transmitter metabolism with vesicle membrane changes is of such importance.

Other Considerations

The frog motor nerve terminal may thus be differentiated into regions adjacent to the dense bands for transmitter release, where vesicles line up to contact and coalesce with the plasma membrane, and adjacent regions beneath the Schwann processes for membrane retrieval, where a distinct filamentous coat pinches off vesicles from the plasma membrane. The repeating association of such areas along the length of the terminal to form morphological synaptic units, noted by others as well (55), may indicate that this synapse is differentiated into a series of relatively self-main-

taining mechanisms for transmitter release, each perhaps similar to a single central nervous system bouton (63, 80). Conversely, the intermittent contact of astrocytic processes with calyceal synaptic endings in the central nervous system (53) may indicate a differentiation of this synapse into functional zones similar to those in the motor nerve terminal.

The scheme presented here implies that a rapid, local recycling of intact synaptic vesicle membrane influences the short-term dynamics of transmitter release at this synapse, by controlling the immediate availability of vesicles. However, this scheme does not consider how long-term exchange of membrane along the axon, between the synapse and the cell body, might interact with the local membrane recycling to influence the long-term behaviour of the synapse. This interaction could take the form of replenishment of synaptic vesicle membrane by anterograde transport of axonal endoplasmic reticulum or removal of old synaptic vesicle membrane by retrograde transport of multivesicular bodies, and thus might control the size of the total stock of synaptic vesicles in the resting terminal.

It now seems clear that the morphology of synapses can be reproducibly altered by intense stimulation delivered under the appropriate conditions. It is possible that as the morphological changes become better recognized and the conditions for stimulation determined, this will become a useful method for tracing functional pathways in the central nervous system.

The technical assistance of Mr. Frank D. Nolan is gratefully acknowledged.

Received for publication 2 August 1972, and in revised form 11 December 1972.

REFERENCES

1. AMSTERDAM, A., I. OHAD, and M. SCHRAMM. 1969. Dynamic changes in the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle. *J. Cell Biol.* **41**:753.
2. ANDRES, K. H. 1964. Mikropinozytose um Zentralsnervensystem. *Z. Zellforsch. Mikrosk. Anat.* **64**:63.
3. ANDRES, K. H., and M. DURING. 1966. Mikropinozytose in motorischen Endplatten. *Naturwissenschaften.* **53**:615.
4. ATWOOD, H. L., F. LANG, and W. A. MORIN. 1972. Synaptic vesicles. Selective depletion in crayfish excitatory and inhibitory axons. *Science (Wash. D. C.)*. **176**:1353.
5. BENNETT, H. S. 1956. The concepts of membrane flow and membrane vesiculation as mechanisms for active transport and ion pumping. *J. Biophys. Biochem. Cytol.* **2** (4, Suppl.):99.
6. BIRKS, R. I. 1966. The fine structure of motor nerve endings at frog myoneural junctions. *Ann. N. Y. Acad. Sci.* **135**:8.
7. BIRKS, R. I., H. E. HUXLEY, and B. KATZ. 1960. The fine structure of the neuromuscular junction of the frog. *J. Physiol. (Lond.)*. **150**:134.
8. BITTNER, G. D., and D. KENNEDY. 1970. Quantitative aspects of transmitter release. *J. Cell Biol.* **47**:585.
9. BOWERS, B. 1964. Coated vesicles in the pericardial cells of the aphid. *Protoplasma.* **59**:351.
10. BRIGHTMAN, M. W. 1965. The distribution within the brain of ferritin injected into cerebrospinal fluid compartments. II. Parenchymal distribution. *Am. J. Anat.* **117**:193.
11. BRIGHTMAN, M. W., and T. S. REESE. 1969. Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* **40**:648.
12. CECCARELLI, B., W. P. HURLBUT, and A. MAURO. 1972. Depletion of vesicles from frog neuromuscular junctions by prolonged tetanic stimulation. *J. Cell Biol.* **54**:30.
13. CLARK, A. W., W. P. HURLBUT, and A. MAURO. 1972. Changes in the fine structure of the neuromuscular junction of the frog caused by black widow spider venom. *J. Cell Biol.* **52**:1.
14. COUTEAUX, R., and M. PECOT-DECHAVEASSINE. 1970. Vesicules synaptiques et poches au niveau des "zones actives" de la jonction neuromusculaire. *C. R. Hebd. Seances Acad. Sci. Ser. D Sci. Nat. (Paris)*. **271**:2346.
15. DEL CASTILLO, J., and B. KATZ. 1954. Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol. (Lond.)*. **124**:574.
16. DEL CASTILLO, J., and B. KATZ. 1956. Biochemical aspects of neuro-muscular transmission. *Progr. Biophys. Biophys. Chem.* **6**:121.
17. DOGGENWEILER, C. F., and J. E. HEUSER. 1967. Ultrastructure of the prawn nerve sheaths. Role of fixative and osmotic pressure in vesiculation of thin cytoplasmic laminae. *J. Cell Biol.* **34**:407.
18. DOUGLAS, W. W., J. NAGASAWA, and R. A. SCHULTZ. 1970. Electron microscopic studies on the mechanism of secretion of posterior pituitary hormones and significance of microvesicles ("Synaptic vesicles"): evidence of secretion by exocytosis and formation of microvesicles as a by-product of this process. *Mem. Soc. Endocrinol.* **19**:353.

19. DOUGLAS, W. W., J. NAGASAWA, and R. A. SCHULZ. 1971. Coated microvesicles in neurosecretory terminals of posterior pituitary glands shed their coats to become smooth 'synaptic' vesicles. *Nature (Lond.)*. **232**:340.
20. DUN, F. T. 1955. The blockage of motor impulses in an asynchronized volley at the neuromuscular junction. *J. Cell Comp. Physiol.* **46**:348.
21. EICHBERG, J., V. P. WHITTAKER, and R. M. C. DAWSON. 1964. Distribution of lipids in subcellular particles of guinea pig brain. *Biochem. J.* **92**:91.
22. FATT, P. 1950. The electromotive action of acetylcholine at the motor end plate. *J. Physiol. (Lond.)*. **111**:408.
23. FRYE, L. D., and M. EDIDIN. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* **7**:319.
24. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubule of the mouse kidney; ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.
25. GRAY, E. G., and R. W. GUILLERY. 1966. Synaptic morphology in the normal and degenerating nervous system. *Int. Rev. Cytol.* **19**:111.
26. GRAY, E. G., and H. L. PEASE. 1972. On understanding the organization of the retinal receptor synapse. *Brain Res.* **35**:1.
27. GRAY, E. G., and R. A. WILLIS. 1970. On synaptic vesicles, complex vesicles, and dense projections. *Brain Res.* **24**:149.
28. GREEN, K. 1966. Electron microscopic observations on the relationship between synthesis of synaptic vesicles and acetylcholine. *Anat. Rec.* **154**:351.
29. GRYNZSPAN-WINOGRAD, O. 1971. Morphological aspects of exocytosis in the adrenal medulla. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:291.
30. HACKENBROCK, C. R., and A. I. CAPLAN. 1969. Ion induced ultrastructural transformations in isolated mitochondria. The energized uptake of calcium. *J. Cell Biol.* **42**:221.
31. HEUSER, J. E. 1971. Evidence for recycling of membrane accompanying transmitter release at the frog neuromuscular junction. Abstracts of the Society for Neuroscience First Annual Meeting, Washington D.C. 112.
32. HEUSER, J. E. 1971. Structural changes with stimulation of frog neuromuscular junctions. Abstracts of Papers Presented at the 11th Annual Meeting of the American Society for Cell Biology New Orleans. 124.
33. HEUSER, J. E., B. KATZ, and R. MILEDI. 1971. Structural and functional changes of frog neuromuscular junctions in high calcium solutions. *Proc. R. Soc. Lond. B Biol. Sci.* **178**:407.
34. HEUSER, J. E., and R. MILEDI. 1971. Effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proc. R. Soc. Lond. B Biol. Sci.* **179**:247.
35. HEUSER, J. E., and T. S. REESE. 1972. Stimulation induced uptake and release of peroxidase from synaptic vesicles in frog neuromuscular junctions. *Anat. Rec.* **172**:329.
36. HOLTZMAN, E., A. R. FREEMAN, and A. KASHNER. 1971. Stimulation-dependent alteration in peroxidase uptake at lobster neuromuscular junctions. *Science (Wash. D. C.)*. **173**:733.
37. HOSEI, R. J. A. 1965. The localization of adenosine triphosphatases in morphologically characterized subcellular fractions of guinea-pig brain. *Biochem. J.* **96**:404.
38. HUBBARD, J. I., and S. KWANBUNBUMPEN. 1968. Evidence for the vesicle hypothesis. *J. Physiol. (Lond.)*. **194**:407.
39. HUBBARD, J. I., R. LINÁS, and D. M. J. QUASTEL. 1969. Electrophysiological Analyses of Synaptic Transmission. Edward Arnold Ltd., London.
40. HURLBUT, W. P., H. B. LONGENECKER, and A. MAURO. 1971. Effects of calcium and magnesium on the frequency of miniature end plate potentials during prolonged tetanization. *J. Physiol. (Lond.)*. **219**:17.
41. JAMIESON, J. D., and G. E. PALADE. 1971. Synthesis, intracellular transport, and discharge of secretory proteins in stimulated pancreatic exocrine cells. *J. Cell Biol.* **50**:135.
42. JOHNSON, D. G., N. B. THOA, R. WEINSHILBOUM, J. AXELROD, and I. J. KOPIN. 1971. Enhanced release of dopamine- β -hydroxylase from sympathetic nerves by calcium and phenoxybenzamine and its reversal by prostaglandins. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2227.
43. JONES, S. F., and S. KWANBUNBUMPEN. 1970. The effects of nerve stimulation and hemicholinium on synaptic vesicles at the mammalian neuromuscular junction. *J. Physiol. (Lond.)*. **207**:31.
44. KANASEKI, T., and K. KADOTA. 1969. The "vesicle in a basket." *J. Cell Biol.* **42**:202.
45. KATZ, B. 1962. The transmission of impulses from nerve to muscle, and the subcellular unit of synaptic action. *Proc. R. Soc. Lond. B Biol. Sci.* **155**:455.
46. KATZ, B. 1969. The Release of Neural Transmitter Substances. Liverpool University Press, Liverpool.
47. KATZ, B., and R. MILEDI. 1965. Propagation of electric activity in motor nerve terminals. *Proc. R. Soc. Lond. B Biol. Sci.* **161**:453.

48. KIRSHNER, N., and A. G. KIRSHNER. 1971. Chromogranin A, dopamine- β -hydroxylase, and secretion from the adrenal medulla. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**: 279.
49. KORNELIUSSEN, H. 1972. Ultrastructure of normal and stimulated motor end plates, with comments on the origin and fate of synaptic vesicles. *Z. Zellforsch. Mikrosk. Anat.* **130**:28.
50. KRISTENSSON, K., Y. OLSSON, and J. SJÖSTRAND. 1971. Axonal uptake and retrograde transport of exogenous proteins in the hypoglossal nerve. *Brain Res.* **32**:399.
51. KRNJEVIC, K., and R. MILEDI. 1958. Failure of neuromuscular propagation in rats. *J. Physiol. (Lond.)* **140**:440.
52. LA VAIL, J. H., and M. M. LA VAIL. 1972. Retrograde axonal transport in the central nervous system. *Science (Wash. D. C.)* **176**:1416.
53. LENN, N. J., and T. S. REESE. 1966. The fine structure of nerve endings in the nucleus of the trapezoid body and the ventral cochlear nucleus. *Am. J. Anat.* **118**:375.
54. MARTIN, A. R. 1955. A further study of the statistical composition of the end plate potential. *J. Physiol. (Lond.)* **130**:114.
55. McMAHAN, U. J., N. C. SPITZER, and K. PEPER. 1972. Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc. Lond. B Biol. Sci.* **181**:421.
56. MILEDI, R., and L. T. POTTER. 1971. Acetylcholine receptors in muscle fibers. *Nature (Lond.)* **233**:599.
57. MILEDI, R., and R. THIES. 1971. Tetanic and post-tetanic rise in frequency of miniature end plate potentials in low-calcium solutions. *J. Physiol. (Lond.)* **212**: 245.
58. NAGASAWA, J., and W. W. DOUGLAS. 1972. Thorium dioxide uptake into adrenal medullary cells and the problem of recapture of granule membrane following exocytosis. *Brain. Res.* **37**:141.
59. NAGASAWA, J., W. W. DOUGLAS, and R. A. SCHULZ. 1970. Ultrastructural evidence of secretion by exocytosis and of "synaptic vesicle" formation in posterior pituitary glands. *Nature (Lond.)* **227**:407.
60. NAGASAWA, J., W. W. DOUGLAS, and R. A. SCHULZ. 1971. Micropinocytotic origin of coated and smooth microvesicles ("synaptic vesicles") in neurosecretory terminals of posterior pituitary glands demonstrated by incorporation of horseradish peroxidase. *Nature (Lond.)* **232**:341.
61. PALADE, G. E. 1959. Functional changes in the structure of cell components. In *Subcellular Particles*. T. Hayashi, editor. The Ronald Press Company, New York. 64.
62. PALADE, G. E., and H. R. PORTER. 1954. Studies on the endoplasmic reticulum. I. Its identification in cells in situ. *J. Exp. Med.* **100**:641.
63. PALAY, S. L. 1958. The morphology of synapses in the central nervous system. *Exp. Cell Res. Suppl.* **5**:275.
64. PALAY, S. L. 1963. Alveolate vesicles in Purkinje cells of the rat's cerebellum. *J. Cell Biol.* **19**:89A. (Abstr.)
65. PEACHEY, L. D. 1964. Electron microscopic observations on the accumulation of divalent cations in intramitochondrial granules. *J. Cell Biol.* **10**:95.
66. PERRI, V., O. SACCHI, E. RAVIOLA, and G. RAVIOLA. 1972. Evaluation of the number and distribution of synaptic vesicles at cholinergic nerve-endings after sustained stimulation. *Brain Res.* **39**:526.
67. PEZARD, A., and R. M. MAY. 1937. Les terminaisons nerveuses du muscle couturier de la genouille et la question de sa partie aneurale. *C. R. Soc. Biol. Fil.* **124**:1081.
68. POTTER, L. T. 1970. Synthesis, storage, and release of ^{14}C acetylcholine in isolated rat diaphragm muscles. *J. Physiol. (Lond.)* **206**: 145.
69. PYSH, J. J., and R. G. WILEY. 1972. Morphologic alterations of synapses in electrically stimulated superior cervical ganglia of the cat. *Science (Wash. D. C.)* **176**:191.
70. ROBERTSON, J. D. 1960. Electron microscopy of the motor end plate and the neuromuscular spindle. *Am. J. Phys. Med.* **39**:1.
71. ROSENBLUTH, J. 1963. Contrast between osmium-fixed and permanganate-fixed toad spinal ganglia. *J. Cell Biol.* **16**:143.
72. ROSENBLUTH, J., and S. L. WISSIG. 1964. The distribution of exogenous ferritin in toad spinal ganglia and the mechanism of its uptake by neurons. *J. Cell Biol.* **23**:307.
73. SCHUELER, F. W. 1960. The mechanism of action of the hemicholiniums. *Int. Rev. Neurobiol.* **2**:77.
74. SINGER, S. T., and G. L. NICOLSON. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D. C.)* **175**:720.
75. SMITH, A. D. 1971. Summary up: some implications of the neuron as a secretory cell. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:423.
76. SMITH, A. D. 1971. Secretion of proteins (chromogranin A and dopamine- β -hydroxylase) from a sympathetic neuron. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:363.
77. SMITH, R. E., and M. G. FARQUHAR. 1963. Preparations of thick sections for cytochemistry and electron microscopy by a nonfreezing technique. *Nature (Lond.)* **200**:691.
78. TAKEUCHI, A. 1958. The long lasting depression

- in neuromuscular transmission of frog. *Jap. J. Physiol.* **8**:102.
79. UTTENTHAL, L. O., B. G. LIVETT, and D. B. HOPE. 1971. Release of neurophysin together with vasopressin by a Ca^{++} dependent mechanism. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:379.
 80. WESTRUM, L. E. 1965. On the origin of synaptic vesicles in cerebral cortex. *J. Physiol. (Lond.)* **179**:4P.
 81. YAMAMOTO, T. 1963. On the thickness of the unit membrane. *J. Cell Biol.* **17**:413.
 82. ZACHS, S. I., and A. SAITO. 1969. Uptake of exogenous horseradish peroxidase (HRP) by coated vesicles in mouse neuromuscular junctions. *J. Histochem. Cytochem.* **17**:161.