MORPHOLOGICAL STUDIES OF STIMULATED ADRENERGIC
AXON VARICOSITIES IN THE MOUSE VAS DEFERENS

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ABSTRACT

The postganglionic axons of sympathetic neurons innervating the mouse vas deferens were stimulated transmurally in vitro by passing square pulses between two platinum electrodes. The ultrastructural appearance of the adrenergic nerve terminals was compared in samples fixed immediately after 30 min of stimulation and in samples allowed to recover for 2 h before fixation. The contralateral vasa deferentia served as controls, and these were incubated in Krebs solution for the same period as stimulated muscles.

For each of four experiments, the mean number of large and small dense-core vesicles per square micrometer was calculated, as were the mean area and perimeter of the axon varicosities in each group. It was found that the number of small vesicles per square micrometer decreased by 60% during the stimulation period, but returned almost to control levels 2 h later. Large vesicles did not change in number during the stimulation or recovery periods. The proportion of vesicles containing cores was also determined for each group and found to decline just after stimulation in the small vesicle population, but to remain constant in the large vesicle population. The core depletion was partly reversed after 2 h.

The vesicle recovery process was studied by use of the extracellular tracer horseradish peroxidase (HRP). When HRP was present in the extracellular space during stimulation, large numbers of vesicles contained the marker after recovery from stimulation. Thus, it is proposed that adrenergic axon varicosities recycle vesicle membrane through the plasma membrane in a manner similar to that already described for cholinergic nerve terminals.

KEY WORDS neurosecretion · dense-core vesicles · postganglionic sympathetic axons · vas deferens · membrane recycling

An outstanding problem in studies of the autonomic nervous system is the mechanism by which neurotransmitter is released from terminal axons. Fluorescence histochemical methods such as that of Falck (21) and Furness and Costa (26) reveal that amineergic transmitter is concentrated mainly in the terminal segments of sympathetic axons. The dilated portions of these axons fluoresce most intensely for norepinephrine (NE) and correspond, when viewed in the electron microscope, to dilated "varicosities" containing many dense-core vesicles. Such vesicles are the morphological representation of NE storage particles (3) isolated from sympathetic nerve homogenates.

Because they store NE, the dense-core vesicles in sympathetic axon varicosities are also likely to be involved in the release of NE. Indirect evidence comes from physiological recordings from smooth
nerve endings in skeletal muscle (18). Each storage particle is roughly the correct size to store and release one quantum of NE (for discussion, see reference 25). In fact, it is highly probable that NE is discharged from sympathetic nerve terminals by exocytosis involving these particles. The best evidence for this so far has been the finding that stimulation-evoked NE release is accompanied by the release of several other components of NE storage particles, including ATP (50), dopamine-β-hydroxylase (DBH) (28, 47, 59), and chromogranin (47). Cytoplasmic molecules are not released during stimulation, suggesting that vesicles discharge directly into the extracellular space via exocytosis (20).

Electron micrographs of cholinergic nerves show synaptic vesicles undergoing exocytosis (14, 31), and provide evidence for local recycling of vesicle membrane (32, 43, 44). Similar data are not available for adrenergic nerves. Morphological data could, however, establish whether one or both of the two sizes of dense-core vesicles in adrenergic nerves undergo exocytosis. The larger vesicles (800-1,200 Å) are thought to originate in the cell body and to flow down the axon to supply new enzymes, as well as NE, to the terminal. This concept was derived from studies showing that DBH accumulated proximal to an axonal ligation (7, 27) with the same time-course that NE (16) and large granular vesicles (36) also accumulated. The small vesicles (400-600 Å) are found in greatest numbers in the terminal portions of the adrenergic axons (see, however, references 29, 52, and 53) and collect in the varicosities. Their origin is uncertain.

Both types of vesicle contain a rich store of NE as well as ATP and protein (46-48). The NE-protein complex is thought to produce the dense cores within vesicles, when oxidized by EM fixatives such as OsO₄ (5, 33, 54). Both types of vesicle have the capacity to take up amines in the presence of Mg and ATP, and this property is easily visualized by exposing sympathetically innervated tissues to intensely staining NE analogues such as 5-hydroxydopamine (11, 55).

In view of the obvious similarities between large and small vesicles, it is interesting that evidence exists for functional differences between the two types of vesicles. Biochemical studies of NE storage particles obtained by fractionating spleen (2) and heart (41) have shown that stimulation of the sympathetic nerve supply to these two organs significantly reduced the amine content of the light particle (small vesicle) fraction but did not lower the amine content of the heavy particle (large vesicle) fraction. Thus, the small vesicles appeared to contain a more labile store of amines than the large vesicles, and on this basis might figure more directly in transmitter release evoked by nerve stimulation. Loss of NE from both the heavy and light particle fractions has been reported under conditions of extreme NE depletion (23), suggesting that the more stable pool of transmitter in large vesicles may be mobilized by intense stimulation. In general, the large vesicles contain much more protein relative to NE than is released upon nerve stimulation, and for that reason they are considered not to play a major role in short-term release of NE (59).

Only a few morphological studies have investigated dense-core vesicles in different functional states of sympathetic nerves. Van Orden et al. (57) found that stimulation of the guinea pig vas deferens reduced the proportion of small vesicles with dense cores by 20%. Optimal conditions were not, however, used to retain dense cores in the nerve endings, probably resulting in somewhat lower values than one could find with stronger oxidizing fixatives such as permanganate or dichromate. Moreover, the study did not consider changes in the absolute numbers of small and large vesicles, and therefore provided no indication of the relative importance of the different types of vesicle. A short paper by Coté et al. (13) did report a selective decrease in the number of small dense-core vesicles after stimulation of the guinea pig vas deferens, but contained no photographs.

If large vesicles undergo exocytosis, they do not seem to do so in large numbers. Their function in the varicosities is indeed unclear, but it is probable that the membrane and proteins which they deliver to the varicosities is transferred to the small vesicles and ultimately used in the process of secretion.

Dahlstrom (17) proposed that the large dense-core vesicles discharge only part of their contents during exocytosis and, by doing so repeatedly, are reduced to the size of small vesicles. Other authors, considering other types of nerves, have imagined that large vesicles discharge completely, just once, and then their components are retrieved in the form of small vesicles in order to be used...
again (40). One can, in fact, imagine a variety of ways in which large vesicles could be partitioned into small vesicles once inside the nerve terminal.

In this paper, we describe morphological data bearing on the roles of small and large dense-core vesicles, obtained by EM observation of adrenergic nerve terminals before and after stimulation. We describe experiments designed to deplete the stores of amine, and reveal which sort of synaptic vesicle becomes depleted in the process. In accordance with previous observations, we have found that it is primarily the small dense-core vesicles which become depleted during stimulation. Also, we have obtained evidence from horseradish peroxidase uptake experiments that these small dense-core vesicles recycle locally in the adrenergic nerve terminal and thus may engage in transmitter storage and secretion over and over again. In contrast, we have found no indication that the large dense-core vesicles undergo exocytosis or engage in vesicle membrane recycling. This, in fact, leaves the role of the large vesicles an enigma.

Additionally, EM analysis of stimulated sympathetic nerves has offered a unique opportunity to study the interdependence of vesicle membrane recycling and transmitter renewal because, unlike the cholinergic synapses which have been studied so far, the transmitter in the adrenergic synaptic cleft and attached to a strain gauge, Muscle contraction was monitored by feeding the signal from the strain gauge into a pen recorder. The sympathetic nerves in the bath were stimulated transmurally by 1-ms square pulses. Pulses were delivered at 10 Hz for 0.5 s, alternating with 0.5 s of rest, for 30 min. Because the bath resistance was very low, large currents were needed to stimulate the vas effectively. We used three times rheobase, typically almost 1 A. This large current inevitably produced some electrolysis of the solution around the electrodes. To minimize the effects of electrolysis, we reversed the polarity with each pulse and exchanged the bath solution every 30 s by continuous flow of Krebs solution through the chamber.

During stimulation, the contralateral muscle rested in oxygenated Krebs solution in which 6 mM MgCl₂ replaced CaCl₂ in an effort to avoid depolarization-evoked release of amines. At the completion of stimulation, the stimulated muscle was divided into three parts. One was immediately fixed in a fixative modified from Woods (61) consisting of 2.5% acrolein in 0.06 M sodium dichromate, pH 6.8, and the other two were placed in recovery baths. These baths consisted of L-15 tissue culture medium which contained electrolytes, amino acids, and energy sources (39). MgCl₂ (4 mM) and ethylene glycol-bis(β-aminoethyl(ether))N,N',N''-tetraacetate (4 mM) were added to prevent transmitter release. One of the recovery baths in each experiment contained drugs to suppress uptake and synthesis of NE during recovery (see "Drugs" below). After a 2-h recovery period, the muscles were fixed in the acrolein-dichromate mixture. All muscles remained in the fixative for 15-18 h, after which time they were rinsed in 0.06 M dichromate buffer and postfixed in a mixture of 2% OsO₄ and 0.06 M dichromate, pH 7.4. Specimens were then rinsed in dichromate buffer and transferred to 1% uranyl acetate in 50 mM sodium acetate buffer, pH 5.0, for 2 h in the dark at room temperature. Specimens were then dehydrated in graded ethanols, embedded in Araldite, and sectioned at 300-600 μm on a Porter-Blum MT-1 ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.). To insure a constant section thickness for purposes of quantitation, only sections showing a silver-gray interference color were selected. Sections were grid-stained with 10% uranyl acetate in 50% methanol, followed by 0.4% lead citrate in 0.15 N NaOH. Sections were viewed in a JEOL 100 B electron microscope.

**Drugs**

To facilitate the release of transmitter, phenoxybenzamine (dibenzyline 10⁻⁶ M) was present in all experiments described below. This drug was intended to inhibit the negative feedback of NE on axonal α receptors which normally acts to suppress NE release after the first few impulses (15, 49, 60). To inhibit renewal of NE, the experiments were done in the presence of desipramine (DMI) (Pertofrane, 10⁻⁶ M; USV Pharmaceutical Corp., Tuckahoe, N. Y.), which was intended to block uptake of NE already released by the axons. An NE-synthesis inhibitor, α-methyl-p-tyrosine (ampt) (5 × 10⁻⁵ M), was also used to prevent renewal of NE stores after initial stores were released by stimulation. Control muscles were exposed to the three drugs in the same concentrations.

**Tracer Studies**

For horseradish peroxidase (HRP) uptake experiments, muscles were dissected and stimulated in the same solutions as before, to which was added 10 mg/ml HRP (type VI; Sigma Chemical Co., St. Louis, Mo.).
Details of the timing of stimulation and rest are described in Results. The muscles were fixed for 12-15 h with 2% glutaraldehyde and 3% paraformaldehyde in 60 mM sodium chloride, 30 mM HEPES buffer at pH 7.2, and 5 mM CaCl₂; then the muscles were chopped transversely into 50-μm disks with a Smith-Farquhar tissue chopper (TC-2, DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) and subjected to the usual diaminobenzidine-hydrogen peroxide incubation for HRP histochemistry (32). Finally, the muscles were post-fixed with 2% OsO₄ in sodium barbital buffer, pH 7.4, block-stained with uranyl acetate, embedded in Araldite, and sectioned according to standard methods. Sections were viewed in the electron microscope without grid stain.

Quantitative Methods

On electron micrographs at a uniform final magnification of 75,000, we measured first the perimeter of each axon profile, by tracing around it with a map reader, and second, the area of each profile, by cutting it out and comparing its weight to that of a 1-μm² standard. We also counted the number of large and small vesicles in each profile, and determined what percentage contained dense cores. Means and standard errors were calculated for each of these values, in each of four experiments, and t tests were performed to test for significant differences. Because, however, the large dense-core vesicles showed an asymmetric frequency distribution, the nonparametric test of Kolmogorov-Smirnov (45) was used to test for differences between these samples.

RESULTS

Before starting the stimulation experiments, it was necessary to standardize the baseline conditions from which stimulation effects could be measured. Vasa deferentia fixed directly after dissection from the animal typically showed a wide variation in their vesicle content and in the proportion of vesicles that contained dense cores. We assume that this was because of inadvertent nerve stimulation during anesthesia and dissection. It was our impression that this variability was reduced when we allowed control muscles to rest for 50 min in calcium-free Krebs solution containing 4 mM MgCl₂ before fixation, in confirmation of Iwayama and Furness (34).

To avoid stimulating secretion by the depolarizing effect of the aldehyde fixative (1), our first series of control muscles was fixed in acrolein-dichromate containing 10 mM MgCl₂ instead of the CaCl₂ that we usually add to EM fixatives. This precaution did not increase the number of dense-core vesicles present in control muscles. Moreover, it made cytoplasmic filaments so dense that it became difficult to count synaptic vesicles. Therefore we returned to using 10 mM CaCl₂ in all our fixatives.

The selection of the acrolein-dichromate fixative itself was based on a quantitative comparison of a variety of fixatives. The dichromate fixative originally described by Woods (61) produced the largest number of dense cores of any fixative tried, without severely degrading the overall quality of fixation of other cell structures. Presumably, the advantages of this fixative are the small size of the acrolein molecule, allowing rapid diffusion to the amine storage site, and the high redox potential of dichromate, facilitating the oxidation of monoamines.

Control Incubation in Magnesium Ringer

The varicosities in muscles incubated in calcium-free Krebs for 50 min before fixation contained an average of 51 small vesicles per μm² (50.67 ± 2.08, N = 182, E = 4) and an average of four large vesicles per μm² (3.73 ± 0.29, N = 186, E = 4). So, at rest, large vesicles comprise ~7% of the total vesicle population. 73% of the small (73 ± 1.0%, N = 187, E = 4) and 95% of the large (95 ± 2%, N = 186, E = 4) vesicles contained electron-opaque cores. In the small vesicles, the cores varied in size from ~50-80% of the vesicle diameter, whereas the cores of the large vesicles were more uniform in size and typically measured ~80% of the vesicle diameter.

Most varicosities contained several mitochondria and small amounts of smooth endoplasmic reticulum. Also, amorphous filamentous material adhered here and there to areas of the plasma membrane, but we could find no discrete presynaptic densities of the sort that mark the "active zones" of other types of synapse. Microtubules and neurofilaments were poorly preserved by the dichromate fixative, but were seen in abundance in glutaraldehyde-fixed tissues in which they were most numerous in the constricted regions of the axons between the vesicle-laden varicosities, or in preterminal regions of the axon. Figs. 2, 4, and 6 show axons and muscle cells in control samples.

30 min of Stimulation

The threshold for eliciting muscle contraction with a single train of shocks was ~8 V, or slightly <0.1 A. The response to a single train was a brief twitch. During prolonged stimulation, the muscle produced a brief twitch lasting <1 s followed by
tetanus which gradually declined to resting tension over the stimulation period of 30 min. This is illustrated in Fig. 1.

The varicosities in the muscle segments fixed immediately after stimulation showed a 60% reduction in the number of small vesicles per μm², (20.04 ± 1.16, N = 175, E = 4, P < 0.00001) but no change in the number of large dense-core vesicles per μm² (3.11 ± 0.29, N = 189, E = 4, P = 0.17). Moreover, among the small vesicles which remained, only 31% had dense cores (31 ± 2%, N = 195, E = 4); and in most of these the core was clearly smaller than normal, typically measuring less than half the vesicle internal diameter. Because of the proliferation of unusual membrane forms in stimulated varicosities, it was necessary to strictly define large vesicles as ovals measuring 800-1,200 Å in diameter. The proportion of large vesicles containing dense cores was unchanged by stimulation. Varicosities fixed immediately after stimulation are shown in Figs. 3, 5, and 7.

To make sure that the observed change in vesicle concentration represented a true depletion of vesicles and not simply an apparent change secondary to axon edema induced by the intensive stimulation, we compared the mean area of stimulated axonal profiles (0.44 ± 0.03 μm², N = 185, E = 4) with the area of control profiles (0.49 ± 0.02 μm², N = 191, E = 4). The lack of change in these area measurements (P = 0.16) indicated that swelling did not occur during stimulation and that the vesicle depletion was real.

To determine whether the loss of synaptic vesicle membrane during stimulation was matched by an expansion of the plasma membrane, as would be expected if the missing synaptic vesicles had undergone exocytosis, we measured the perimeters of stimulated and control axon profiles. We assumed that a change in perimeter would accurately reflect an expansion of the plasma membrane because we had no reason to suppose that the length of the axons would be changed and total surface area = perimeter × length. The mean perimeter of stimulated axons was 2.97 μm (2.97 ± 0.11, N = 168, E = 3), which was not significantly greater (P < 0.14) than the mean control perimeter of 2.77 μm (2.77 ± 0.08, N = 191, E = 3).

Recovery after 30 min of Stimulation

The varicosities in muscle segments which rested in culture medium for 2 h after stimulation showed an almost complete recovery in the number of small vesicles to a mean value of 45 per μm² (45.28 ± 1.91, N = 309, E = 4), a value not significantly different (P = 0.07) from that of control nerves. Moreover, the percentages of small vesicles containing dense cores increased by 17%, during the 2 h after stimulation, to a mean of 48% (48 ± 1%, N = 330, E = 4). But this did not represent a recovery to the control value which was 73% dense cores. Furthermore, many of the cores of the small vesicles were small and eccentrically located inside the vesicles, unlike those in control nerves.

The number of large vesicles seen in recovered nerves was similar to values obtained for both control and stimulated samples (3.99 ± 0.26, N = 302, E = 4), and the proportion of large vesicles containing cores was similarly unchanged.

The mean perimeter of recovered varicosities was 2.93 μm (2.93 ± 0.09, N = 221, E = 3), which was not significantly different from that of either stimulated or control nerves. The mean areas of recovered varicosities was similarly unchanged. A varicosity fixed after stimulation and recovery is shown in Fig. 8.

Recovery in the Presence of Inhibitor Drugs

The nerves which rested in L-15 culture medium with 3 × 10⁻⁵ M αmpt to inhibit NE synthesis, and 10⁻⁵ M DMI to inhibit neuronal uptake of NE, also showed full recovery of synaptic vesicle numbers within 2 h (52.80 ± 4.23, N = 90, E = 3), and, surprisingly, a degree of recovery of dense cores in these vesicles (52.0 ± 3.0%, N = 89, E = 3) which was comparable to that observed in the absence of inhibitors (P < 0.19). As was the case in the noninhibited recovery, the percentage of cores in the small vesicle population rose to midway between stimulated and control values. Similarly, the cores of the small vesicles were much smaller than those in
control axons and were eccentrically placed within the vesicles, occupying much less than 50% of the vesicle interior. Thus, the concentrations of synthesis and uptake inhibitors that we used did not reduce the efficiency of membrane recycling or of transmitter renewal.

As was the case in the noninhibited recovery condition, the large vesicles and their core content showed no quantitative differences from that of other samples. Similarly, varicosity areas and perimeters were no different from those in other samples. A varicosity fixed after stimulation and
Two large varicosities in a muscle fixed immediately after stimulation are in the center of the field and a third is to the right. All have electron-transparent cytoplasm and a paucity of synaptic vesicles. Among the few remaining vesicles, dense cores are most common in the large (800-1,200 Å) vesicles. The small varicosity to the right contains a swollen mitochondrion and a coated invagination of the plasma membrane (see inset). Both are common features of stimulated varicosities. × 30,000. Inset: × 105,000.

Tracer Studies

Controls: Varicosities in unstimulated muscles exposed to HRP for up to 80 min did not show significant uptake of the tracer. This interval matched the time spent in tracer by stimulated muscles. These muscles were also washed in HRP-free Krebs solution for 60 min, as were stimulated
One of the large varicosities in Fig. 2 is shown here at higher magnification. This was from an unstimulated muscle placed in 0-calcium Krebs solution for 50 min. × 78,000.

A varicosity fixed immediately after 30 min of stimulation shows the variety of membrane forms in vesicle-depleted axons. Arrow points to coated vesicle. × 78,000.

The labeling of these resting varicosities was usually confined to a single vesicle per profile, when tracer was visible at all. Most varicosities showed no evidence of tracer uptake.

BRIEF STIMULATION: Three muscles were stimulated in HRP for only 2 min, then immediately fixed, to permit observation of the initial stages of HRP entry into stimulated varicosities. The muscles had been incubated in HRP for 20 min before stimulation to insure infiltration of the tracer into the vicinity of the varicosities. After brief stimulation, the tracer was present in coated invaginations of the varicosity membrane (see Fig. 10) or in one or two vesicles per profile.

PROLONGED STIMULATION AND REST: Five muscles were stimulated intermittently after a 20-min period of incubation with HRP. The stimulation consisted of three 7-min stimula-
tion periods alternating with two 3-min rest periods. The muscles were then allowed to remain in the tracer medium for 30 min, during which time additional uptake might occur. Then muscles were washed in large volumes of HRP-free, Krebs solution for 60 min before fixation to remove HRP from the extracellular spaces. Ca++ was replaced by Mg++ in the wash solution to prevent exocytosis of labeled vesicles. Varicosities in these muscles showed abundant uptake of the tracer, in many synaptic vesicles, as well as in larger membrane-bounded profiles, which may correspond to the cisternae described by Heuser and Reese (32). A small number of oval profiles in the size range of large vesicles was labeled. Dense cores were not visible in these profiles, and their identity is uncertain. In most varicosities, large vesicles were distinct and did not contain HRP. Figs. 11 and 12 show varicosities in muscles subjected to prolonged stimulation and rest in HRP.

Figs. 13-16 present, in graphic form, changes in the number of vesicles and dense cores that occurred as a result of electrical stimulation. These changes are summarized diagrammatically in Fig. 17.

DISCUSSION

The selective depletion of small dense-cored vesicles following nerve stimulation supports the conclusion drawn from earlier biochemical studies (2, 12, 23, 41) that it is the small, dense core vesicles that release NE in response to nerve stimulation. There has been one report of preferential NE depletion from the large vesicle pool (38). We have found no morphological evidence for the involvement of the large, dense-core vesicles in either NE release or recycling of vesicle membrane after stimulation. The frequency of large vesicles was statistically the same in stimulated, resting, and recovering nerves, indicating that they are not depleted during stimulation and do not divide or proliferate during recovery.
Most of our observations of recovering nerves were made 2 h after stimulation. By this time, the 60% reduction in the small vesicle pool immediately after stimulation had been completely reversed; this requires an addition of 15 new vesicles (60% of the resting number) to an average varicosity of 0.5 μm² in 2 h. The time-course of this recovery was so rapid as apparently to rule out replenishment of the vesicles by axonal transport and to suggest that local vesicle formation occurs in the varicosities. This conjecture is based on a study of vesicle turnover in the spleen (19) in which it was calculated that only 2.5% of the vesicle pool is replaced each hour by axoplasmic flow from the cell soma.

The events leading to the transient disappear-
FIGURE 10 This varicosity was fixed immediately after 2 min of stimulation in HRP. The tracer is engulfed by a coated invagination of the plasma membrane (lower right). Paraformaldehyde-glutaraldehyde fixation. × 76,000.

FIGURE 11 This varicosity was stimulated in HRP intermittently for 30 min, remained in HRP for 30 additional min, and was fixed after a 60-min wash in Krebs solution. Many vesicles and two larger membranous structures contain the tracer. Paraformaldehyde-glutaraldehyde fixation. × 76,000.

FIGURE 12 Same as Fig. 11. × 76,000.

ance and subsequent reappearance of small vesicles can best be inferred from the experiments involving the extracellular tracer HRP. This large molecule can only enter cells by a bulk transport mechanism such as endocytosis. Thus, the presence of HRP-filled vesicles in stimulated axons
The concentration of vesicles per μm² of varicosity area is indicated for each experiment. Small vesicles are represented by the open bars and large vesicles are represented by the stippled bars.

The percentage of vesicles containing dense cores is indicated for each experiment. Small vesicles are represented by the open bars and large vesicles are represented by the stippled bars.

indicates that the vesicle membrane was in contact with the extracellular space during HRP incubation, and that it subsequently invaginated and pinched off, bringing with it the tracer. One way in which this might have occurred is that a vesicle undergoing exocytosis released its NE and engulfed HRP before bouncing back into the vesicle pool. Alternatively, a vesicle undergoing exocytosis may have opened out completely so that its components coalesced with the membrane (32), to re-enter later by invagination, pinching off a bolus of HRP in the process.

The available evidence argues for the latter possibility, since the large transient depletion of small vesicles could not have occurred if each vesicle returned to the vesicle pool immediately after NE release. We therefore conclude that vesicle membrane recycles through the plasmalemma of adrenergic varicosities, just as the membrane of cholinergic vesicles recycles at the frog neuromuscular junction (32), the preganglionic endings in sympathetic ganglia (43), and the turtle photoreceptor (44).

We calculated the increment in perimeter that
FIGURE 15 The alterations in both the number of small vesicles and the number of vesicles containing cores; data pooled from four experiments. The concentration of vesicles per $\mu$m$^2$ of varicosity area is indicated by the open bars for total small vesicles and by the stippled bars for small vesicles with cores.

FIGURE 16 The relative lack of effect of stimulation on large vesicles; data pooled from four experiments. The concentration of vesicles per $\mu$m$^2$ of varicosity area is indicated by the open bars for total large vesicles and by the stippled bars for large vesicles with cores.

FIGURE 17 Summary of the changes in numbers of large and small dense-core vesicles immediately after stimulation and 2 h later.

would result from the addition of 60% of the membrane of the small vesicle pool (15-500-Å vesicles) to an average varicosity whose perimeter measured 2.77 $\mu$m. On this basis, one would expect the perimeter of stimulated varicosities to almost double. We in fact found no increase in varicosity perimeters (in general agreement with Coté et al. [13]), and therefore we can only speculate that the vesicle membrane, rather than enlarging the surface of the varicosity itself, migrated to the constricted regions of the axons between the varicosities which we did not measure. At cholinergic synapses (32, 43), it was possible to measure a significant increase in presynaptic surface area after stimulation, but this may only be characteristic of presynaptic elements whose shape and size are confined by contact with adjacent cells and which are thereby forced to sequester added membrane in convolutions of their own surfaces.

To visualize the process of membrane retrieval directly, we examined varicosities which had been briefly stimulated in HRP-containing medium and then immediately fixed. These preparations appeared similar to those fixed at rest, except that there was a larger number of coated pits forming from their plasma membranes. This prompted us to look carefully for coated vesicles at a variety of recovery times, and to try to enhance the preservation of the delicate "coat" material with the fixative of Kanaseki and Kadota (35), as well as by the freeze-substitution methods of Heuser (31). Unfortunately, we did not succeed in stabilizing large numbers of coated vesicles by those means, and thus conclude either that vesicles do not figure importantly in the retrieval process at this synapse, or that we cannot stabilize them in this tissue by the usual methods.

Thus, from a consideration of the number of vesicles in stimulated versus resting axons, and the uptake of HRP into these vesicles, we conclude that NE discharge is accomplished by exocytosis from small dense-core vesicles, and that the membrane of these vesicles is recycled locally. Membrane is retrieved at least in part by coated invaginations of the plasmalemma of the varicosity.

We have also learned something about the renewal of transmitter in a stimulated nerve ending, and about the relation between vesicle turnover and transmitter renewal. However, in making such a correlation on the basis of morphological data alone, we have had to make the assumption that the transmitter content of the tissue is reflected in the appearance of the vesicles' dense cores. Although it is generally agreed that the dense core results from the reduction of metallic
salts in the fixative by monoamines in the vesicles (5, 33, 48, 54), it remains less certain that quantitative assumptions can be made about relative NE concentrations on the basis of the size, number, or density of the cores.

The number of cores is probably a good indicator of catecholamine content because the number of cores can readily be reduced by catecholamine-depleting drugs such as reserpine (6, 42, 62) and α-methyl-p-tyrosine (57), and can readily be augmented by administration of exogenous amines (11, 55). It has recently been demonstrated that core size is also correlated with the NE concentration of the tissue under certain conditions (22, 24, 62). In contrast, the intensity of dense cores seems to be a poor indicator of catecholamine content (37).

One of the factors to which dense core size and number are most sensitive is the type of fixative used (54). For the purposes of this study, we assumed that under similar conditions of fixation and incubation, the number and size of dense cores should be equivalent. In control tissues, a stable baseline for these parameters was in fact obtained. Stimulation, in contrast, in all cases resulted in a decrease in the proportion of vesicles containing dense cores, and also in a reduction in size of the few remaining cores. This core depletion was at least partially reversible. In the tissues we examined 2 h after stimulation, there was a 50% increase in the proportion of vesicles containing cores, although many of these vesicles continued to show a smaller core diameter than controls.

The decline in the proportion of vesicles containing cores just after stimulation may reflect a preferential exocytosis of vesicles containing cores (i.e., transmitter). Alternatively, it may reflect the initial part of the recovery process, in which vesicle membrane has been retrieved from the plasmalemma but not yet filled with new transmitter. In the case of axons recovering in vitro, it seems probable that a latency exists between the reformation of vesicles and the refilling of these vesicles with transmitter. Whether this would also obtain in vivo is not known.

In parallel with the observations that vesicle recycling at the neuromuscular junction continued even when ACh synthesis was blocked by the inhibitor of choline uptake, hemicholinium (9, 32), drugs which inhibit uptake and synthesis of NE (i.e., DMI and ampt, respectively), did not hinder the retrieval of vesicle membrane at this synapse either. Vesicle membrane recycling seemed to proceed independent of a supply of transmitter.

However, we must consider that the drugs DMI and ampt may not have adequately blocked uptake and synthesis of transmitter in our studies, because no difference was apparent between the dense core contents of tissues which were allowed to recover with and without drug inhibition. The drug concentrations used (between 10^-3 and 10^-4 M) were the highest concentrations that could be used without introducing stimulation-like effects (51), yet the residual functions of the synthesis and uptake mechanisms were apparently active enough to produce many stainable cores in the new population of vesicles.

Several attempts have been made to deplete the vas deferens of NE by stimulation (4, 10, 23, 58), and all but one of these (58) have met with little success. Thus, we must reconcile the depletion of vesicles that we have observed with the failure of others to deplete NE stores measured biochemically. Possibly, the discrepancy can be accounted for by the difference in experimental technique. Our intense stimulation in the presence of NE uptake and synthesis inhibitors (augmented by the facilitation of transmitter release by blocking the negative feedback involving axonal α receptors) may produce significant depletion of tissue stores of NE. If depletion does not occur, the loss of small vesicles can be interpreted as the depletion of a readily releasable pool of transmitter, small compared to a stable pool of transmitter in the nerve (56) and muscle cytoplasm and in vesicles which were not released by the stimulation.

In summary, this study provides strong morphological evidence that NE is released from small dense-core vesicles by exocytosis. The membrane of these vesicles is recycled by the varicosities to form new vesicles within a short period of time, and at some time after new vesicles are formed, they become loaded with transmitter. This transmitter is first visible as a small, dense core which would become larger over time to correspond with the size of cores in control nerves.

Many questions remain to be explored. Among these are: What is the role of large dense-core vesicles? Are there mechanisms for membrane retrieval other than coated vesicle formation, and if so, what are they? Is the retrieved membrane identical to the original vesicle membrane, or does
any part of the plasmalemma invaginate and acquire the membrane-bound DBH and other constituents needed for conversion to a synaptic vesicle?

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