# Rapid Changes in Synaptic Vesicle Cytochemistry after Depolarization of Cultured Cholinergic Sympathetic Neurons

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ABSTRACT   Sympathetic neurons taken from rat superior cervical ganglia and grown in culture acquire cholinergic function under certain conditions. These cholinergic sympathetic neurons, however, retain a number of adrenergic properties, including the enzymes involved in the synthesis of norepinephrine (NE) and the storage of measurable amounts of NE. These neurons also retain a high affinity uptake system for NE; despite this, the majority of the synaptic vesicles remain clear even after incubation in catecholamines. The present study shows, however, that if these neurons are depolarized before incubation in catecholamine, the synaptic vesicles acquire dense cores indicative of amine storage. These manipulations are successful when cholinergic function is induced with either a medium that contains human placental serum and embryo extract or with heart-conditioned medium, and when the catecholamine is either NE or 5-hydroxydopamine. In some experiments, neurons are grown at low densities and shown to have cholinergic function by electrophysiological criteria. After incubation in NE, only 6% of the synaptic vesicles have dense cores. In contrast, similar neurons depolarized (80 mM $K^+$) before incubation in catecholamine contain 82% dense-cored vesicles. These results are confirmed in network cultures where the percentage of dense-cored vesicles is increased 2.5 to 6.5 times by depolarizing the neurons before incubation with catecholamine. In both single neurons and in network cultures, the vesicle reloading is inhibited by reducing vesicle release during depolarization with an increased $Mg^{++}/Ca^{++}$ ratio or by blocking NE uptake either at the plasma membrane (desipramine) or at the vesicle membrane (reserpine). In addition, choline appears to play a competitive role because its presence during incubation in NE or after reloading results in decreased numbers of dense-cored vesicles. We conclude that the depolarization step preceding catecholamine incubation acts to empty the vesicles of acetylcholine, thus allowing them to reload with catecholamine. These data also suggest that the same vesicles may contain both neurotransmitters simultaneously.

Sympathetic neurons from rat superior cervical ganglia acquire cholinergic characteristics when grown in vitro under some, but not all, conditions (13, 20, 24, 50). Cultures of these neurons therefore have been used as a model system to study what mechanisms regulate the expression of neurotransmitter genes in autonomic neurons. When grown under conditions fostering primarily adrenergic characteristics, these neurons have synaptic vesicles containing dense cores (20, 26, 30, 44). Dense cores in synaptic vesicles have been demonstrated in vivo in amine-storing neurons including the terminal plexus of the superior cervical ganglion neurons innervat-

ing the iris (17, 46) and are believed to indicate the storage of catecholamines within the vesicles (4). However, when the rat superior cervical ganglion neurons are grown under conditions which promote the acquisition of cholinergic properties, the majority of the synaptic vesicles lack dense cores (26, 30) and appear similar to the clear vesicles found in nonadrenergic neurons, including those of cholinergic neurons within the peripheral nervous system.

That sympathetic neurons need not lose adrenergic properties as they acquire cholinergic properties in culture has been well documented (9, 16, 19, 49). When grown in medium

that contains human placental serum and chick embryo extract, these neurons show a parallel increase in both cholinergic and adrenergic properties. Specifically, the activities of choline acetyltransferase, tyrosine hydroxylase, and dopamine-beta-hydroxylase all show similar increments over time in culture (19). Furthermore, even after several months under these conditions, the superior cervical ganglion neurons stain positively with a specific antibody to tyrosine hydroxylase (16, 19) and are capable of taking up tritiated norepinephrine (NE)[1] (49). In another medium which promotes the development of cholinergic characteristics, i.e., heart-conditioned medium, the quantity of adrenergic function has been reported to decrease as cholinergic function increases (39, 51); however, even in these studies, the data indicate that several adrenergic properties are retained at readily detectable levels in cholinergic sympathetic neurons. Thus, although NE synthesis and tyrosine hydroxylase activity decrease as acetylcholine synthesis increases, both adrenergic characteristics are measurable and most cells are tyrosine hydroxylase positive (39, 51). Moreover, when single neurons are grown on heart muscle islands in heart-conditioned medium, many neurons can be shown to release both NE and acetylcholine upon stimulation; indeed, it has been reported that under these circumstances most neurons exhibit dual physiological function and that purely cholinergic neurons are rather uncommon (13, 43).

Because sympathetic neurons retain adrenergic characteristics as they acquire cholinergic properties in culture, they provide an interesting system to study how such multifunctional neurons load transmitters into their synaptic vesicles. Earlier studies interpreted the presence of clear vesicles in cholinergic sympathetic neurons as evidence that the neuron had lost their capacity to store NE within small vesicles (30). Our present data suggest another interpretation. We report here that cultured cholinergic sympathetic neurons known to contain a predominantly clear vesicle population can, after acute treatment, be shown to contain dense-cored vesicles (DCV). The principal manipulation was depolarization of the neurons by exposure to high $K^+$, resulting in vesicle release before incubation in NE. We postulate that the $K^+$ acts to empty the vesicles of acetylcholine and allows them to be reloaded with NE. This study demonstrates that cholinergic sympathetic neurons in culture retain the capability of loading NE into their vesicles and do so whether the neurons are grown in medium that contains human placental serum and embryo extract or in heart-conditioned medium.

## MATERIALS AND METHODS

*Tissue Culture:* Neurons were dissociated from the superior cervical ganglia of perinatal rats using previously described methods (23). The neurons were then plated either at low density onto small collagen drops for the island cultures (see below) or at a density of 2,000–3,000 neurons per dish for network cultures. Island cultures were fed a medium containing 62% Eagle's minimum essential medium (Gibco Laboratories, Grand Island, NY), 24% human placental serum, 10% chick embryo extract, 1% 200 mM glutamine, 3% 1.1 M glucose, and 25 biological units/ml of nerve growth factor (47). Four separate network culture series were also fed this standard medium and studied for comparison with the island culture. To study vesicle reloading under another condition that promotes cholinergic function, two series of network cultures were established and maintained on 60% heart-conditioned medium prepared using the method detailed by Hawrot and Patterson (14). All cultures were fed

three times a week and incubated in a 5% $CO_2$ humidified atmosphere at 35°C. All cultures in both types of media were exposed to 5-fluorodeoxyuridine and uridine (both at 10 μM) for 2 d to eliminate nonneuronal cells.

The preparation of collagen-coated dishes for the network cultures has been described (23). For the island cultures, a method was adapted from that reported by Furshpan et al. (12). A 25-gauge needle was used to place collagen drops in a grid of 9–12 (500–2,000 μm) drops onto the same Aclar dishes (33C, 5 mil, Allied Chemical Corp., Morristown, NJ) used for the network cultures. The dishes were then exposed to ammonia vapors as previously described (23) and a second application of smaller collagen drops was air dried onto the initial collagen islands. Serial dilutions of a suspension of dissociated neurons were then added to the dishes. The attachment of neurons onto the collagen islands was monitored visually and the cultures were rinsed at appropriate times (30–90 min) to obtain islands with either single (Fig. 1 a) or multiple (3–7) neurons.

To study whether vesicle reloading might occur in synapses of nonsympathetic cholinergic neurons, cultures were used in which spinal cord cross-sections were grown adjacent to superior cervical ganglion explants (36). In similar cultures with dissociated superior cervical ganglion neurons, the spinal cord provides a cholinergic synaptic input to sympathetic neurons (27).

*Electrophysiological Methods:* Conventional electrophysiological techniques which have been previously described (16) were used to obtain intracellular recordings from neurons on collagen islands. Cultures were perfused with a balanced salt solution (BSS) (pH 7.2) which contained 124 mM NaCl, 12 mM NaOH, 30 mM HEPES, 2 mM $CaCl_2$, 5 mM KCl, 0.5 mM $MgCl_2$, and 11 mM dextrose. Acetylcholine synthesis was promoted by the addition of 30 μM choline chloride. The cultures were equilibrated in the choline-containing BSS 30–45 min before the start of the intracellular recordings.

*General Methods before Fixation:* All the island cultures had been in the standard BSS with 30 μM choline for up to 60 min before and during the electrophysiological recordings. Some cultures were rinsed briefly in BSS without choline and incubated for 30 min in 10 μM NE in BSS (5 mM KCl) with 0.2 mg/ml ascorbate. To test whether depolarization would allow the formation of DCV, other cultures were incubated for 45 min in BSS with 80 mM KCl (reducing the NaCl to 49 mM from the usual 124 mM). After several brief rinses in BSS (5 mM KCl), including one that contained 10 μM NE, the cultures were incubated in NE as detailed above. Network cultures were either rinsed briefly in BSS to remove the culture medium or were pre-incubated for 45 min in choline-containing BSS to be comparable to the island cultures used for electrophysiology. As with island cultures, some network cultures were incubated directly in 10 μM NE; others were first depolarized in 80 mM KCl before exposure to NE.

In several network culture series grown either on standard medium or heart-conditioned medium, 10 μM 5-hydroxydopamine (5OHDA) was used instead of NE for loading.

To reduce vesicle release during depolarization, we incubated both island and network cultures in BSS that contained 80 mM $K^+$, low $Ca^{++}$ (0.1 mM), and high $Mg^{++}$ (10 mM). Incubation in 2 μM reserpine (Ciba Pharmaceutical Co., Summit, NJ) (10) for 1–3 h before depolarization in 80 mM $K^+$ or in 5 μM desipramine (Geigy Pharmaceuticals, Ardsley, NY) (2, 21), 15 min before and during NE incubation, was used to block the uptake of NE at either the vesicle membrane or plasma membrane, respectively.

*Electron Microscopy:* Following the incubation protocols detailed above, all cultures were fixed in 3% $KMnO_4$ with the same procedures used in prior experiments (26). After embedding, the islands studied physiologically were identified from photomicrographs taken before the intracellular recordings. Those with single neurons or, in the initial experiments, a few with multiple neurons which had survived the incubation and fixation procedures, were mounted and sections were cut in a plane parallel to the collagen. Blocks from the network cultures that contained neurons and nerve fiber bundles were similarly mounted and sectioned. Without further staining, the sections were studied and photographed using a Philips 300 electron microscope.

*Vesicle Classification and Analysis:* The vesicle classification was done by a procedure similar to that reported previously (26). A minimum of 15 vesicle-containing varicosities were obtained for each island culture and 25 from each network culture. For some of the island cultures and for all of the network cultures, the sampling at the electron microscopic level and the vesicle counts were done by two observers. Because close agreement has been shown for two similarly treated cultures (26), in most experiments only one network culture per treatment group was analyzed. From prints at the same magnification (× 102,000), the vesicles were classified as previously described (26). Data from two observers again proved to be in close agreement (~5–8% discrepancy). Averages were thus obtained to give percent of vesicles considered to be dense cored for each treatment group. In addition, for each group a distribution of varicosities that contain increasing percentages of DCV could be generated as a histogram (see Fig. 3).
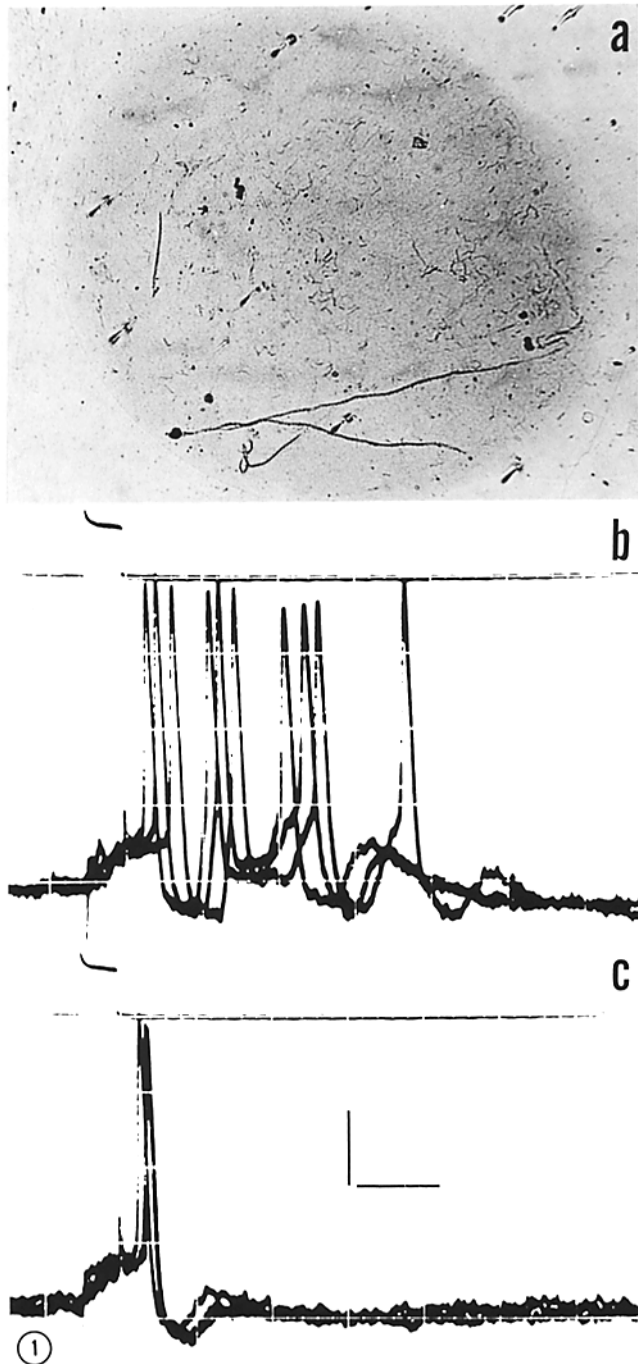
FIGURE 1 (a) Single sympathetic neuron grown on a small collagen island in medium that contained human placental serum and embryo extract. Two bundles of neurites extend from the neuronal soma. Photographed after fixation in KMnO₄ and embedding. 19 d in vitro. × 55. (b and c) Intracellular recording from the neuron in a in the absence (b) and in the presence (c) of 100 μM hexamethonium. In each of these records, the top trace shows the duration of the depolarizing current pulse (0.2 nA) used to evoke action potentials in this neuron. The bottom trace is a record of the transmembrane potential; in both b and c the responses to three successive depolarizations have been superimposed. In the absence of hexamethonium (b), each of the three action potentials that was directly evoked by intracellular stimulation was followed by a series of supra- and sub-threshold depolarizations. Thus, this neuron appeared to have formed one or more synapses upon itself. In the presence of hexamethonium (c), a nicotinic, cholinergic antagonist, each of the three depolarizing stimuli evoked only a single action potential; synaptic potentials were either absent or greatly reduced

## RESULTS

### General Observations in Culture

The development of dissociated embryonic neurons from the superior cervical ganglia in the network culture has been described in detail elsewhere (26). By 2 wk in vitro, these cultures had well-developed fascicles of nerve fibers connecting single or small groups of neurons. They were subsequently cultured for a total of 5–9 wk in vitro because prior studies using this culture system have demonstrated that substantial cholinergic function (16, 19, 24, 26) has developed by this time.

The island cultures showed a similar pattern of development. Neurons were observed to extend processes in the first 24 h of culture, and when an island supported more than one neuron a network of nerve fiber bundles was present after several weeks. The processes from isolated single neurons tended to turn upon themselves and form one or more fascicles; thus, a single neuron often had several large bundles of fibers coming from the soma (Fig. 1 a). This configuration facilitated the electron microscopic sampling of vesicle-containing varicosities. Because the collagen islands tended to lift off the Aclar dishes after 3–4 wk in vitro, the island cultures were studied between day 16 and day 21 in vitro.

### Excitatory Synapses in Island Cultures

Intracellular recordings were obtained from 68 neurons on collagen islands to determine whether they had formed excitatory synapses. In a few initial experiments, islands bearing several (≤7) neurons were used; in subsequent experiments, only islands with a single neuron were chosen. 85% ($n = 57$) of the neurons tested were found to have formed synapses; when stimulated, they caused excitatory synaptic potentials to appear in either themselves (Fig. 1 b) or in another neuron. Previous studies have shown that, when maintained in the culture medium used in this study, embryonic sympathetic neurons form nicotinic cholinergic synapses (28, 35). In agreement with these earlier findings on network cultures, we found that, in all cases tested ($n = 12$), hexamethonium (50–100 μM) inhibited synaptic transmission among neurons on collagen islands (Fig. 1 c).

Only those islands on which all of the neurons were found to have formed excitatory synapses were processed for electron microscopy. Of the 57 excitatory neurons, 31 (54%) subsequently yielded suitable electron microscopic data for analysis (Table I).

### Vesicle Analysis on Island Cultures

The number of DCV given as a percentage of the total vesicles (30–70 nm in diameter) for the various groups studied in the island cultures is summarized in Table I. Only 6% of the vesicles had dense cores in cultures incubated in NE without prior depolarization. An example of a varicosity from this group that contained a predominance of clear vesicles is shown in Fig. 2 a. When the incubation in NE followed depolarization, 82% of the vesicles now contained dense cores

in amplitude. When hexamethonium was removed from the perfusate, synaptic potentials reappeared within 5 min (not shown). The vertical bar represents 20 mV; the horizontal bar represents 20 ms.

219

TABLE I. *Summary of the Percentage of DCV in Neuronal Island Cultures*

| | Number of neurons | Number of varicosities | Number of vesicles | % DCV |
|---|---|---|---|---|
| 5 mM K⁺ → NE | 17* | 95 | 2,584 | 6% |
| 80 mM K⁺ → NE without choline | 4 | 60 | 1,408 | 82% |
| 80 mM K⁺ → NE + choline[‡] | 1 | 39 | 406 | 5% |
| 80 mM K⁺ (↓Ca⁺⁺, ↑Mg⁺⁺)[§] → NE | 2 | 30 | 252 | 6% |
| Reserpine[∥] → 80 mM K⁺ → NE | 4 | 60 | 1,216 | 12% |
| 80 mM K⁺ → NE + Desipramine[¶] | 3 | 45 | 951 | 3% |

* This number includes three islands with 3, 4, and 7 neurons each and 3 single neurons.
[‡] Concentration of choline was 30 μM.
[§] Concentration of Ca⁺⁺ was 0.1 mM and of Mg⁺⁺ was 10 mM.
[∥] Reserpine was added at 2 μM for 1–3 h before electrophysiological analysis.
[¶] Desipramine was added at 5 μM 15 min before and during NE incubation.

(Fig. 2b). The effects of depolarization on the loading of NE into vesicles required exocytosis as only a small percent of DCV were observed in cultures exposed to 80 mM K⁺ in the presence of an elevated Mg⁺⁺/Ca⁺⁺ ratio (Table I). Furthermore, uptake of NE after depolarization appeared to involve two specific transport mechanisms. Only 3% of the vesicles were dense cored when NE uptake at the neuronal membrane was reduced in the presence of desipramine (2, 21) (Table I). Blocking NE uptake at the vesicle membrane level by incubating the cultures in reserpine (10) before the start of the experiments also resulted in a low percent of DCV (12%, Table I). A varicosity (from a desipramine-treated neuron) with a clear vesicle population typical of all three of these groups is shown in Fig. 2c. This vesicle-containing varicosity was also similar to those photographed from the single neuron which was exposed to choline during NE incubation. The question asked with this experiment was whether, following depolarization, the presence of choline during NE incubation would inhibit the formation of dense cores within the vesicles. As seen in Table I, choline appeared to compete in some way with NE and the predominant vesicle type was clear (5% DCV).

Fig. 3 shows the distribution of varicosities that contained increasing percentages of DCV for the six treatment groups using island cultures. Of the varicosities from neurons exposed to NE after depolarization, none had <60% DCV. In contrast, neurons from the nondepolarized control, as well as the other groups (reserpine, desipramine, and depolarization with an elevated Mg⁺⁺/Ca⁺⁺ ratio), had few varicosities with >20% DCV and none with >40% DCV. Similarly, if choline was present during NE incubation after depolarization, no varicosity had >40% DCV.

## Vesicle Analysis on Network Cultures Grown in Human Placental Serum and Chick Embryo Extract

The results of experiments using network cultures generally confirmed and expanded the findings from the island cultures (compare Table I with Table II and Fig. 3). Thus, in four separate culture series, the varicosities of cultures exposed to

NE without prior depolarization had predominantly clear vesicles (see Fig. 4a); only 20 ± 3% (mean ± SEM.) of the vesicles were dense cored (Table II); this confirms earlier work from our laboratory (25, 26). Furthermore, when the varicosities were classified as to the percent of DCV (Fig. 3), the distribution was similar to that of prior studies at comparable times in culture (see Fig. 4, ref. 26). Thus, 71% of the control varicosities had predominantly clear vesicles (i.e., contained <20% DCV) and only 12% had >80% DCV. In contrast, network cultures treated with 80 mM K⁺ before NE incubation had 68 ± 5% (mean ± S.E.M.) of the vesicles classified as dense cored (Table II; see also Fig. 4b); therefore, depolarization prior to NE incubation increased the percentage of DCV by 2.5- to 5.4-fold. Similarly, the distribution of varicosities that contained increasing proportions of dense cores changed; after depolarization, 60% of the varicosities contained >80% DCV (Fig. 3, RELOAD). Unlike island cultures, however, some of the varicosities in network cultures contained predominantly clear vesicles (i.e., some of the varicosities had <40% DCV).

As in island cultures, the effect of K⁺ on reloading in the network cultures appeared to depend on exocytosis and to require specific uptake of NE. Thus, increasing the Mg⁺⁺/Ca⁺⁺ ratio during K⁺ depolarization or treating with desipramine or reserpine decreased the percentage of DCV from 75% to 19%, 6%, and 34%, respectively (Table II). Similarly, if choline was present during NE incubation, the percentage of DCV was low (14 and 15% for two separate culture series, Table II). The varicosity distribution of all of these groups thus resembled the nondepolarized group (Fig. 3).

Because the results in the network cultures paralleled those of the island cultures so closely, a number of experiments were done using only the network cultures. In several experiments in which cultures were fixed immediately following K⁺ depolarization, the percentage of DCV was low (11%). In addition, the number of vesicles per varicosity appeared to be decreased and the size of vesicles appeared to be smaller than in control nondepolarized cultures; however, rigorous quantitation of these observations has not been done (see, however, reference 8). Two protocols were used to determine how readily the population of DCV that had been generated by depolarization and reloading with NE could be reversed to a population of clear vesicles. If a second K⁺ depolarization followed NE incubation and the cultures were then incubated in choline, the percentage of DCV was now 16% (compared to 21% for control and 52% for the depolarized and NE-reloaded neurons, series 1471, Table II). Finally, simply incubating the neurons for 45 min in 30 μM choline after NE incubation resulted in control levels of DCV (21% vs. 26%, series 1514, Table II). Thus, a low (control) percentage of DCV are found if choline is present (a) during NE incubation, or (b) after reloading with NE (with or without a second depolarization in 80 mM K⁺).

## Vesicle Analysis on Network Cultures Grown in Heart-conditioned Medium

The results using network cultures grown for 7 or 14 wk in heart-conditioned medium were the same as those from cultures grown in human placental serum and embryo extract (Table III). Without prior depolarization, the percentage of DCV was low (16%, 14%, 11%) when the incubation was done in either NE or 5OHDA. A representative varicosity that
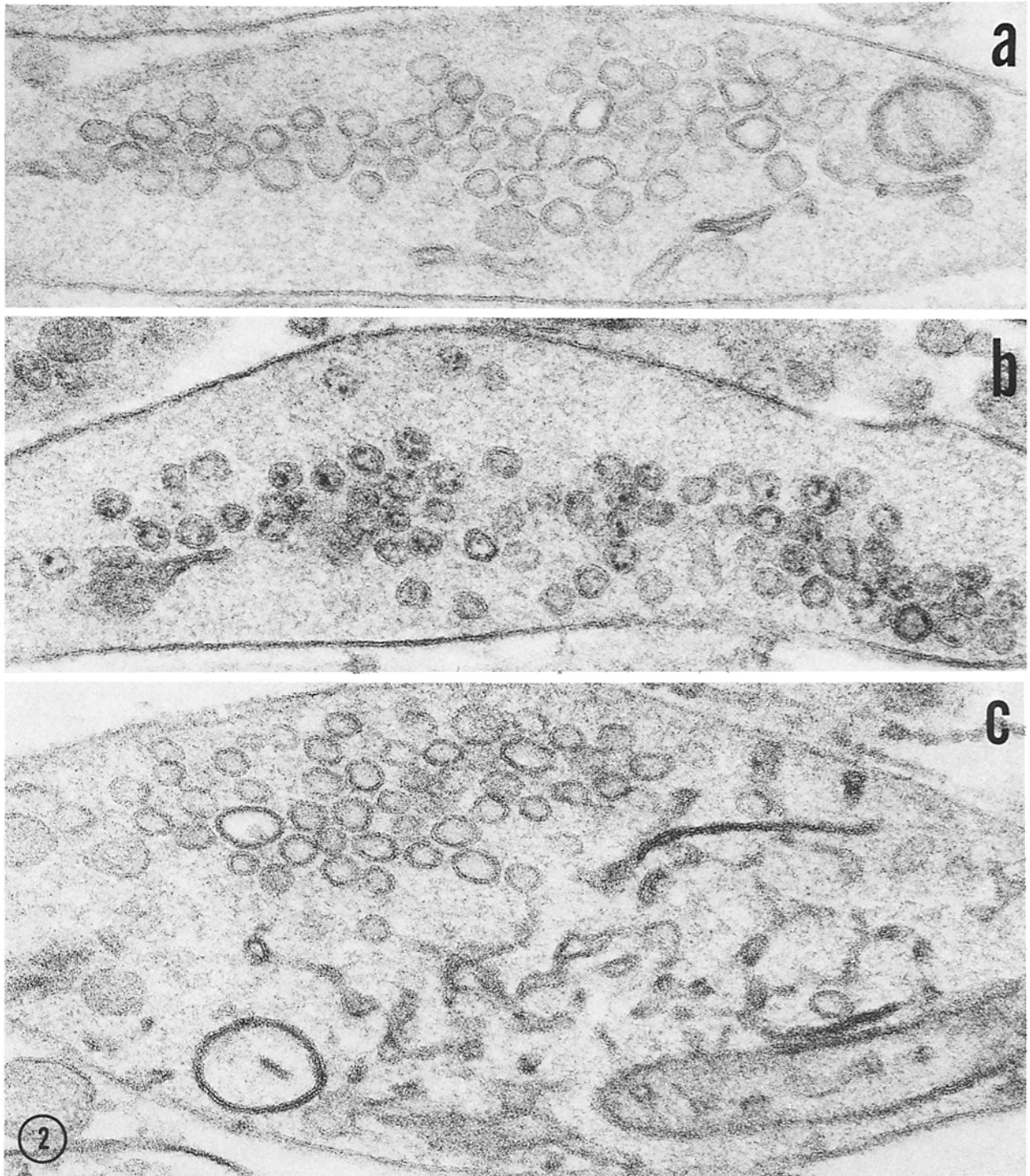
FIGURE 2  Electron micrographs of vesicle-containing varicosities from dissociated rat superior cervical ganglia neurons grown on collagen islands in human placental serum and embryo extract. All neurons were found to be cholinergic with intracellular recordings. (a) Varicosity from a control neuron incubated in NE before fixation in $KMnO_4$. The vesicles are predominantly clear. (b) Varicosity from a neuron which was depolarized with 80 mM $K^+$ before it was incubated in NE. The majority of the vesicles contain dense cores. (c) Varicosity from a neuron which was also depolarized in 80 mM $K^+$ after physiological study but was treated with desipramine before and during incubation in NE. Similar to the control (a) which was not depolarized, the vesicle population is predominantly clear. This varicosity also resembles those from neurons treated with an increased $Mg^{++}/Ca^{++}$ ratio during incubation in 80 mM $K^+$, those from neurons treated with reserpine, and those from the single neuron treated with 30 $\mu M$ choline simultaneously with NE (see Fig. 3). Each of the three varicosities (a, b, and c) are similar to the varicosities found in comparable groups from network cultures (see Tables I and II and Fig. 3). $KMnO_4$ fixation. (a and b) 16 d in vitro. (c) 19 d in vitro. × 115,000.

contained clear vesicles is shown in Fig. 4a. After depolarization, the percentage of DCV was increased 4.7- to 6.5-fold with the percentage of DCV ranging from 69 to 91%. A representative varicosity that contained DCV is illustrated in Fig. 4b. The DCV generated using 50HDA following depolarization were also seen in network cultures grown in the
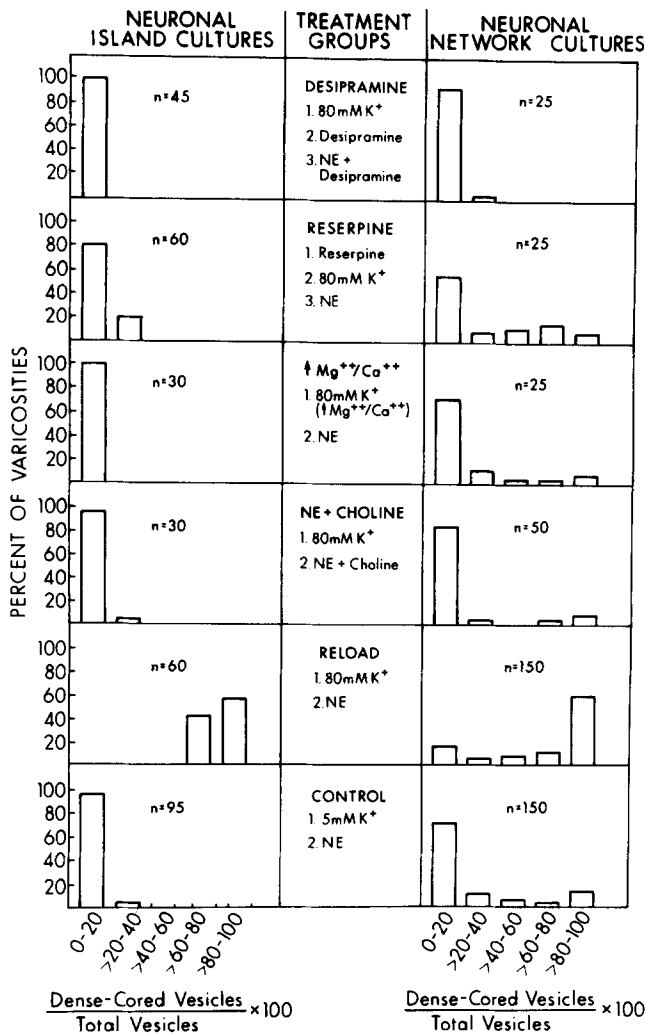
FIGURE 3   Histograms showing the percentage of varicosities that contain increasing percentages of DCV for six treatment groups in both neuronal island and network cultures grown in human placental serum and embryo extract. The data are derived from the same groups shown in Tables I and II and show the number of DCV as a percentage of the total vesicles for each varicosity. The number (n) of varicosities is shown for each group. For the control group (CONTROL) in which NE incubation was done without prior depolarization, the majority of the varicosities contain <40% DCV. With depolarization (RELOAD), the majority contain >60% DCV. When NE uptake was blocked (DESIPRAMINE or RESERPINE) or when vesicle release was reduced during depolarization ($\uparrow$ Mg$^{++}$/Ca$^{++}$), the histogram profiles resemble that of the controls, i.e., the varicosities contain predominantly clear vesicles. Similarly, when choline was present during the incubation in NE (NE + CHOLINE), the synaptic vesicles are predominantly clear.

medium that contained human placental serum and embryo extract (example not shown). Thus, the vesicle population of cholinergic sympathetic neurons in culture appeared to be capable of amine storage when grown in either of two media and when incubated in either of two amines.

## Vesicle Analysis on Spinal Cord–Superior Cervical Ganglia Co-cultures

To determine whether all cholinergic neurons have the capacity to load NE into synaptic vesicle after depolarization or whether it is unique to cholinergic sympathetic neurons, we looked at synaptic vesicles within spinal cord neurons exposed to 80 mM K$^+$ prior to NE incubation. A general description of the morphology of these cultures is given elsewhere (36). Using the same criteria as for the synaptic vesicles of superior cervical ganglion neurons, we judged only 2.7% of the vesicles to be dense cored following K$^+$ and NE (Fig. 5) compared to 2.2% DCV in similar cultures not exposed to depolarizing conditions. Thus we found no evidence for NE-loading into spinal cord neurons providing cholinergic input to superior cervical ganglion neurons in vitro.

## DISCUSSION

Rat sympathetic neurons in culture retain a number of adrenergic characteristics as they acquire cholinergic properties (9, 16, 19, 26, 49, 51). However, even though these cholinergic sympathetic neurons have the enzymes necessary for NE synthesis (19, 26) and possess some store of endogenous NE (20), the synaptic vesicles are clear, i.e., they lack cytochemically detectable stores of NE (26, 29, 30). Moreover, the majority of the vesicles remain clear when neurons are incubated in a catecholamine (26, 29, 30). This latter observation has been somewhat puzzling as these neurons retain a high affinity uptake system for NE (sensitive to desipramine) as they acquire cholinergic function (29, 45, 49). The results of this study show that sympathetic neurons grown in culture and known to contain clear vesicles can, with brief alteration of their culture environment, come to contain DCV. The manipulation used was depolarization in 80 mM K$^+$ followed by incubation in NE. Our hypothesis is that the depolarization-induced vesicle release empties the vesicles of their acetylcholine and allows the subsequent reloading with NE. Thus, if the vesicle release is blocked by increasing the Mg$^{++}$/Ca$^{++}$ ratio during depolarization, reloading with NE does not occur.

Although vesicle release is a necessary condition for the generation of DCV, our data show that specific plasma membrane uptake and vesicle loading mechanisms for NE are also required. Thus the percentage of DCV was low when the

TABLE II.   Summary of the Percentage of DCV in Neuronal Network Cultures Grown in Human Placental Serum and Embryo Extract

| | | | | | | | | | 80 mM K$^+$ | |
| | | | | | | | | | $\rightarrow$ NE $\rightarrow$ | 80 mM K$^+$ |
| | | | | | 80 mM K$^+$ | 80 mM K$^+$ | Reserpine | 80 mM K$^+$ | 80 mM K$^+$ | $\rightarrow$ NE $\rightarrow$ |
| Culture | Weeks | Number of | 5 mM K$^+$ | 80 mM K$^+$ | $\rightarrow$ NE + | ($\uparrow$Mg$^{++}$/ | $\rightarrow$ 80 mM | $\rightarrow$ NE + | $\rightarrow$ 80 mM K$^+$ | choline |
| series | in vitro | varicosities | $\rightarrow$ NE | $\rightarrow$ NE | choline | Ca$^{++}$) | K$^+$ $\rightarrow$ NE | DMI | $\rightarrow$ choline | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1394 | 9 | 100[‡] | 21 | 75 | — | — | — | — | — | — |
| 1451 | 5 | 125 | 14 | 75 | — | 19 | 34 | 6 | — | — |
| 1471 | 6 | 100 | 21 | 52 | 14 | — | — | — | 16 | — |
| 1514 | 5 | 100 | 26 | 68 | 15 | — | — | — | — | 21 |

\* Specific concentrations used were as follows: NE, 10 $\mu$M; choline, 30 $\mu$M; Mg$^{++}$/Ca$^{++}$, 10 mM/0.1 mM; DMI, 5 $\mu$M; reserpine, 2 $\mu$M.
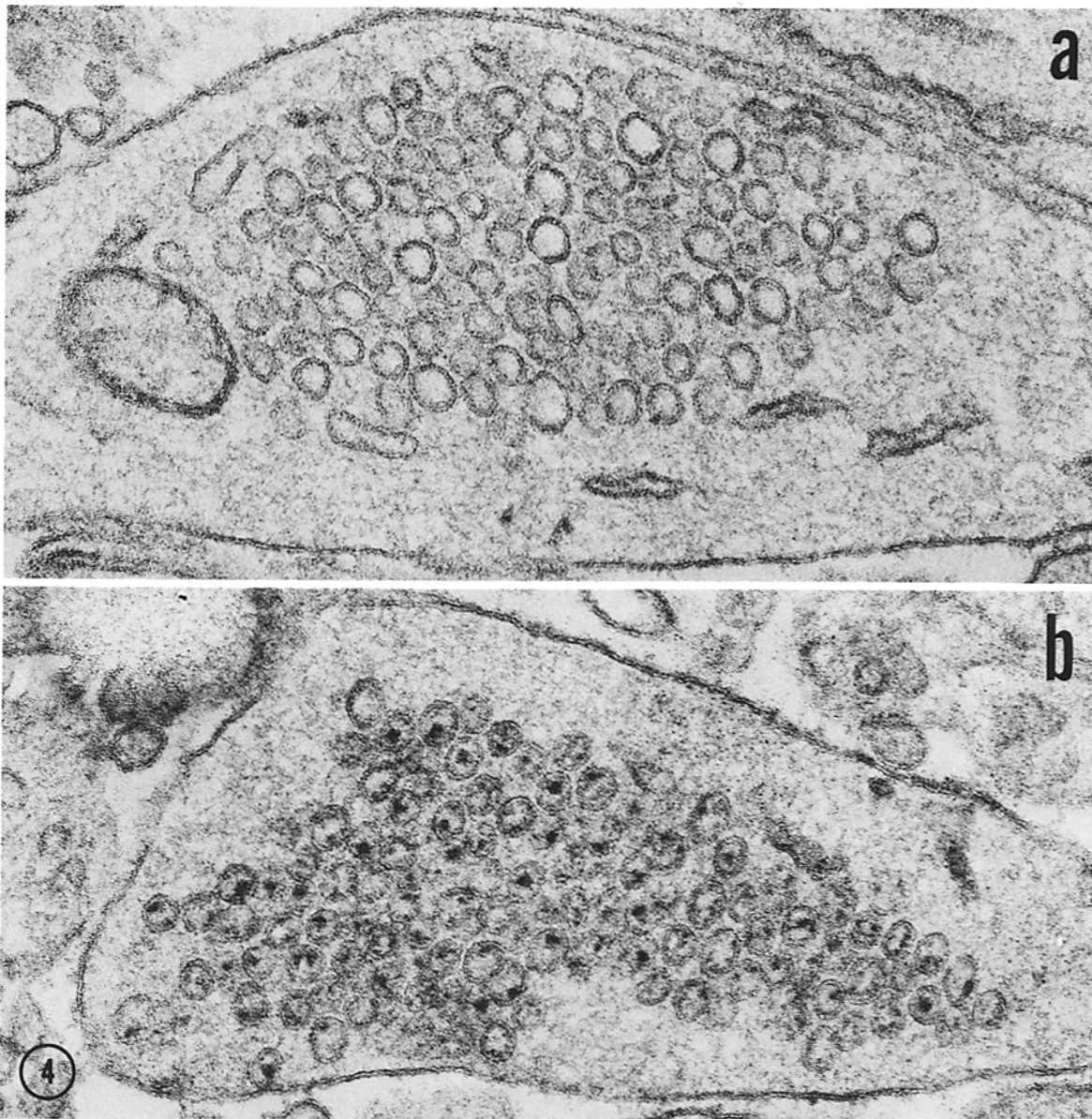‡ Each group had 50 varicosities in series 1394; all other series had 25 varicosities per group.

FIGURE 4  Electron micrographs of vesicle-containing varicosities from dissociated rat superior cervical ganglion neurons grown as network cultures in 60% heart-conditioned medium. (a) A varicosity from a neuronal culture (series 1542, Table III) incubated in 50HDA without prior depolarization. The vesicles are clear. (b) A varicosity from a sister culture that had been exposed to 80 mM K⁺ prior to 50HDA incubation. The majority of vesicles now contain dense cores. 7 wk in vitro. KMnO₄ fixation. × 115,000.

TABLE III.  Summary of the Percentage of DCV in Neuronal Network Cultures Grown in Heart-conditioned Medium

| Series | Weeks in vitro | Treatment group | % DCV |
|---|---|---|---|
| 1542 | 7 | 5 mM K⁺ → NE* | 16 |
| | 7 | 80 mM K⁺ → NE | 75 |
| | 7 | 5 mM K⁺ → 50HDA* | 14 |
| | 7 | 80 mM K⁺ → 50HDA | 91 |
| 1568 | 14 | 5 mM K⁺ → 50HDA | 11 |
| | 14 | 80 mM K⁺ → 50HDA | 72 |
| | 14 | 80 mM K⁺ → NE | 69 |

* Both NE and 50HDA were used at a concentration of 10 μM.

transport of NE across the plasma membrane was inhibited by desipramine or when the uptake into the vesicle was inhibited by reserpine (Fig. 3, Tables I and II). Moreover, synapses formed by spinal cord neurons upon principal neurons of superior cervical ganglion explants contained few

DCV after depolarization and incubation in NE because the spinal cord presynaptic endings lack the specific uptake system for catecholamines and do not accumulate cytochemically demonstrable amounts of catecholamine after incubations in rather high concentrations (10 μM) of such transmitters. Cholinergic vesicles isolated from Torpedo do, in fact, take up catecholamines (34). Thus, the cytochemical demonstration of NE in synaptic vesicles after depolarization does not represent NE passively enclosed within recycling membrane as the vesicles are formed.

Although the dense cores of small granular synaptic vesicles have been generally accepted as an indication of catecholamine storage (17, 46), this correlation has been questioned (for discussion, see reference 11). Recent studies, however, have provided evidence that such a correlation exists. In vitro incubation of rat vas deferens in high K⁺ resulted in a parallel decrease in NE and the number of small DCV (42). Similarly, fractions of small synaptic vesicles isolated from the seminal

FIGURE 5   Electron micrograph of a synapse formed by a spinal cord neuron upon a superior cervical ganglia neuron (N) in co-culture. The culture was depolarized in 80 mM K⁺ prior to incubation in NE. Despite this, the vesicle population is predominantly clear. Only 2.7% of the vesicles were considered dense cored in 15 total varicosities studied. 5 wk in vitro. × 115,000.

ducts of castrated rats and subjected to varying incubation conditions have shown a correlation between NE content and the presence of dense cores (11). Intravesicular densities have also been reported to result from fixation in the divalent ion calcium (5 to 90 mM) (6, 7, 37, 41). Electron-dense particles as indicators of calcium binding sites of synaptic vesicles have thus been reported in neuromuscular junctions (37, 41) and in electric organs (6, 7), as well as in isolated vesicles (6) and in mitochondria (see reference 41). That calcium deposition is contributing to the appearance of dense cores in our study is unlikely for several reasons. The concentration of calcium was no greater than 2 mM compared to at least 5 mM and more often the 50 to 90 mM reported for visualization of calcium binding sites (6, 7, 37, 41). Secondly, the calcium concentration was constant for both nondepolarized and depolarized cultures in our studies. Lastly, the electron densities indicating calcium binding sites are reduced with stimulation or depolarization (7, 37). In the present study, depolarization was required to increase the numbers of DCV.

The reloading of vesicles was observed in cultures in which cholinergic function was induced by either of two different media (human placental serum with embryo extract or heart-conditioned media) and with two different catecholamines (NE and 50HDA). The results therefore do not appear to be idiosyncratic to a particular set of culture conditions. Moreover, in both of the culture media used (Table I and Higgins, D., unpublished data), most (>85%) of the neurons have already formed cholinergic synapses by day 16 in vitro; yet the vesicles of such neurons could be reloaded as efficiently after 14 wk as after 2½ wk in vitro (Tables I and III). It would appear that cholinergic sympathetic neurons in vitro retain the capacity to take up and store NE for at least several months after they have begun to form cholinergic synapses.

Although the data obtained from island cultures were similar to that of network cultures, some quantitative differences were observed. The percentage of DCV in the island cultures which had not been depolarized was lower (6%) than in the network cultures (21%). This difference is not surprising in that only those neurons (85%) shown to have cholinergic function (and therefore containing clear vesicles) were selected from the island cultures for analysis. A population (15%) in which cholinergic function was not observed was excluded in the island cultures but was included in the network cultures. Although previous studies have emphasized the fact that the majority of the perinatal superior cervical ganglion neurons are capable of acquiring cholinergic properties (16, 26, 30), these studies have also revealed that there is heterogeneity in the response of the neurons to the culture conditions and, even under conditions where considerable cholinergic function (e.g., choline acetyltransferase activity) is demonstrable, some of the varicosities contain predominantly DCV (see Fig. 3, *NEURONAL NETWORK CONTROL*, and ref. 26). The heterogeneity with respect to neurotransmitter status has also been shown using physiological analyses (13, 43). In the present study, even though the neurons were selected have cholinergic function, the island cultures appeared to reload more effectively. Thus, 82% of the vesicles were dense cored after reloading (Table I) and no varicosity containing <60% DCV was found (Fig. 3). In contrast, some 29% of the varicosities in network culture contained substantial numbers of clear vesicles (≥40%) after depolarization and reloading. These differences are probably due to technical differences in the way the two types of cultures were handled. Because of both the very low cell density and their fragility, the island cultures were only very briefly rinsed after NE incubation before fixation. In contrast, the networks with their substantial

cell density and thick neuritic bundles received rinses over a 10–15-min period before fixation. We know from very recent studies that incubation in BSS for 30 min even without choline results in a significant decrease (i.e., 33%) in the percentage of DCV (unpublished results). Thus, the network cultures very likely had some reversal of loading during the standard rinses before fixation and, therefore, showed greater numbers of clear vesicles.

Both immunocytochemical and physiological studies give evidence that multiple transmitters can be present within a single neuron (5, 18, 22) in vivo. The presence of multiple transmitters within a single neuron immediately introduces the question of how a neuron regulates the synthesis, storage, and release of the various different transmitters. When dual function in superior cervical ganglion neurons grown in vitro was demonstrated (12), the discussion began as to whether the synaptic endings or the vesicles themselves were dual in function (13). Our data confirm that a single neuron can generate vesicles for storage of either of two transmitters. It also suggests that some form of competition exists between acetylcholine and NE for loading into vesicles. If 30 $\mu$M choline is present with NE in the incubation medium after the $K^+$ depolarization (at a time when vesicles are presumably being formed and reloaded), the percentage of DCV remains low (Tables I and II; Fig. 3). In addition, if, after reloading with NE, the neurons are simply placed in BSS that contains choline for 45 min, the vesicle population becomes clear (series 1514, Table II). Because recent evidence indicates that acetylcholine is specifically preferred over choline for uptake into vesicles (1, 38), we assume that it is ultimately acetylcholine competing with NE. Depolarization with vesicle release thus appears to give for at least a short time the advantage to NE over acetylcholine in terms of vesicle loading.

Our present study further suggests that two transmitters may be stored in the same vesicle and that the *same* population of vesicles that are clear in control conditions are dense cored after depolarization and NE incubation. Studies by other investigators (3, 8) have shown that a substantial portion of NE-reloaded DCV may in fact be vesicles which have been recyled. Vesicle recycling has been demonstrated at the neuromuscular junction (15) and recent studies have provided evidence that a similar mechanism operates in the varicosities of sympathetic neurons. In two different sympathetic systems, a significant decrease in the number of small dense-cored synaptic vesicles occurred when depolarization was accomplished either by electrical stimulation (3) or by elevated $K^+$ (8). Furthermore, in both studies, if horseradish peroxidase was present during both the depolarization-induced vesicle release and the recovery period, substantial numbers of vesicles contained the tracer. These experiments indicate that many of the DCV that we found were recycled vesicles observed to be clear before depolarization and reloading with NE. In addition, from our data, the substantial numbers of clear vesicles seen after merely reincubating the neurons in choline-containing BSS without a second exposure to depolarizing conditions (Table II) would also argue that the same population of vesicles which are dense cored after the usual depolarization and reloading protocol become clear.

Obtaining direct evidence for the simultaneous presence of NE and acetylcholine in the same vesicle is hampered by the lack of a specific and sensitive morphological assay for localization of acetylcholine. One possibility that we cannot presently resolve is that NE remains within the vesicle as acetyl-

choline accumulates but that under these conditions the presence of catecholamine can no longer be demonstrated by $KMnO_4$ fixation. The use of isolated vesicle preparations from cultures of cholinergic sympathetic neurons to test relative loading of radiolabeled NE and acetylcholine could provide valuable information concerning not only the simultaneous presence of the two transmitters but also the proposed competition. Immunocytochemical evidence of two transmitters in one vesicle is available for terminals in the raphe nuclei and dorsal horn of the spinal cord of the rat where some vesicles have been shown to have both substance P and serotonin (40). Similarly, secretory granules of the adrenal medulla are known to contain both catecholamines and enkephalins (48). In both instances, however, the vesicles are larger ($\geq 60$ nm) and not of the small vesicle size studied here. In yet another instance, however, evidence suggests that different transmitters may be stored in different vesicles; thus vasoactive intestinal peptide is contained in large DCV and acetylcholine in small, clear vesicles (33). Because the variety of neurotransmitters now known include not only NE and acetylcholine but a number of amino acids and peptides, more than one mechanism of handling multiple transmitters within a neuron might be expected.

Assuming that clear vesicles in fact indicate decreased intravesicular NE (see above), the reason that the cholinergic system may be more effective in commanding intravesicular space for its neurotransmitter, acetylcholine, than the catecholamine system for NE is not known. A possible hypothesis might be summarized as follows. The superior cervical ganglion neuron in vitro responds to environmental cues and develops increasing cholinergic function (i.e., choline acetyltransferase activity). As the activity of the enzyme increases, so does the intracellular concentration of acetylcholine. Acetylcholine then competes for intravesicular storage and causes NE to be shifted elsewhere within the neuron. Increased norepinephrine storage in a nonvesicular compartment could result in a feedback inhibition of tyrosine hydroxylase and this would cause a decrease in NE synthesis. Morphologically, in the early stages of increasing cholinergic function, the dense cores observed within vesicles become smaller and less dense and clear vesicles appear in varicosities that increasingly contain a mixed population of clear vesicles and DCV (26, 30). The numbers of clear vesicles then increase as cholinergic function becomes well established. A similar sequence of events may occur in vivo during the development of the cholinergic sympathetic neuron (31, 32). Neurons innervating the sweat glands of the foot pad can be shown initially to demonstrate adrenergic characteristics (formaldehyde-induced fluorescence, small DCV). Postnatally, cholinergic characteristics including acetylcholinesterase staining and clear vesicles are increasingly demonstrable. Nevertheless, even in the adult rat, at least one adrenergic characteristic, the catecholamine uptake system, is retained in cholinergic sympathetic axons. Although the hypothesis proposed above would be difficult to test in vivo, studies are currently underway to obtain further information concerning the presence of competition between the two neurotransmitter systems within cholinergic sympathetic neurons in vitro.

In conclusion, we have shown that the cytochemistry of synaptic vesicles can be acutely altered in sympathetic neurons that are functionally cholinergic. This is true for each of two media used to induce cholinergic function. These observations may bear more generally on the questions surrounding the

regulation of multiple transmitters within a single neuron.

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