

CIRCULATION AND TURNOVER OF SYNAPTIC VESICLE MEMBRANE IN CULTURED FETAL MAMMALIAN SPINAL CORD NEURONS

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ABSTRACT

Intact neurons in cultures of fetal rodent spinal cord explants show stimulation-dependent uptake of horseradish peroxidase (HRP) into many small vesicles and occasional tubules and multivesicular bodies (MVB) at presynaptic terminals. Presynaptic terminals were allowed to take up HRP during 1 h of strychnine-enhanced stimulation of synaptic transmitter release and then "chased" in tracer-free medium either with strychnine or with 10 mM Mg^{++} which depresses transmitter release. Tracer-containing vesicles are lost from terminals under both chase conditions; the loss is more rapid (4–8 h) with strychnine than with 10 mM Mg^{++} (8–16 h). There is a parallel decrease in the numbers of labeled MVB's at terminals. Loss of tracer with 10 mM Mg^{++} does not appear to be due to the membrane rearrangements (exocytosis coupled to endocytosis) that presumably lead to initial tracer uptake; terminals exposed to HRP and Mg^{++} for up to 16 h show little tracer uptake into vesicles. Nor is the decrease likely to be due to loss of HRP enzyme activity; HRP is very stable in solution. During the chases there is a striking accumulation of HRP in perikarya that is far more extensive in cultures initially exposed to tracer with strychnine than 10 mM Mg^{++} regardless of chase conditions. Much of the tracer ends up in large dense bodies. These findings suggest that synaptic vesicle membrane turnover involves retrograde axonal transport of membrane to neuronal perikarya for further processing, including lysosomal degradation. The more rapid (4–8 h) loss of tracer-containing vesicles with strychnine may reflect vesicle membrane reutilization for exocytosis.

Recent work on neuromuscular junctions by Holtzman et al. (33), by Ceccarelli et al. (12, 13) and by Heuser and Reese (29) has established that, in these nerve terminals, processes resembling endocytosis accompany synaptic transmitter re-lease. Stimulation of synaptic transmission at neuromuscular junctions in the presence of extracellular tracers (horseradish peroxidase [HRP] or dextrans) leads to the appearance of tracer in numerous vesicles morphologically resembling

small synaptic vesicles (12, 13, 29, 33). Similar events probably occur in other neuronal systems (25, 56). The eventual fate of the vesicles that are formed in this way is not clear. An attractive and widely held view is that many of these vesicles are reutilized for transmitter storage and release, and some important indirect evidence supports this notion (13, 29). However, there is also considerable evidence indicating that synaptic vesicles have a finite half-life and are eventually turned over (8, 23, 35, 42, 56, 64).

Most previous work on membrane circulation in neurons has been restricted by the fact that the experiments were done on freshly isolated preparations in which the axonal connection between neuronal perikarya and terminals was cut. Thus, the overall pattern of membrane transport throughout the neurons could not be examined. The present report concerns a study of intact neurons in cultures of fetal rodent spinal cord explants. We have demonstrated the same kind of stimulation-dependent accumulation of tracer into presynaptic vesicles in this central nervous system tissue as is found at peripheral neuromuscular junctions. In addition, our analysis of the behavior of tracer-labeled vesicles strongly suggests that synaptic vesicle membrane turnover involves transport of membrane back to neuronal perikarya for eventual degradation. This process appears to be relatively slow, requiring hours to produce a significant decline in the population of tracer-containing vesicles in terminals. Over shorter periods of time there is a more rapid stimulation-dependent depletion of tracer-filled vesicles; this finding is consistent with the evidence from neuromuscular junctions that has been interpreted as supporting vesicle reutilization (13, 29).

Preliminary reports of these studies have been published (32, 35, 60, 61).

MATERIALS AND METHODS

Preparation of Cultures

Explants of 14-day fetal rat and mouse spinal cords were prepared and maintained as described previously (50, 51). In brief, transverse sections of spinal cord (approximately 0.5 mm thick) were explanted onto collagen-coated (7) coverslips and maintained in sealed Maximow double-coverslip assemblies at 34.5°C for 1.5–6 wks. The culture medium was composed of 33% human placental serum, 53% Eagle's minimal essential medium, 10% chick embryo extract in Simms' balanced salt solution (BSS), and 600 mg/100 ml glucose. The BSS contained the following salt concentrations: 137 mM

NaCl, 2.7 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.36 mM Na₂PO₄, 0.15 mM NaH₂PO₄, 6.0 mM NaHCO₃, and 5.5 mM glucose in glass-distilled water.

Electrophysiology and Pharmacology

For neurophysiological studies, cultures were transferred to special sealed chambers designed for long-term electrical stimulation and recording using Ag-AgCl electrodes which contacted the tissue via saline-filled glass pipettes (3–8 μm tips) positioned by magnetically coupled micromanipulators (14, 15). An Ag-AgCl wire in the periphery of the bath served as a ground electrode. Bioelectric signals were recorded with high input impedance preamplifiers and an oscilloscope. Temperature was maintained at about 34°C with infrared lamps, and pH was stabilized at about 7.2 by flowing 3% CO₂-air through the chamber. The recordings were carried out with the culture submerged in 0.5 ml of BSS. Spontaneous discharges of the synaptic networks in the cord explants were enhanced by introduction of strychnine (10⁻⁶–10⁻⁵ M) for periods of 15 min, 1 h, and 8 h. Strychnine appears to competitively block glycine-mediated inhibitory synapses (21) and thereby facilitates synchronous activation of extensive excitatory synaptic networks in the cord explants (16, 17). Some strychnine-treated cultures were stimulated electrically at 0.1–1/s (0.2-ms pulses applied via 10-μm saline-filled pipettes) although many strychnine-treated preparations showed repetitive spontaneous discharges without electric stimulation (Fig. 1).

The synaptic activity of other cultures was depressed by increasing the Mg⁺⁺ concentration from 1 to 10 mM (for 15 min to 16 h) while maintaining normal 1 mM Ca⁺⁺ levels. An increase in the ratio of Mg⁺⁺ to Ca⁺⁺ has been shown to depress transmitter release at presynaptic sites (16, 18, 37, 38). In addition, some cultures were treated with 100 μg/ml Xylocaine, an agent that also suppresses neuronal network activity in our cultures (16, 18).

Since the cultures could not be optimally maintained for more than 8 h in BSS, during longer term experiments all the explants were exposed to pharmacologic agents in complete medium.

Tracer Experiments

For tracer studies, cultures were first presoaked for 10 min in 1% HRP Type II (Sigma Chemical Co., St. Louis, Mo.) in either BSS or BSS with 10 mM Mg⁺⁺. The following experiments were then performed: (a) cultures were exposed to HRP and strychnine for 15 min, 1 h, 4 h, and 8 h; (b) cultures were exposed to HRP with 10 mM Mg⁺⁺ for 15 min, 1 h, 4 h, 8 h, and 16 h; (c) cultures were exposed to HRP and strychnine for 1 h, washed free of extracellular tracer by repeated rinses with BSS containing 10 mM Mg⁺⁺ for 30 min, and then "chased" in tracer-free nutrient medium or BSS either with strychnine (10⁻⁶ M) for 4 h and 8 h, or with 10 mM Mg⁺⁺ for 4

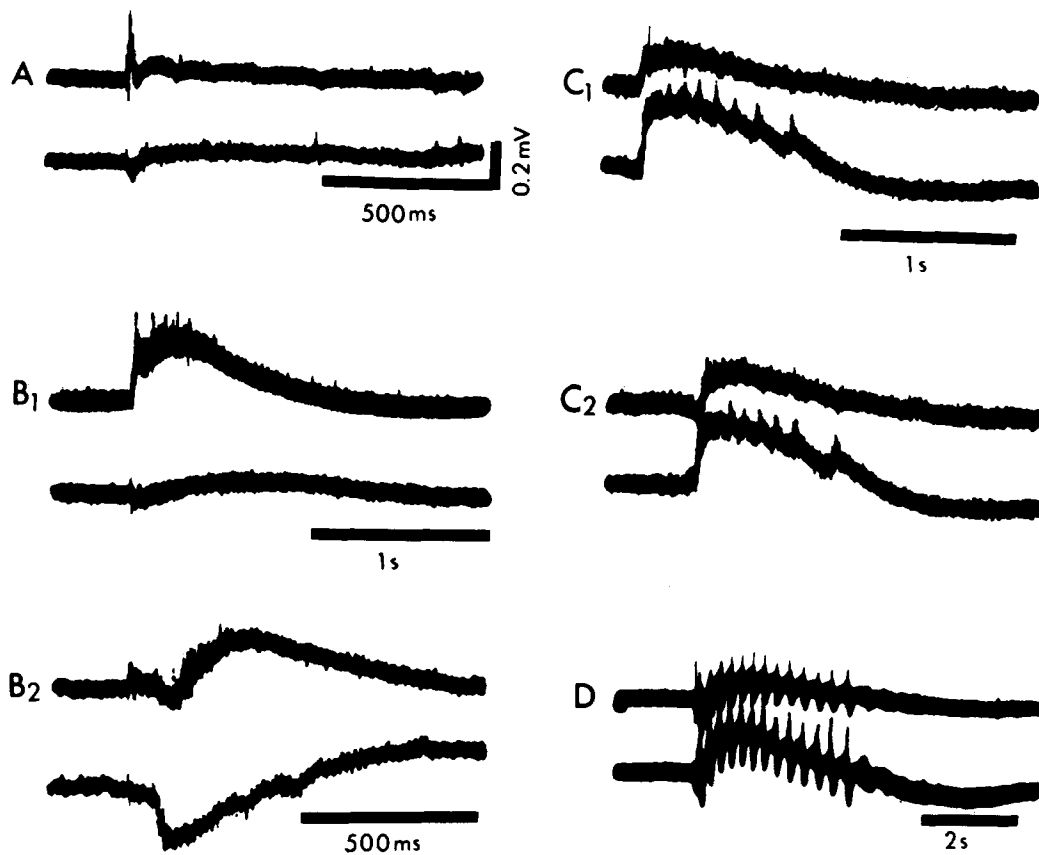


FIGURE 1 Typical evoked and spontaneous synaptic network discharges recorded in fetal rodent spinal cord explants during exposure to peroxidase before fixation for electron microscope study (14-day fetal tissues; 12–14 days in vitro). (A) Bioelectric response evoked at two sites in fetal rat spinal cord explant by single electric stimulus applied to third site. Note relatively short duration and small amplitude of these discharges in BSS with 1% horseradish peroxidase (HRP) as in BSS alone. (B₁) After introduction of strychnine (10^{-5} M), along with the HRP, response becomes much larger and lasts more than 1 s. (B₂) Cord responses are still quite prominent after stimulating at 1/s for 30 min. (C₁) Complex repetitive network discharges evoked in two regions of another rat cord explant in 10^{-5} M strychnine plus 1% HRP. (C₂) Similar elaborate discharges also occur spontaneously at both recording sites. (D) Longer-lasting (4 s) repetitive discharge sequence evoked at two sites in a fetal mouse cord explant during exposure to 10^{-5} M strychnine and 1% HRP. Note that all of these synaptically mediated slow waves and repetitive spike barrages are blocked after increasing the Mg^{++} concentration of the BSS from 1 to 10 mM or by Xylocaine (100 μ g/ml). Time and amplitude calibrations in Fig. 1 A apply to all succeeding records, unless otherwise noted. Upward deflection indicates negativity at active recording electrodes, and onset of stimuli is indicated by first sharp pulse or break in baseline of each sweep (see technical details in reference 14).

h, 8 h, and 16 h; (d) cultures were exposed to HRP and 10 mM Mg^{++} for 1 h, washed free of extracellular tracer, and then chased with strychnine or 10 mM Mg^{++} for 4 h and 8 h; and (e) cultures were exposed to HRP in BSS or nutrient medium for 1 h and in nutrient medium for 16 h with no pharmacological treatments.

During these tracer experiments many of the cultures were monitored for the presence or absence of synaptic network discharges with the electrophysiological techniques described above.

Fixation

HRP-treated cultures were rinsed for 1 min or 30 min in BSS with 10 mM Mg^{++} and then fixed by immersion in 2.5% glutaraldehyde (55) (Fisher Scientific Co., Springfield, N.J.) in 0.1 M cacodylate buffer (pH 7.2) with 1% sucrose and 10 mM Mg^{++} (4, 5) at room temperature for 30 min and on ice for an additional 30 min. They were then rinsed overnight in cold 0.1 M cacodylate buffer containing 7% sucrose. In one experi-

ment cultures were monitored for synaptic activity during the fixation procedure; none was detectable.

Cytochemistry

Fixed explants were frozen in 7.5% sucrose for 30 s on the head of a freezing microtome, dissected off the coverslip in cold 0.1 M cacodylate buffer with 7% sucrose, sliced into small fragments with a sharp razor blade, soaked in the diaminobenzidine (DAB) medium of Graham and Karnovsky (28) without substrate (H_2O_2) for 15 min, and then incubated in complete medium for 45 min at room temperature. Preparations incubated in H_2O_2 -free medium served as controls; these showed no reaction product. Cultures not exposed to exogenous peroxidase but incubated in the full cytochemical medium also showed no reaction product. Enzyme incubations were stopped by rinsing with cold 7.5% sucrose.

Electron Microscope Preparation

Tissue was postfixed in cold 1% OsO_4 in 0.1 M cacodylate buffer, stained *en bloc* (27) with uranyl acetate, dehydrated in a graded series of ethanols, and embedded in Epon (Ladd Research Industries, Inc., Burlington, Vt.) (46). Silver to gray sections were cut with a diamond knife on a Porter-Blum MT-2B ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.), stained with lead citrate (63) or uranyl acetate and lead citrate (65) and examined on an RCA EMU 3F or a JEOL JEM-100 electron microscope. Micrographs were taken at initial magnifications of $\times 4$ -15,000.

Light Microscopy

1-2- μm Epon thick sections were examined either unstained, by phase microscopy, or lightly stained with toluidine blue (53).

Biochemistry

A 1% solution of HRP in culture medium was maintained at 37°C and assayed for peroxidase activity (Worthington Enzyme Manual, Worthington Biochemical Corp. Freehold, N.J.) with *O*-dianisidine (Schwarz/Mann Div., Becton Dickinson & Co., Orangeburg, N.Y.), After 0 h, 4 h, 8 h, 24 h, and 48 h.

RESULTS

General

Experiments were routinely focused on spinal cord explants after 12-14 days in culture, and, unless otherwise noted, descriptions refer to such cultures. Morphological and electrophysiological details of fetal rodent spinal cord explants have been described in detail elsewhere (9, 17, 19, 20, 50, 51). In brief, the explants contain many

neurons, glial cells, an elaborate surface neuropil, and numerous synapses. They generate complex, organotypic bioelectric activity. Presynaptic endings contain the usual organelles, electron-lucent (500 Å diameter) synaptic vesicles, mitochondria, a few dense-cored vesicles, some elements of smooth endoplasmic reticulum (59), and occasional multivesicular bodies (MVB) (Figs. 4, 6, and 9). Perikarya also contain the usual organelles, (49) including much rough endoplasmic reticulum, a prominent Golgi apparatus, and numerous lysosomes (Fig. 14).

Cytochemical experiments suggest that most of the explant is accessible to HRP although demonstration of the tracer is strongly affected by the amount of extracellular HRP present at the time of fixation. Thus, in cultures exposed to tracer for up to 1 h and then rinsed in BSS with 10 mM Mg^{++} for 30 min before fixation, HRP reaction product is found in intracellular membrane-delimited bodies throughout the explants, but only a little is demonstrable in the extracellular space. By contrast, when cultures are rinsed for only 1 min before fixation, HRP reaction product is confined to the periphery of the fragments that we prepare for incubation. In these cultures, much reaction product is found both in the extracellular space and in the intracellular bodies. Our experiments indicate that in explants that have not been thoroughly rinsed before fixation, reaction product for HRP extends to an approximately equal depth from the surface all around the periphery of the incubated fragment. This is true regardless of the region of the explant from which the fragment was taken or the thickness of the fragment. These findings suggest that the enzymatic activity of extracellular HRP can deplete the incubation medium of H_2O_2 or DAB rapidly enough so that penetration of adequate quantities of these chemicals to deep regions of the tissue is prevented, and hence cytochemical reaction product is not formed.

Both extensively rinsed and briefly rinsed explants were studied for the present work. The briefly rinsed explants are useful since one can unequivocally ascertain that a given ending was surrounded by tracer during the experiment. The extensively rinsed ones provide a more adequate quantitative view of the overall pattern of synaptic activity.

We are dealing with a heterogeneous population of neurons, and the neurons display considerable variation in tracer accumulation under all of the

experimental conditions studied. Even in preparations where conditions produced maximal uptake of HRP, as many as 20–25% or occasionally even more of the presynaptic terminals showed little or no uptake of tracer (Fig. 2). Thus, all of our evaluations are based on “blind” experiments in which the investigator was unaware of the physiological treatment of the explants. No results were accepted as significant unless they could be reproduced three times. It is clear that electron microscopy of the complex cellular arrays used in this study permits semiquantitative comparisons only when differences in HRP labeling are marked.

Short-term tracer studies were done on both mouse and rat cultures, whereas in all longer-term experiments we used mouse explants only.

Extracellular electrical recordings showed that in the presence or absence of HRP the cultures display strychnine-enhanced, spontaneous and electrically evoked, long-lasting synaptic network discharges (Fig. 1). This electrical activity is readily blocked by elevated (10 mM) Mg^{++} levels or by Xylocaine (100 $\mu g/ml$). See the legend of Fig. 1 for details of the electrical behavior.

Presynaptic Terminals

SHORT-TERM TRACER EXPOSURE: After exposure of the cultures to HRP for up to 1 h in the presence of strychnine, with or without simultaneous electrical stimulation (Fig. 1), peroxidase is demonstrable in numerous small vesicles in presynaptic endings (Fig. 3). Fig. 2 summarizes the

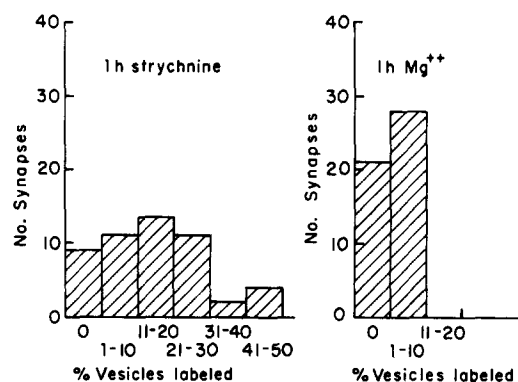


FIGURE 2 Histograms showing the frequency distribution of labeled vesicles in a pair of cultures exposed for 1 h to HRP with strychnine (left) or 10 mM Mg^{++} (right). For each culture a typical region was located in the thin sections and the first 50 terminals encountered were scored as to the percent of the synaptic vesicles that contained reaction product.

frequency distribution of such tracer-containing vesicles in a typical experiment. With strychnine, peroxidase also accumulates in tubules and in MVB's (Fig. 4). MVB's are seen in 5–10% of the terminals in a given thin section, and after a 1-h exposure to HRP with strychnine almost all of these are labeled.

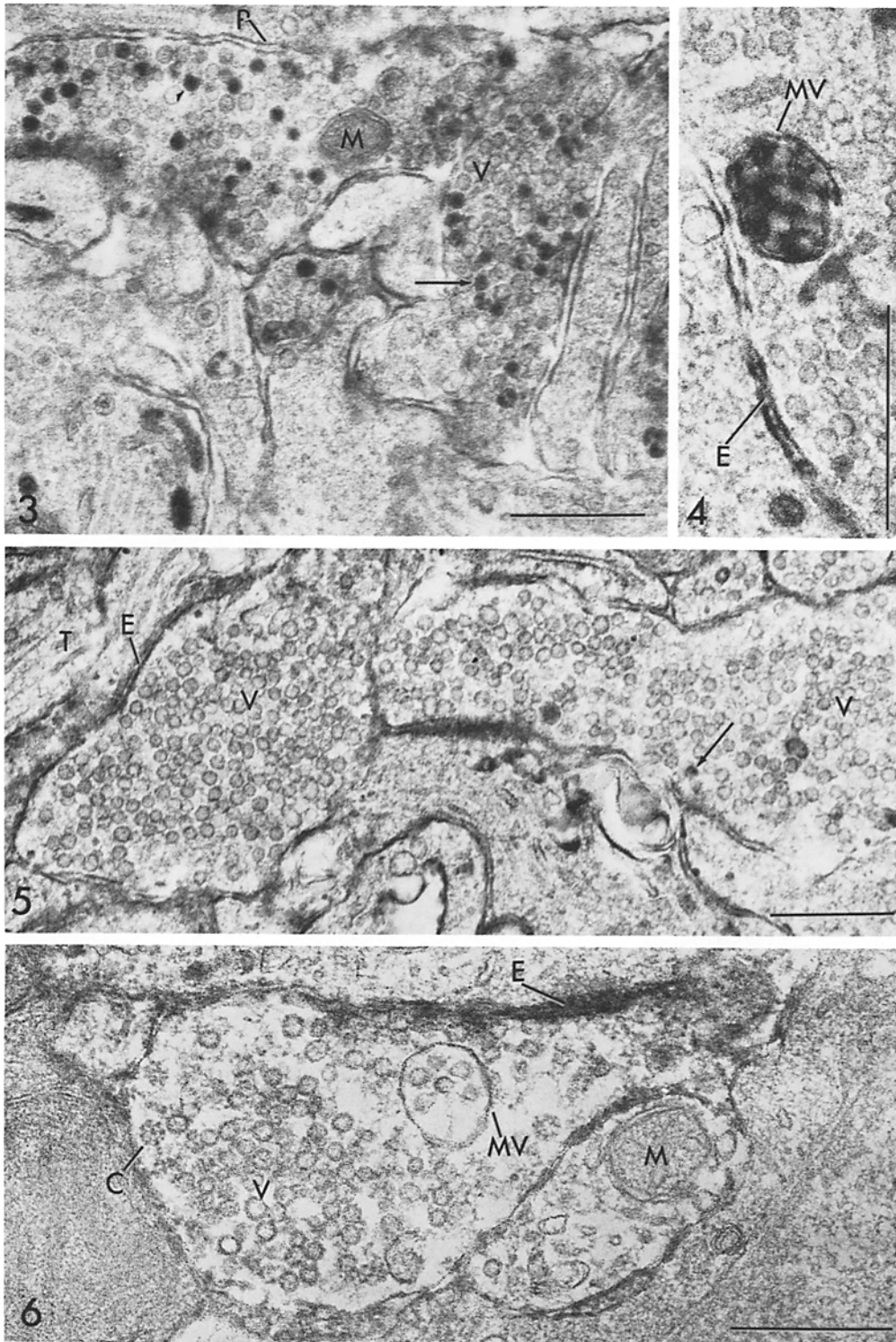
When cultures are exposed to HRP for 1 h (or longer) in the presence of 10 mM Mg^{++} , very few vesicles contain demonstrable HRP (Figs. 5 and 6). In these preparations, labeled MVB's are seen in only 1% of the terminals; most MVB's show no reaction product (Fig. 6).

Since one may overlook some MVB's in terminals when they lack tracer, we cannot confidently assess the total frequency of such bodies (labeled plus unlabeled) under varying physiological conditions. However, we have the impression that the total frequency of MVB's is lower in preparations exposed to Mg^{++} for 1 h than in those exposed to strychnine for 1 h.

Very few vesicles or MVB's containing tracer were seen when synaptic activity was depressed by 100 $\mu g/ml$ Xylocaine instead of 10 mM Mg^{++} .

A few cultures were incubated in peroxidase for 1 h without pharmacological or electrical stimulation. These showed uptake of tracer at presynaptic terminals at levels higher than those seen with 10 mM Mg^{++} , but markedly lower than those obtained with strychnine. The uptake in such cultures is quite variable; this is not surprising, since spontaneous electrical activity of the explants is known to vary greatly from preparation to preparation (14, 16, 17).

CHASE EXPERIMENTS: When cultures are exposed to HRP for 1 h in strychnine to “label” a population of vesicles with tracer, and then chased in the absence of HRP, there is a decline in the frequency of tracer-containing vesicles, tubules, and MVB's in the presynaptic terminals. Fig. 7 summarizes the frequency distribution of tracer-containing vesicles in a typical chase experiment. When the chase is carried out in a medium with 10^{-6} M strychnine, the decline in HRP-containing bodies is evident by 4 h, and by 8 h, relatively few vesicles present in the terminals show demonstrable peroxidase (Figs. 7 and 9). When 10 mM Mg^{++} is present during the chase, the frequency of labeled vesicles is still quite high at 4 h; by 8 h (Figs. 7 and 8) some decline is evident, and by 16 h, the number of HRP-containing vesicles in the terminals has dropped drastically (Figs. 7 and 10). The decline in labeled vesicles is paralleled by a



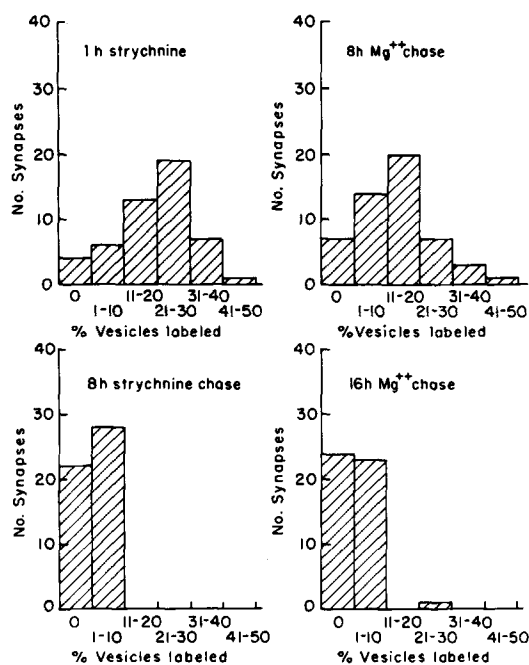


FIGURE 7 Histograms summarizing the results of a chase experiment. The enumerations were done as with Fig. 2. All cultures were exposed to HRP with strychnine for 1 h. One was fixed without a subsequent chase (upper left). The other three were all chased as indicated on the histogram.

noticeable decrease in the frequency of labeled MVB's and an increase in the frequency of unlabeled MVB's (Fig. 9). As far as we can tell, the total frequency of MVB's does not change noticeably during the chases.

Cultures exposed to tracer with 10 mM Mg⁺⁺

and then chased with strychnine medium or high Mg⁺⁺ for up to 8 h show little or no HRP reaction product in presynaptic vesicles or MVB's.

LONG-TERM PEROXIDASE EXPERIMENTS: Preparations maintained in HRP for 8 h in the presence of strychnine show many tracer-containing vesicles in their presynaptic terminals, whereas those kept in HRP with high Mg⁺⁺ for as long as 16 h show only one or two such vesicles. By 8–16 h, most MVB's in terminals of preparations treated with HRP and strychnine or 10 mM Mg⁺⁺ show reaction product.

Tracer molecules still appear to be enzymatically active 16 h after administration; considerable peroxidase is demonstrable in glial cells and in neuronal perikarya (see below). Furthermore, in cultures that are not specially treated pharmacologically, but maintained in HRP for up to 16 h, there are appreciable numbers of HRP-containing vesicles present in the terminals. In the absence of degradative enzymes, HRP is quite stable in solution (cf. 57); in our biochemical analysis, more than 90% of initial HRP activity was retained after the HRP was kept in culture medium at 37°C for 48 h.

Axons

After a 1-h tracer exposure, only a little HRP was seen in axons. During chase experiments and long-term tracer experiments HRP was more frequently seen in axons (Fig. 11) within membrane-delimited vesicles, tubules, and occasional MVB's that often appear elongated or tubular (5, 43, 62). No marked differences were apparent in

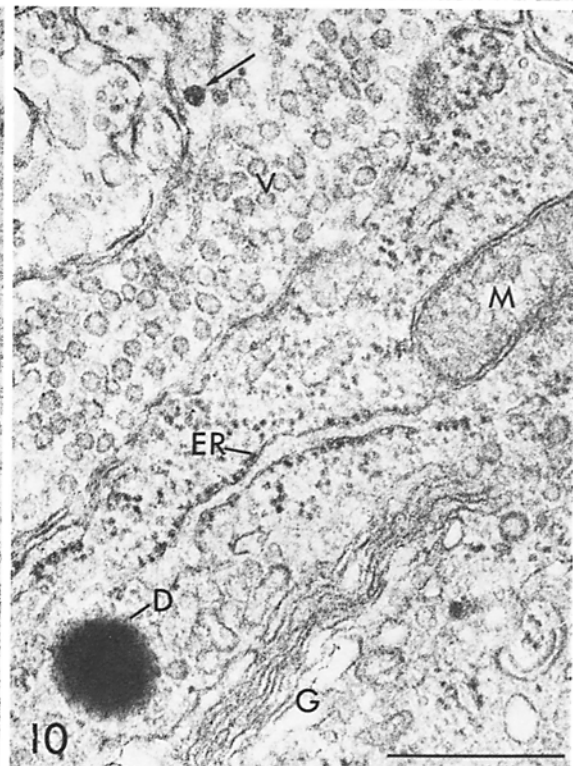
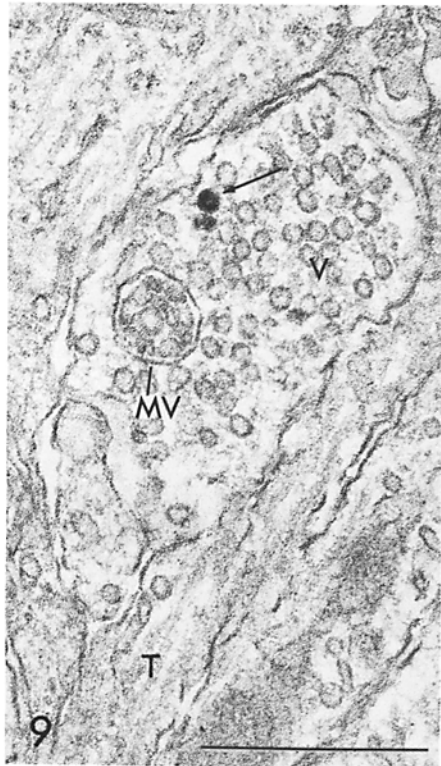
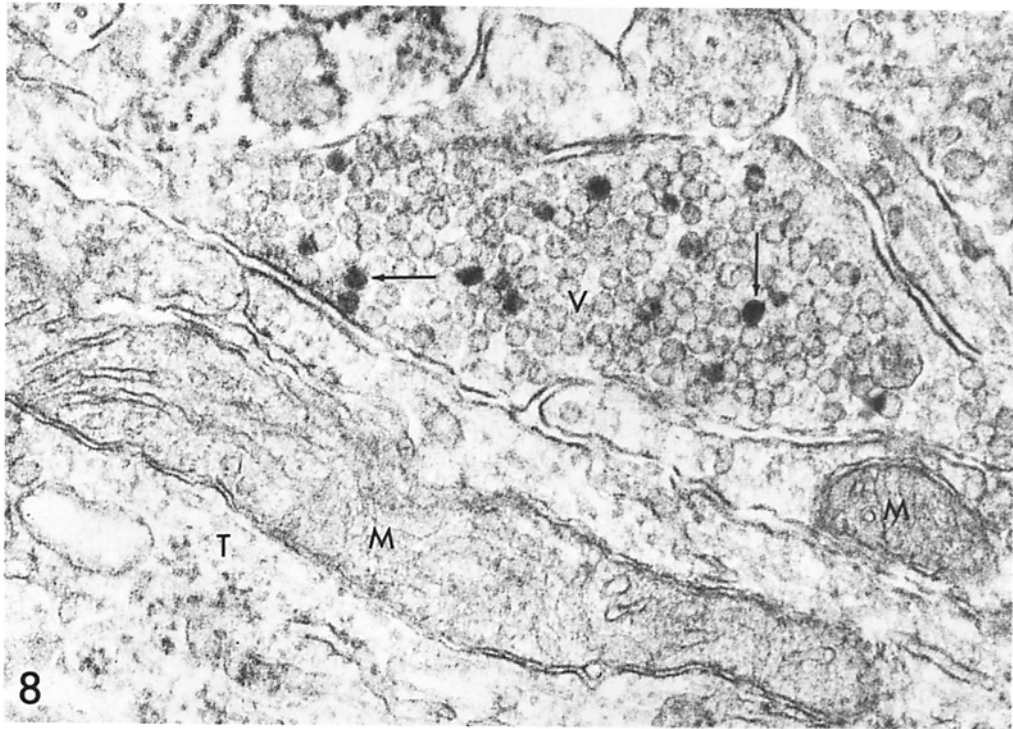
FIGURES 3–6 Electron micrographs from 14-day-old fetal rodent spinal cord explants. Bar length is 0.5 μ m. All electron micrographs are from material stained with lead citrate.

FIGURE 3 Electron micrograph of a presynaptic ending from a culture treated for 1 h with 10⁻⁵ M strychnine and 1% HRP. Many small vesicles (*V*) contain reaction product (*arrows*). Plasma membrane is seen at *P*, a mitochondrion at *M*. \times 45,000.

FIGURE 4 Presynaptic ending from a culture treated as in Fig. 3. A multivesicular body filled with HRP reaction product is seen at *MV*. Extracellular space is seen at *E*. \times 74,000.

FIGURE 5 Presynaptic ending from a culture exposed for 4 h to 1% HRP with 10 mM Mg⁺⁺. HRP reaction product is seen in the extracellular space (*E*) and in an occasional vesicle (*arrow*). Most of the synaptic vesicles (*V*) show no peroxidase reaction product. Microtubules are seen at *T*. \times 40,000.

FIGURE 6 Presynaptic ending from a culture exposed for 1 h to 1% HRP and 10 mM Mg⁺⁺. Demonstrable HRP is confined to the extracellular space (*E*). A multivesicular body without reaction product is seen at *MV* (see Fig. 4). Synaptic vesicles are seen at *V*, a coated vesicle at *C*, and a mitochondrion at *M*. \times 53,000.



the HRP accumulation found in axons under the various chase conditions examined.

Perikarya

LIGHT MICROSCOPY

Peroxidase accumulation in neuronal perikarya was evaluated by extensive light microscope study. This was done in blind experiments in which phase microscopy or lightly stained toluidine blue sections were used. In these preparations it is not difficult to see the characteristic intracellular bodies containing HRP in neuronal perikarya or glia.

SHORT-TERM TRACER EXPOSURE: A few small tracer-containing bodies were seen in neuronal perikarya of cultures exposed to HRP for up to 1 h with strychnine or 10 mM Mg⁺⁺ (Fig. 12). No differences in tracer accumulation between Mg⁺⁺ and strychnine preparations were apparent. Under these conditions, many large peroxidase-containing bodies are found in glial cells.

CHASE EXPERIMENTS: During the chase period, much HRP accumulates in neuronal perikarya of cultures initially exposed to tracer with strychnine-induced stimulation and then chased in strychnine or Mg⁺⁺ medium (Fig. 13). Markedly less peroxidase accumulates in neuronal perikarya during the chase when initial exposure to tracer is with 10 mM Mg⁺⁺. We have not observed striking differences in perikaryal accumulation dependent upon the chase conditions.

ELECTRON MICROSCOPY

SHORT-TERM TRACER EXPOSURE: In neuronal perikarya of cultures exposed to HRP during 1 h of strychnine or 10 mM Mg⁺⁺ treatment,

reaction product is seen in small vesicles, elongate tubules, and a few MVB's.

CHASE EXPERIMENTS: In neuronal perikarya of explants exposed to HRP for 1 h with strychnine and then chased with strychnine or elevated Mg⁺⁺, tracer is predominantly found in large dense bodies (lysosomes) and in occasional tubules and MVB's (Fig. 14). In neuronal perikarya of cultures exposed to HRP with 10 mM Mg⁺⁺ and chased with either strychnine or elevated Mg⁺⁺, tracer also eventually accumulates in large dense bodies.

DISCUSSION

Membrane Retrieval

Our results confirm, for intact mammalian central nervous system neurons, the relation between synaptic transmitter release and the retrieval of membrane in the form of small vesicles, previously demonstrated at invertebrate and amphibian neuromuscular junctions (12, 13, 29, 33). When spinal cord synapses are actively releasing neurotransmitter, HRP uptake by endocytosis is fairly extensive. By contrast, little accumulation of tracer into synaptic vesicles is seen when the cord synaptic network is depressed either by Mg⁺⁺ or Xylocaine, both of which probably act by impeding transmitter release (16, 37, 38). These latter agents do not appear to have a general massive inhibitory effect on endocytosis; in their presence, perikarya, dendrites, and glial cells continue to take up considerable HRP by endocytosis.

Certain limitations should be borne in mind when interpreting these experiments. Agents such as strychnine or excess Mg⁺⁺ may have complex effects on cells. And, it is not yet feasible to draw

FIGURES 8-10 Electron micrographs from 14-day-old fetal rodent spinal cord explants. Bar length is 0.5 μ m. All electron micrographs are from material stained with lead citrate.

FIGURE 8 Presynaptic ending from a culture treated for 1 h with 10⁻⁵ M strychnine plus 1% HRP and then maintained for 8 h in HRP-free medium with 10 mM Mg⁺⁺. A number of small vesicles (V) contain demonstrable HRP (arrows). Mitochondria are seen at M, microtubules at T. \times 57,000.

FIGURE 9 Presynaptic ending from a culture treated for 1 h with 10⁻⁵ M strychnine plus 1% HRP and then maintained for 8 h in HRP-free medium with strychnine. Most small vesicles (V) do not contain demonstrable HRP. An unlabeled multivesicular body is seen at MV (See Figs. 4 and 6), microtubules at T. One small vesicle with HRP reaction product is seen at arrow. \times 60,000.

FIGURE 10 Presynaptic ending from a culture treated for 1 h with 10⁻⁵ M strychnine plus 1% HRP and then maintained for 16 h in HRP-free medium with 10 mM Mg⁺⁺. Most small vesicles (V) do not contain demonstrable HRP; one labeled vesicle is seen at arrow. In an adjacent cell, endoplasmic reticulum is seen at ER, mitochondria at M, Golgi apparatus at G, and an HRP-filled dense body at D. \times 47,000.

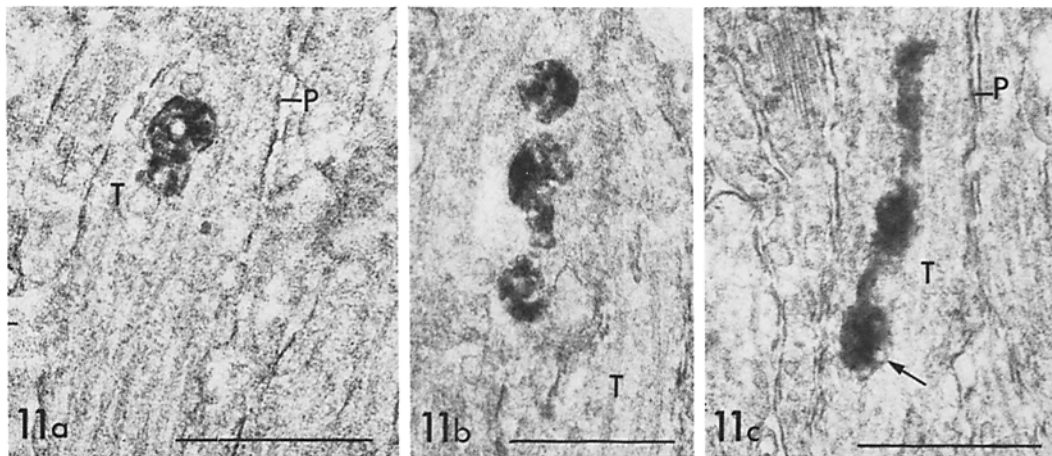


FIGURE 11 Portions of axons from a Mg^{++} chase experiment (8 h of chase). The bodies in Fig. 11 *a* and *b* are multivesicular bodies containing reaction product for peroxidase. The one in Fig. 11 *c* illustrates an observation made occasionally: a peroxidase-containing tubular structure shows internal vesicles (*arrow*) suggesting that it may be a distorted multivesicular body. Microtubules are seen at *T*, plasma membrane at *P*. Electron micrograph from a 14-day-old spinal cord explant, stained with lead citrate. Bar, $0.5 \mu m$. 11 *a*, $\times 52,000$; 11 *b*, $\times 44,000$; 11 *c*, $\times 44,800$.

conclusions about the quantitative relations between the number of tracer-containing vesicles that form in a terminal and the number of quanta (37) of transmitter released. For example, we still lack information about the relative rates of exocytosis and compensating endocytosis (6, 30), the efficiency of tracer uptake, and the sensitivity of our cytochemical techniques. Also, our extracellular recording methods monitored the synchronous synaptic discharges of large neuronal populations, not the activities of individual neurons. Also, we do not yet understand the extent to which retrieved membrane resembles the membrane of unused synaptic vesicles.

On the other hand, HRP does not have dramatic effects on the electrical behavior of our cultures. Dextran has been used in place of HRP to demonstrate stimulation-dependent uptake at neuromuscular junctions (13), suggesting that the particular tracer used is not of central importance, and recent work on the amphibian retina (56) indicates that synaptic uptake of HRP at photoreceptor terminals is markedly responsive to light-induced changes in physiology, giving us added confidence that we are dealing with widespread phenomena.

An outstanding uncertainty is whether cells handle an HRP-containing structure differently from a comparable structure lacking tracer. In prior work on membrane retrieval in insulin-stimulated adrenal medulla (1), the same dra-

matic changes in the frequency of endocytotic structures (MVB's) etc. were noted in the presence or absence of HRP. In our studies on neurons we have not detected differences in the frequency of pertinent structures related to the presence of HRP, although the possibility of subtle effects obviously cannot be excluded.

Differences in detail may exist between the membrane retrieval process seen in intact mammalian spinal cord neurons and neuromuscular junction systems. For example, at frog neuromuscular junctions, it has been suggested that tracer uptake occurs via small "coated" vesicles which fuse to form intracellular cisternae that ultimately break up by budding into small synaptic vesicles (29). Neither cisternal intermediates nor large numbers of coated vesicles are prominent at stimulated presynaptic endings in our spinal cord studies. We do see occasional coated vesicles, and it is quite possible that variations of this type depend largely upon differences in rates of coat loss (coated vesicles do seem to lose their coats in many cell types [27]) or on experimental details.

MVB's appear to be quite infrequent in neuromuscular junctions (13). But in our preparations, 5–10% of the terminals in a given thin section contain such a body; extrapolating to three dimensions, this indicates that MVB's are fairly common in the presynaptic endings of these spinal cord neurons.

The fact that intact neurons within large aggre-

gates show the expected variation of HRP uptake with stimulation suggests that tracer uptake may provide a means for monitoring synaptic activity in cells difficult to approach by more conventional means. The studies on the retina alluded to above (56) point in the same direction.

Fate of Retrieved Membrane

Biochemical studies on the turnover of membrane constituents in brain have been interpreted as supporting the viewpoint that synaptic vesicles have half-lives of many days (cf. references 22, 26, 47, 54). However, one cannot obtain unambiguous information about membrane half-lives for synaptic vesicles, or other secretory structures, from the usual pulse-chase labeling experiments; precursor membranes present in neuronal perikarya and axons may well provide a pool that continues to contribute label to the vesicle population for some time after the low molecular weight precursor molecules have disappeared (31, 35). Studies of the apparent rates at which new vesicle-associated enzymes are supplied to noradrenergic terminals suggest fairly short half-lives, on the order of 1 or 2 days (8, 23, 41).

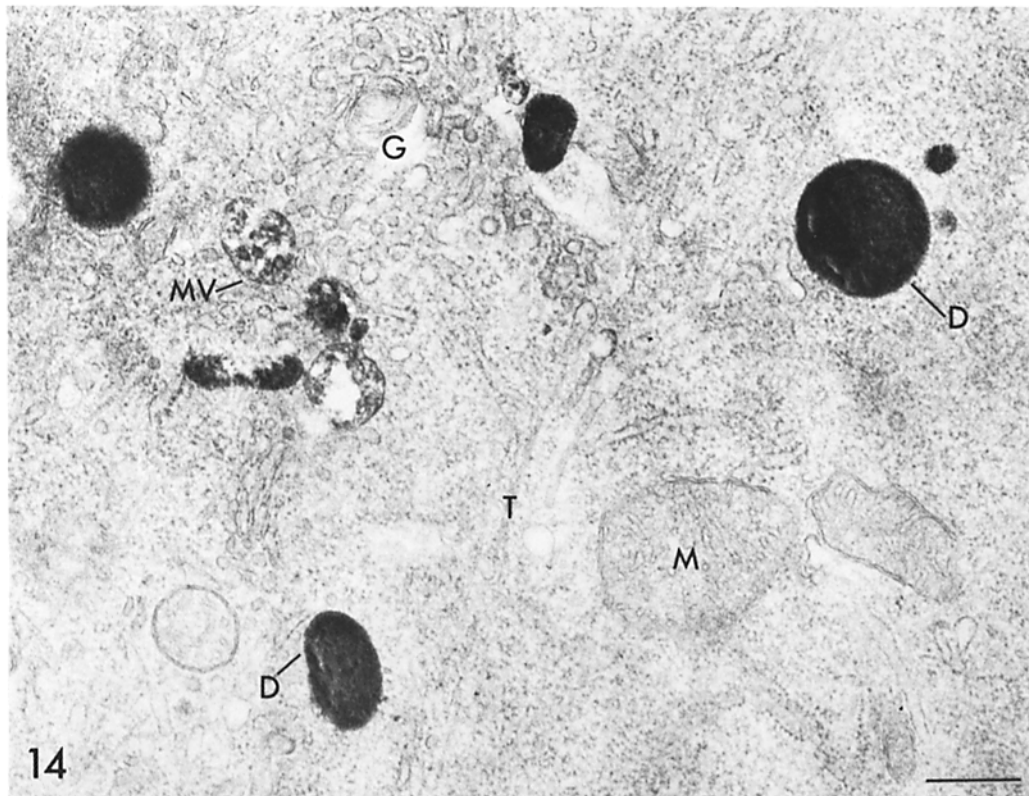
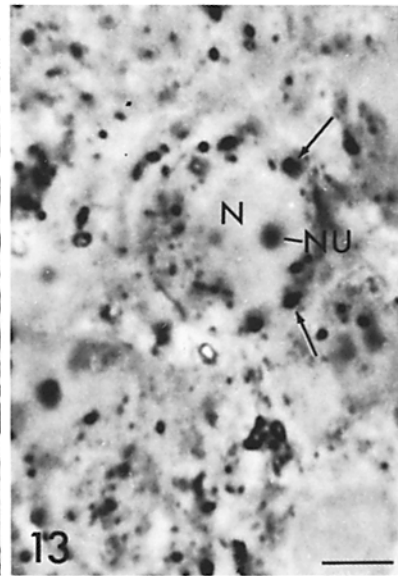
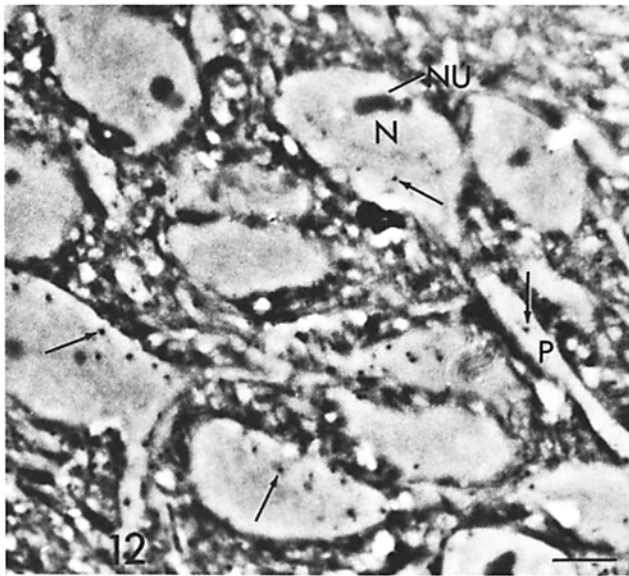
Our findings indicate that vesicles and other structures labeled with HRP in response to stimulation disappear from the endings through processes that require several hours to produce a marked decrease in the frequency of labeled vesicles. The number of labeled bodies at terminals decreases, at an appreciable rate, even when cultures are maintained in 10 mM Mg^{++} without tracer. Under these conditions, it is unlikely that much loss of tracer occurs through processes such as those associated with transmitter release that account for the initial accumulation of tracer. (Presumably the latter processes are exocytosis coupled with endocytosis.) Cultures kept in HRP with 10 mM Mg^{++} for periods as long as the most extensive chases (8–16 h) show few labeled vesicles, whereas such vesicles would be expected to form by “compensating” endocytosis if the endings were active in transmission by exocytosis (3, 6, 12, 13, 29, 32, 33, 35, 45, 52, 67, 68). Furthermore, the enzyme activity of HRP that is required for its cytochemical demonstration is quite stable, and, HRP-labeled vesicles and other structures can be demonstrated in neurons and other cells maintained in the tracer under appropriate conditions for quite long periods. Thus, it seems most improbable that the HRP is simply inactivated during the chase.

We propose that much of the depletion of label from terminals during a chase with elevated Mg^{++} reflects retrograde intraneuronal transport (2, 5, 39, 40, 43) of vesicles as well as MVB's and tubules to which the vesicles contribute membrane (1, 5, 31, 34, 35, 43, 62), presumably by fusion. The fact that perikarya continue to accumulate HRP for many hours during chase experiments apparently, in part, reflects this transport. Prior studies on retrograde transport have not focused on its relevance to turnover of synaptic components.

Most of the tracer in our study eventually is found in lysosomes. This is consistent with the likely role of these organelles in membrane degradation (31).

Perikaryal tracer accumulation during chases is much more dramatic when the initial exposure to HRP is with strychnine than when the tracer exposure occurs with elevated Mg^{++} . In other words, the amount of tracer that eventually ends up in neuronal perikarya is related to the degree of synaptic activity during the initial loading of presynaptic endings with peroxidase. Such observations carry obvious implications for the use of retrograde transport methods in neuronal mapping (2). Our methods are not sensitive enough to determine whether different chase conditions result in different rates of tracer accumulation in neuronal perikarya. The technical problems in visual evaluation are in part due to cellular heterogeneity and in part to the fact that there is appreciable direct uptake of tracer by neuronal perikarya and dendrites, and some uptake by axons (34). We are also conservative in our evaluations because of considerations such as the fact that the rinses employed before a chase probably cannot remove all extracellular HRP, particularly if some of it binds to membranes.

In many cell types (cf. references 1, 27, 30, 35) endocytosis results in the appearance of tracers in tubules, some of which are attached to MVB's. Usually, one can readily see that these structures are distinct from endoplasmic reticulum, especially if the latter system is studded with ribosomes. In axons, however, the agranular reticulum (cf. references 30, 59) and the peroxidase-containing tubules can resemble one another fairly closely. At present, we are inclined to believe that relatively little peroxidase actually enters the axonal endoplasmic reticulum under the conditions we have studied. Some tubules with HRP seen in axons are fairly short, and others are actually elongate MVB's or dense bodies (see also references 5, 43,



66). In this study and in prior work, we have not seen tracer in some characteristic portions of the reticulum such as the branched networks often present at terminals (35, 59). Thus, to us, it is likely that many of the tracer-containing tubules in axons are a category of structure separate from the agranular reticulum. However, it certainly would be very premature to rule out some entry of HRP into the reticulum in our material, and the situation under other circumstances, such as anterograde transport, needs further study (36, 43). This is especially true since the axonal reticulum can play a role in lysosome formation (30, 31, 35, 66) and lysosomes are major terminals for endocytosed material. One might imagine, for example, that there exist physically separate or otherwise specialized portions of the reticulum responsible for lysosome formation, transport of synaptic materials (see below and references 30, 59), and other functions.

To account for their finding that HRP taken into vesicles during one round of stimulation is lost under some conditions of subsequent stimulation in the absence of extracellular tracer, Heuser and Reese (29) have argued that tracer can be lost from terminals by exocytosis related to the reutilization of vesicle membrane for neurotransmission. The analysis of Ceccarelli et al. (13) emphasized that such re-use is the simplest explanation of the ability of neuromuscular junctions to reaccumulate transmitter after stimulation. Work by Zimmerman and Whittaker (67, 68) on *Torpedo* neuromuscular junctions may provide support for this view. It may also be pertinent that, in their studies on peroxidase uptake by photoreceptor cells in frog retina, Schacher et al. (56) noted the eventual clustering of tracer-containing

vesicles along synaptic ribbons which are present at sites where transmitter release is thought to occur. Also, there have been recent findings that presynaptic exocytosis can occur in the absence of transmitter release (11), although the physiological significance of this remains to be evaluated.

Since little HRP accumulates at the cut endings of the axons of their frog neuromuscular junctions, Heuser and Reese (29) have argued that retrograde flow is unlikely to be of major quantitative significance in explaining the stimulation-related loss of tracer from neuromuscular endings. At present, it is difficult to predict how much HRP should turn up at a given axonal region within a given period of time if retrograde flow does occur; if the observations by Heuser and Reese do in fact reflect the absence of such flow, it would suggest significant differences between these neuromuscular and spinal cord preparations. Also, of uncertain significance are the indications that depletion of previously labeled vesicles from the frog neuromuscular junctions (29) appears to continue during the rest period after stimulation in the absence of extracellular HRP.

In the light of initial biochemical findings suggesting that stimulation enhances the rate of retrograde transport of macromolecules (44), the observations on neuromuscular junctions and our own observations require further analysis. Nevertheless, re-use of membrane for exocytosis seems to be the simplest explanation for the more rapid depletion of tracer-containing vesicles that we observe in our experiments when we use strychnine medium for the chase. Our point in the present paper is that other mechanisms, notably transport of membrane back to neuronal perikarya for lysosomal degradation, probably also make major

FIGURE 12 Phase-contrast micrograph of several neuronal perikarya from a culture exposed for 1 h to 10^{-8} M strychnine and 1% HRP. HRP reaction product is seen in several small bodies (arrows) in the perikaryon and in a neuronal process (P). A nucleus is seen at N, nucleolus at NU. Bar, 5 μ m. \times 1700.

FIGURE 13 Phase-contrast micrograph of a neuronal perikaryon from a culture exposed for 1 h to 10^{-8} M strychnine plus 1% HRP and then chased for 8 h with strychnine. HRP reaction product is seen in many bodies (some of which are noticeably larger than the ones in Fig. 12) distributed around the nucleus seen at N. A nucleolus is seen at NU. Bar, 5 μ m. \times 1900.

FIGURE 14 Electron micrograph of a portion of a neuronal perikaryon from a culture treated for 1 h with 10^{-8} M strychnine plus 1% HRP and then maintained for 8 h in HRP-free medium with strychnine. HRP reaction product is seen in several dense bodies (D) and in a multivesicular body (MV). Golgi apparatus is seen at G, a mitochondrion at M, microtubules at T. Electron micrograph from 14-day-old spinal cord explant, stained with lead citrate. Bar length is 0.5 μ m. \times 25,000.

contributions to the turnover of synaptic vesicles. This argument may imply that the membrane the cell uses to form synaptic vesicles must be replaced in the synapses at a fairly rapid pace. From our data on Mg^{++} chases we estimate that the half-life of the membranes of the vesicles we label in the presynaptic endings of our preparations is on the order of 10–20 h. The caveat concerning the possible effects of the presence of tracer in the vesicles should be recalled, but we are encouraged that our figures are not widely discordant with estimates from very different techniques (8, 23, 41). Some new synaptic vesicles apparently are made in neuronal perikarya (see reference 35 for review); however, the axons and presynaptic terminals themselves contain another probable source of vesicles, the agranular reticulum. That the reticulum can produce new synaptic vesicles is indicated by studies on noradrenergic neurons showing continuity between agranular reticulum and small vesicles that can accumulate the catecholamine 5-hydroxydopamine (59). Supplementary evidence comes from autoradiographic studies indicating that some axoplasmic transport from neuronal perikarya is mediated by the reticulum (10, 26). Interestingly, some of the larger vesicles with dense cores seen in synapses may have an endocytotic origin (5); some may represent a type of MVB. (It should be borne in mind that the data we have obtained relates to the timing of turnover in the terminals proper; we have no direct information on the total lifespan of vesicle membrane from initial assembly to final degradation of the constituents.)

We are grateful to Ms. Fe Reyes, Ms. Bea Crain, Ms. Ellen Lory, Ms. Blossom Kutscher and Ms. Dale Bloom for their excellent technical assistance.

This study was supported by National Institutes of Health Grants NS09475 to Dr. Holtzman, NS06545 to Dr. Crain, NS08770 to Ms. Peterson, NS12148 to Dr. Teichberg, and by a postdoctoral fellowship to Dr. Teichberg from the Interdepartmental Training Grant in the Behavioral and Neurological Sciences at Albert Einstein College of Medicine (5T1 MH 6418).

Received for publication 3 January 1975, and in revised form 12 May 1975.

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