

Structure of the synapse

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CHAPTER CONTENTS

Structure of the Presynaptic Active Zone
Recycling of Synaptic Vesicles
Vesicle Turnover and Transmitter Metabolism
Vesicles and Transmitter in Adrenergic Synapses
Ultimate Origin and Fate of Synaptic Vesicles
Development and Degeneration of Synaptic Terminals
Glial and Schwann Cells Next to Synaptic Terminals
Structure of the Postsynaptic Active Zone

THE TERM SYNAPSE was used originally by Sherrington (108) to refer to the point of functional contact between two nerve cells, the point at which the electrical activity of one is brought to bear on the other. Such contacts have been magnified electron microscopically until their finest structural details have come into view, and thereby evidence has been provided that presynaptic secretion and postsynaptic reception of the chemicals that mediate synaptic transmission occur only in certain small regions of the contact. The term synapse, however, has been used for so long to refer to the whole contact formed by a nerve terminal that it has become desirable to find a new term for the specialized regions within the synapse where the chemical is actually transmitted. These regions were recognized to be morphologically unique and were named synaptic complexes before their function was known (210). Recently, Couteaux & Pecot-Dechavassine (56) have suggested that instead they should be called the "active zones" of the synapse. As morphological data on synapses continue to accumulate, this term seems more and more apt.

In this chapter current information on the location and structure of the active zones at chemical synapses is presented. We focus particularly on the morphological features of chemical synapses that can help to explain how the presynaptic neuron secretes transmitter and how the postsynaptic neuron senses and responds to it. The approach to these problems is

from a general point of view, without considering in detail the morphological differences among various types of chemical synapses, unless they point to significant differences in the mode of operation of these synapses.

STRUCTURE OF THE PRESYNAPTIC ACTIVE ZONE

The first clear electron micrographs of synapses provided a wealth of new morphological information and formed the basis for most of the ideas about synaptic function that are still prevalent. De Robertis & Bennett (71) named the small membrane-bound vesicles that are abundant in presynaptic nerves the "synaptic vesicles" and proposed that they were secretory droplets of neurotransmitter, analogous to the much larger secretory droplets in adrenal medullary cells. To support their proposal, they pointed out that synaptic vesicles cluster in regions where the pre- and postsynaptic processes are closely apposed (69).

In the first clear pictures of the central nervous system (CNS), Palay (209) showed that small vesicles are a ubiquitous feature of presynaptic nerve terminals and thus establish a morphological polarity at the synapse. His pictures revealed, in addition, that synaptic vesicles cluster over regions where the neighboring membranes of the two neurons forming the synapse appear thicker or more dense than elsewhere, and on this basis Palay proposed that such dense patches represent specialized sites for vesicle discharge. Later, in his influential review of the morphology of synapses in the CNS, Palay (210) named these dense patches, with their clusters of vesicles, the "synaptic complexes," and he reiterated that they could represent the actual sites of impulse transmission across the synapse (Fig. 1).

We shall see how the morphological evidence in favor of this idea has accumulated, until today the synaptic complexes can be considered the active zones of the synapse.

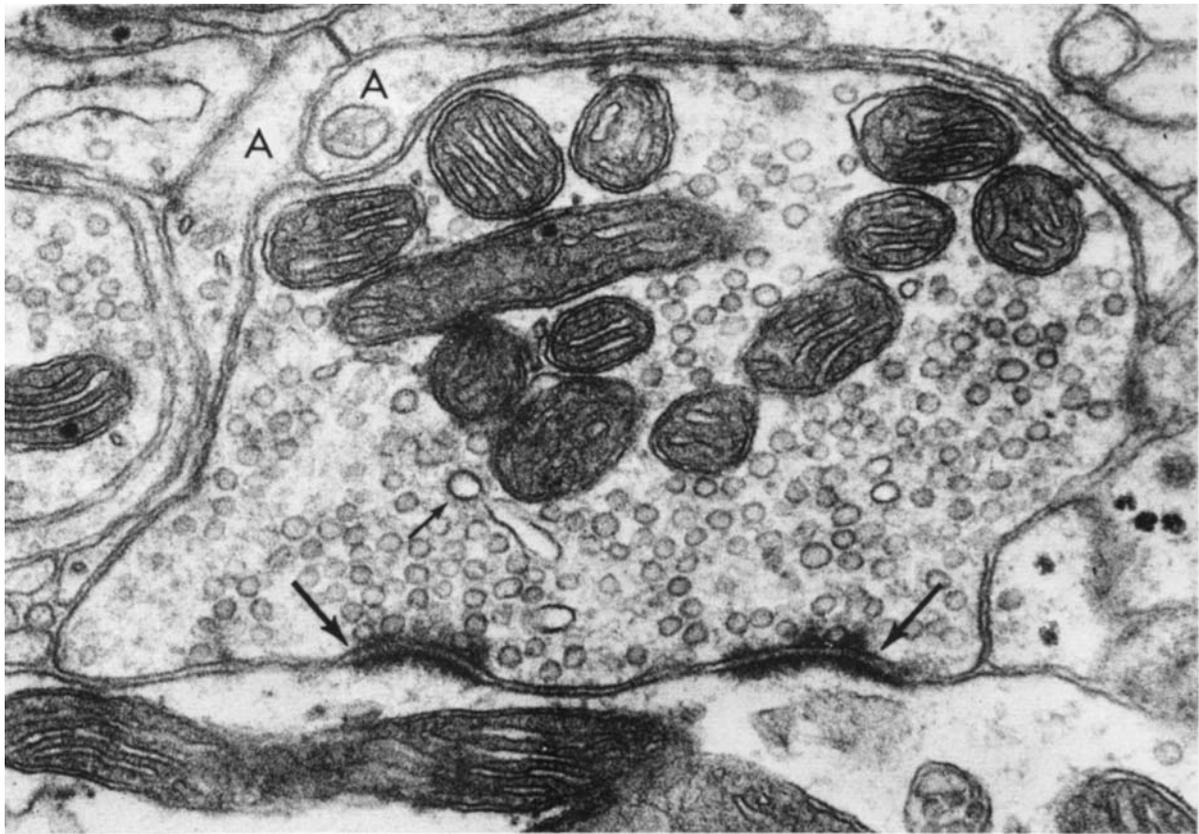


FIG. 1. Thin section of synapse on dendrite in superior olive. Two specializations of the presynaptic membrane are indicated by *large arrows*. Each specialization is composed of several tufts of dense material, around which synaptic vesicles nestle. Opposite the presynaptic specializations are specialized regions of the postsynaptic membrane at which a continuous layer of dense fuzz is applied to its inner surface. Such membrane specializations and associated synaptic vesicles comprise a synaptic complex which has come to be regarded as the active zone of the synapse. Coated vesicles (*small arrow*), small cisternae, and mitochondria are other typical organelles found in presynaptic terminals in the CNS. Many synaptic terminals in the CNS are capped by astrocytic processes indicated here at A. (Micrograph supplied by R. Perkins.) $\times 60,000$.

However, we should consider first another possible function of the membrane specializations at the synaptic complex. When Palade (206) first described the thickened regions of the apposed membranes at the synapse, he compared them to the specialized adherence plaques (i.e., desmosomes) that Porter found between various types of epithelial cells (98, 187, 230). Palay (209) agreed that these synaptic thickenings could be sites of adhesion but pointed out that their fine structure is somewhat different from that of desmosomes. He cautioned against concluding that the synaptic complex is a rigid solder joint that could prevent dynamic rearrangements of the contact between the pre- and postsynaptic membranes (210).

However, since then a great deal of circumstantial evidence has accumulated to support the idea that synaptic membranes adhere to each other at the synaptic complex. Synaptic membranes in these regions adhere to each other when brain tissue is distorted by shrinkage during fixation (116) or by dissection of single neurons (134); even when brain is com-

pletely fragmented by homogenization into isolated nerve terminals or synaptosomes (123), thickened portions of the postsynaptic membrane cling to the presynaptic densities of synaptosomes and can be separated only with strong proteolytic agents (50, 194). Often, when nerve terminals in the brain degenerate and become engulfed by glial cells, such dense portions of the postsynaptic membrane remain adherent to the degenerating synapse and become engulfed as well (48, 282).

Furthermore, as discussed in detail below, synaptic thickenings or densities also occur in neuromuscular junctions, and here too there is evidence that nerve and muscle membranes adhere to each other at these points (see Fig. 4). Couteaux (51) showed that the entire postsynaptic membrane ripped away with the presynaptic terminal when he pulled on the nerve in fixed tissue. McMahan et al. (186) found that the thickened portions of the presynaptic membrane remain attached to the muscle when individual terminal branches are pulled away with microelectrodes.

Even application of collagenase to loosen connective tissue over the neuromuscular junction before their manipulation was not sufficient to break the adhesion of nerve and muscle membrane. Betz & Sakmann (13) found that long treatment with proteases is needed to separate them completely. Taken together, these miscellaneous observations are good evidence that pre- and postsynaptic membranes adhere at the dense or thickened regions. However, the tenacity of this adhesion under acute mechanical stress does not rule out more subtle or slower changes. For instance, when certain neurons undergo chromatolysis, presynaptic terminals are released from their surfaces and become surrounded by glia (24, 134, 235). This is associated with the disappearance of postsynaptic densities in the chromatolytic neuron (184).

Important details of the structure of the membrane specializations at the synaptic complex became apparent when Gray examined tissue stained with phosphotungstic acid. He found that staining small blocks of osmium-fixed brain tissue with phosphotungstic acid during alcohol dehydration, a method introduced to stain the contractile proteins in muscles (90, 132), enhanced the electron density of the synaptic thickenings and revealed new structural details that clearly differentiated the pre- and postsynaptic components of the synaptic complex (116). He showed that in spinal cord synapses the presynaptic thickening is composed of a number of discrete dense projections arranged in a more or less triangular array, each of which extends from the presynaptic membrane into the cytoplasm for some distance and is typically surrounded by synaptic vesicles (118). This close spatial relationship suggested to Gray that vesicles might adhere to the dense projections in preparation for discharge.

Bloom & Aghajanian (25, 26) confirmed Gray's observations by developing a method to stain the dense material in the synaptic complex without staining the other membranes. They stained tissues with alcoholic phosphotungstic acid after fixing them with aldehydes, rather than fixing them with osmium tetroxide, which also stains membranes generally. This approach revealed that the dense projections are interconnected at their bases by narrow dense bands. Pfenninger and Akert (2, 221, 222) developed another method for staining the synaptic densities without staining the neighboring membranes, which involved treating aldehyde-fixed tissue with bismuth iodide, which, like phosphotungstic acid, is supposed to react with positively charged proteins. They also saw that presynaptic dense projections are interconnected by fine strands lying on the inside of the membrane and proposed that the entire array of triagonally interconnected dense projections should be called the presynaptic grid (2, 227).

Of particular interest in the organization of the presynaptic grid is the spacing of the adjacent dense

projections just far enough apart so that synaptic vesicles can nestle between them and thus reach the presynaptic membrane. In fact, the spacing between dense projections is narrower in synapses that contain smaller synaptic vesicles (2). The vesicles that reside in the narrow spaces within the grid apparently contact the presynaptic membrane directly, because it has no cytoplasmic coat in these spaces. Thus they are likely to be those that discharge transmitter during synaptic activity.

Although a few images that looked like synaptic vesicles whose membranes had fused with plasma membrane in the act of discharge have been observed in the vicinity of the presynaptic specialization in thin sections (87, 109, 122), they have not been seen with any regularity, and there has been no way to tell whether the vesicles in question were joining the plasmalemma or pinching off from it. In order to observe such a phenomenon, it would be desirable to see large expanses of the presynaptic membrane in a face-on view, rather than the very narrow strips of membrane that can be seen within a thin section. While no technique exists yet to look at the outside surface of cells embedded in tissues at the necessary resolution, the freeze-fracture technique has offered a useful alternative approach to this problem.

In this procedure, frozen tissue is fractured or broken open in a high vacuum and the freshly exposed surface is replicated by evaporating platinum and carbon onto it (193). The advantage of this method is that fractures tend to track along membranes in frozen tissue and thus expose broad panoramas of the surfaces of frozen cells (192). In fact, there is now a great deal of evidence that fractures actually split frozen membranes through their hydrophobic interiors and thus expose not the true surface of the cells, but internal views of split plasma membranes (30). In such internal views, deformations of the plasma membrane, such as spots where synaptic vesicles are attached, ought to be readily apparent and easily mapped.

The first results of the pioneering freeze-fracture studies of Akert, Pfenninger, Sandri, and Moor (2, 223, 224) were indeed informative. In spinal cord, the same tissue where Gray discovered the triangular arrays of presynaptic dense projections, they could identify the presynaptic plasmalemma and see on it circular areas, approximately the diameter of synaptic specializations, which were marked by clusters of small dimples or protuberances on the appropriate internal views of the plasma membrane (Figs. 2 and 3).

These deformations in the fracture plane were thought, for several reasons, to be sites of attachment of synaptic vesicles. First, they resembled the freeze-fracture images of vesicles fused with the plasmalemma that had been found in other cells, such as capillary endothelia where they appear as protuberances on the outer leaflet and dimples on the internal

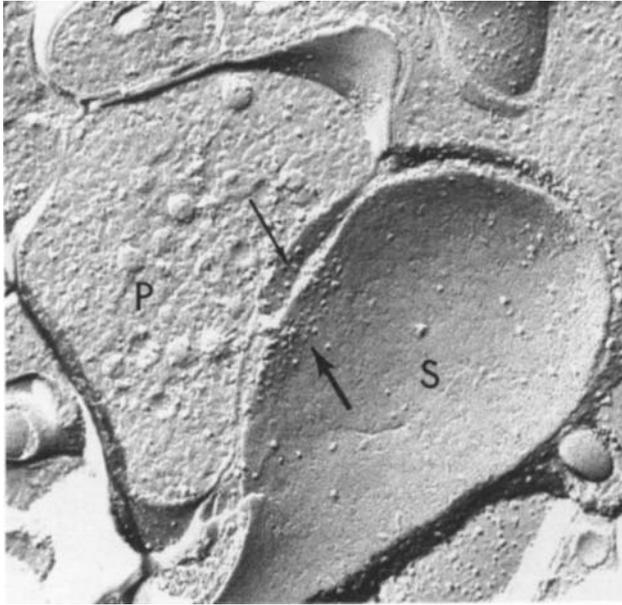


FIG. 2. Freeze-fractured synapse of a parallel fiber (*P*) onto the spine (*S*) of a Purkinje cell dendrite in the molecular layer of the cerebellum. The cluster of particles (*large arrow*) in this platinum replica of the remaining external leaflet of the fractured spine membrane (*S*) is thought to represent the postsynaptic active zone of this synapse. Opposite this postsynaptic specialization the axoplasm of the cross-fractured parallel fiber (*P*) contains a number of synaptic vesicles and the cytoplasmic leaflet of its presynaptic membrane displays a cluster of particles in the narrow region where it has been split (*smaller arrow*). Platinum deposits are *dark*, leaving light shadows. $\times 80,000$.

leaflet of the fractured plasma membrane (255). Occasionally, enough deformations were found in a single terminal to recognize that they tended to cluster in hexagonal arrays with about the same spacing as the vesicles associated with the presynaptic grid (223, 224). Two different spacings were present in spinal cord synapses, which were thought to reflect the different sizes of synaptic vesicles found there (2). Finally, fractures that broke completely through such pock-marked membranes revealed synaptic vesicles in the underlying cytoplasm, so there was no doubt that these were membranes of nerve terminals (2).

Streit et al. (263) investigated whether these membrane deformations changed in any way during synaptic activity, by comparing presynaptic membranes from spinal cords fixed under barbiturate anesthesia with cords from animals fixed while awake, on the assumption that the overall level of synaptic activity would be greater in awake animals. The synapses they found in awake animals did not have any more of these deformations in their presynaptic membranes than anesthetized synapses, but they thought that more of them looked "open"; that is, more of the protuberances on the outer leaflets of the presynaptic membranes had little craters at their tops (see Fig. 12). These craters were thought to be membranous necks that had connected synaptic vesicles with the

plasmalemma at the time the tissue was prepared for freeze-fracturing. This interpretation suggested that the deformations are rather persistent attachment sites for synaptic vesicles which can open briefly in order to discharge transmitter.

Pfenninger & Rovainen (226) investigated how such presynaptic membrane deformations are related to discharge of transmitter in the giant axons of the lamprey spinal cord, which has the advantage that it can be kept alive *in vitro* and stimulated either electrically or by ionic manipulations. They showed that after stimulation the number of deformations in the presynaptic membrane increased to 4 times the number at rest but that this increase was prevented if magnesium was substituted for calcium in the bathing medium, a measure that is known to block transmitter release at synapses. They also showed that the proportion of deformations of the open type doubled as a result of stimulation. These authors also concluded that the deformations were vesicle attachment sites but that both their number and their shapes were related to discharge of transmitter.

These studies on CNS synapses made it clear that vesicle attachment sites characterize the presynaptic specialization, but they left the impression that such sites are rather persistent structures which exist in two distinct and probably reversible stages: a closed, resting stage and an open stage related to discharge of transmitter. The total number of vesicle attachment sites appeared to respond only sluggishly to stimulation. Thus it was unclear whether the significant interactions of synaptic vesicles with presynaptic membrane required fusion of the two membranes. It did appear, however, that this interaction, whatever its nature, occurred at the presynaptic vesicular grid. The deformations occasionally seen outside the grid appeared to be sites where coated vesicles were forming from the plasmalemma [Figs. 3 and 12; (171, 226)].

Further evidence for the role of synaptic vesicles in transmitter discharge came from morphological studies of neuromuscular junctions, which proceeded along lines roughly parallel to the studies of CNS synapses just described. In their first description of synaptic vesicles, Palade (206) and Palay (210) reported that vesicles could also be found in motor nerve terminals in rat diaphragm muscle. Subsequently, Robertson (245) found that synaptic vesicles fill the motor nerve terminals in amphibian muscle, and on the basis of this information, del Castillo & Katz (65) proposed that synaptic vesicles could contain the intracellular packets of transmitter, which they had postulated from the way in which these nerve terminals secrete transmitter in discrete multimolecular pulses or quanta.

Just as Palay and Gray had shown in CNS synapses, Birks et al. (17) showed that synaptic vesicles in the frog neuromuscular junction cluster around tufts of dense material on the presynaptic membrane

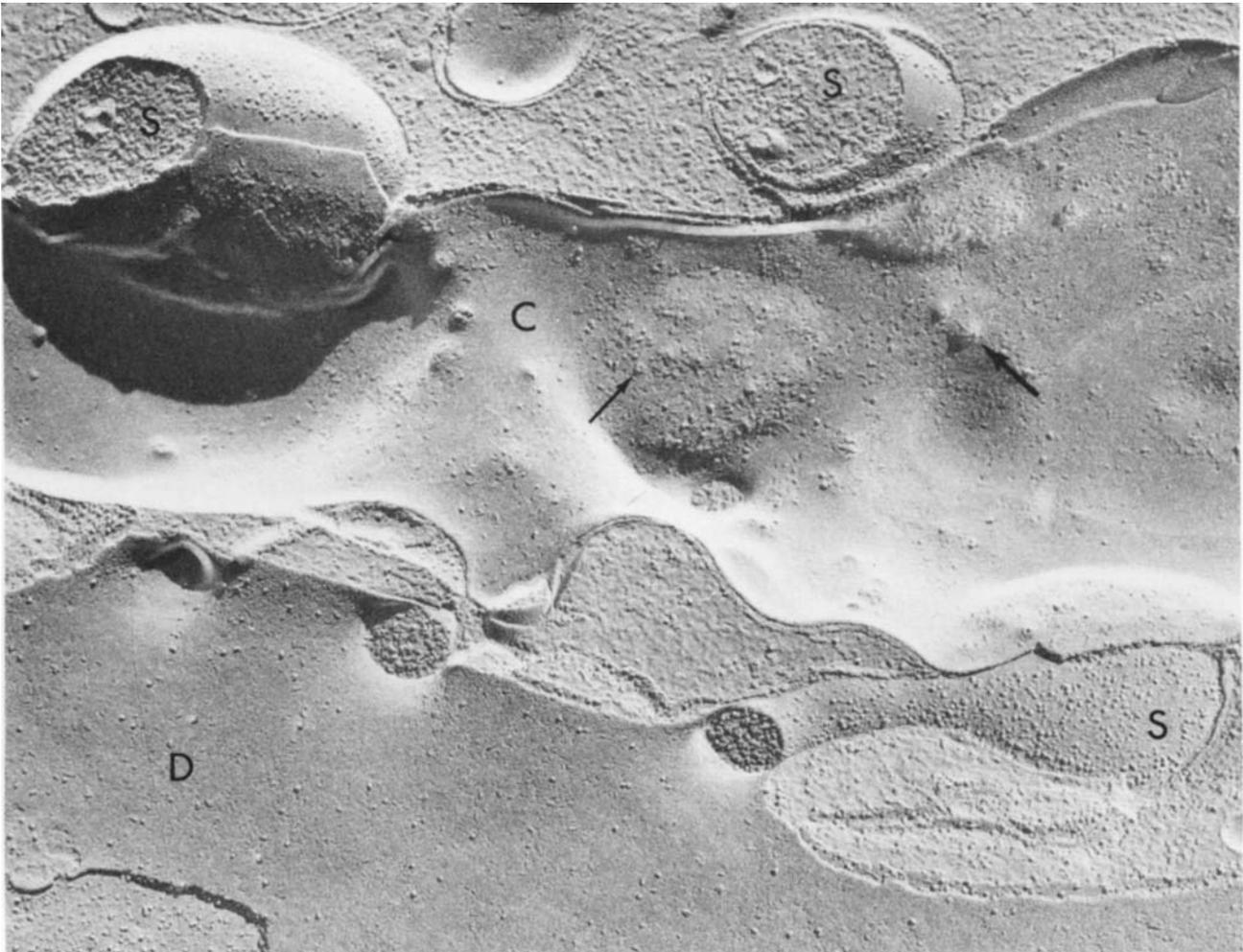


FIG. 3. Replica of a freeze-fracture through cerebellar molecular layer which has struck a climbing fiber (C) where it contacts the spines (S) of a Purkinje cell dendrite (D). One of the dendritic spines (lower right) can be traced to its origin from the dendrite. At sites of synaptic contact, the outer leaflet of the climbing fiber plasmalemma is indented and these indentations display a number of tiny deformations (arrow) which are thought to be sites where synaptic vesicles were caught in the process of exocytosis. Such deformations are characteristic of the active zones of CNS synapses. Surrounding the active zones are larger plasmalemmal deformations which are thought to be stages of coated vesicle formation from the presynaptic membrane (large arrow). Platinum deposits are white in this picture and shadows are black. $\times 120,000$.

which stain with phosphotungstic acid. The only striking way in which the presynaptic specialization of the neuromuscular junction differed from that in CNS synapses was in the geometric arrangement of the presynaptic densities, which were aligned in narrow bands directly across from the deep transverse folds in the muscle membrane [Fig. 4; (17)]. This seemed an admirable arrangement for synaptic transmission, to have vesicles queuing in elongated dense zones strategically located to discharge transmitter where it would have maximum access to the broad expanses of postsynaptic membrane in the folds.

However, in osmium-fixed neuromuscular junctions, Birks and co-workers found very few vesicle-shaped deformations of the presynaptic membrane

and of course could not tell whether those they did find represented vesicle discharge or vesicle formation. They concluded that the chance of finding a discharging vesicle, even if this synapse could be fixed during the peak of transmitter secretion produced by a nerve impulse, was extremely small.

Nevertheless subsequent studies succeeded in revealing, with some regularity, structures that looked like discharging synaptic vesicles. Couteaux & Pecot-Dechavassine (56) illustrated that frog neuromuscular junctions fixed with aldehydes could display a number of small invaginations or deformations in their plasmalemma, just the size of a synaptic vesicle. Since the muscles used for this study were fixed in situ by perfusing fixative through the vasculature of intact animals, it was hard to know whether the

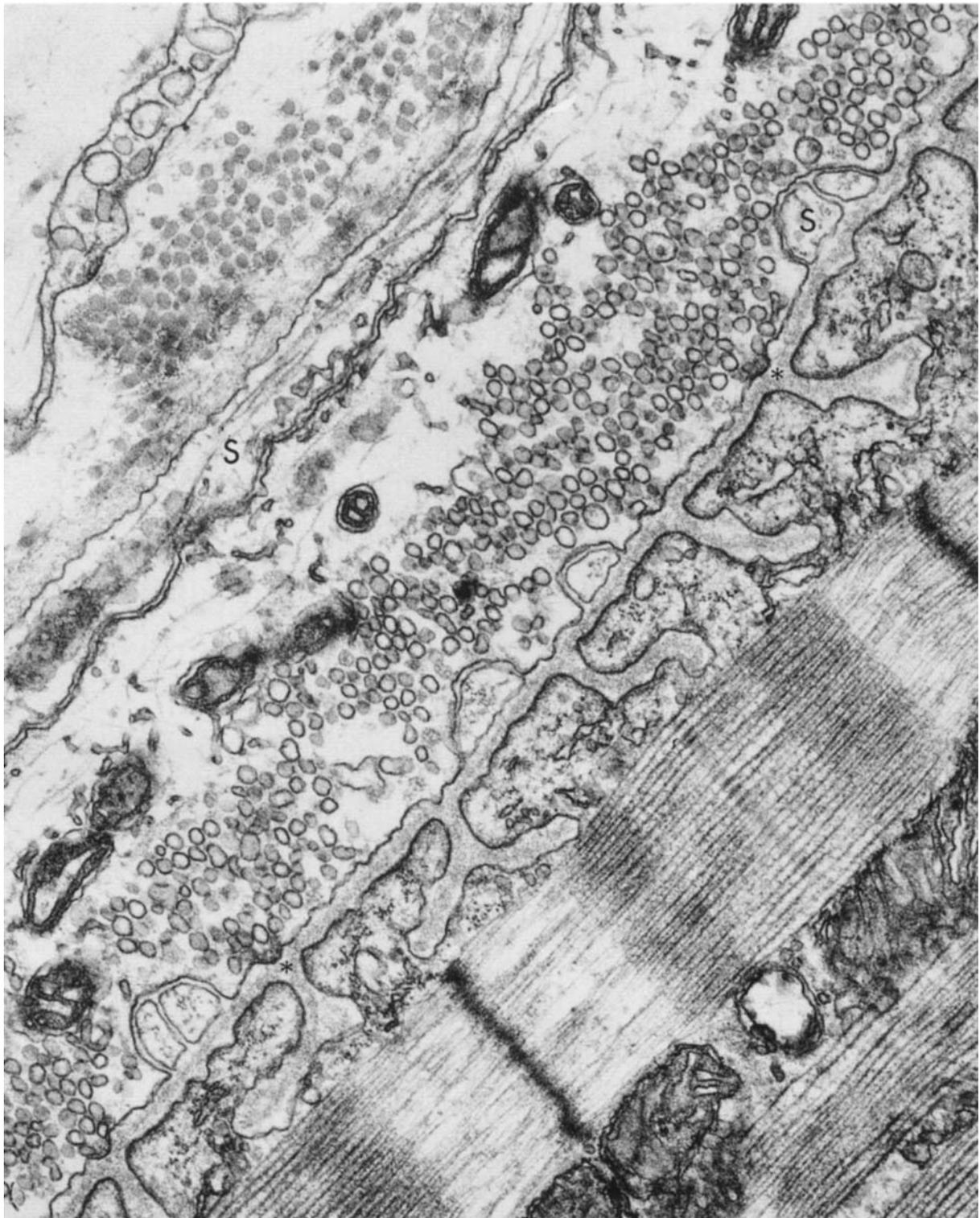


FIG. 4. Longitudinal section through a terminal branch of a neuromuscular junction in frog sartorius muscle. Synaptic vesicles cluster around specialized regions of the presynaptic membrane (*asterisks*) where it protrudes slightly toward the opposing folds in the muscle membrane. These regions of the presynaptic membrane are subtended by a dense cytoplasmic material which forms bands that face the mouths of muscle folds. At the tops of the folds the postsynaptic membrane of the muscle is also underlain by a dense material that may represent its active or receptive zone. Freeze-fracture views of this region are shown in Figs. 5 and 13. The terminal is sheathed on a Schwann process (*S*) which intrudes fingers (*S* at upper right) between the terminal and the muscle. $\times 40,000$.

nerve terminal had been active during fixation. However, the precise localization of the invaginations to the vicinity of the dense bands, where synaptic vesicles are normally lined up (57), supported their suggestion that they represent synaptic vesicles caught in the act of discharging transmitter. Several years earlier, Couteaux had proposed that the presynaptic densities and their clusters of vesicles be called "active zones," in preference to the term "synaptic complex" in order to stress that transmitter discharge probably occurs in these localized areas [cited in (81)]. The discovery of Couteaux & Pecot-Dechavassine that structures that looked like discharging synaptic vesicles are restricted to exactly these zones was remarkable support for this idea.

Subsequently, when Dreyer and co-workers (77, 219) examined the first freeze-fracture replicas of neuromuscular junctions, they found plasmalemmal deformations lined up adjacent to the dense bands, which is exactly the region of the nerve terminal where Couteaux and Pecot-Dechavassine had found invaginations of the plasmalemma. The dense bands were easily identified because they displace the plasmalemma slightly toward the mouths of the subjacent transverse folds in the muscle. Along both margins of each ridge were one or two rows of unusually large intramembranous "particles" (Fig. 5).

Particles, either randomly dispersed or aggregated at membrane specializations such as intercellular junctions, are a ubiquitous feature of freeze-fractured membranes and are thought to be sites where protein components of the cell membrane invade the lipid bilayer (31, 32). The distinguishing feature of the particles at the ridges in the neuromuscular junction was their large size and precise arrangement. Also their constant relationship to the plasmalemmal deformations suggested to Dreyer et al. (77) that they might be preexisting sites in the plasmalemma that could initiate vesicle attachment.

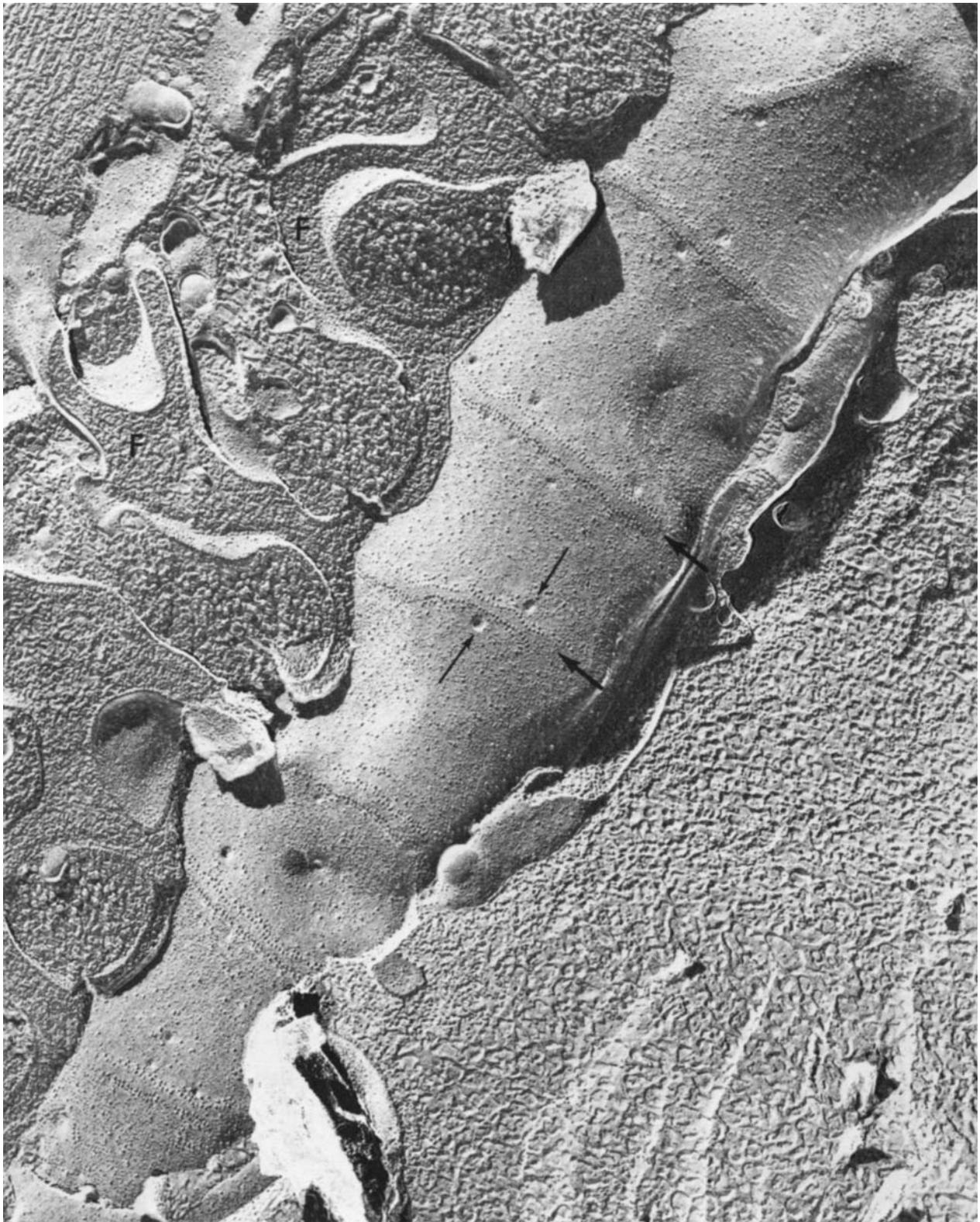
Heuser et al. (144) subsequently used the freeze-fracture technique to study the frog neuromuscular junction in different functional states. They found that the deformations seen in the presynaptic membrane by earlier workers are present only during synaptic activity. Terminals that were presoaked in Ringer's solution containing magnesium instead of calcium, and then fixed in aldehydes containing magnesium in order to suppress transmitter secretion as much as possible, displayed practically none of these plasmalemmal deformations. At least at the frog neuromuscular junction, such deformations do not seem to be long-lasting sites in the plasmalemma that persist in the absence of transmitter release; rather they seem to be transient distortions which occur at the moment of vesicle discharge. This is the case in frog sympathetic ganglia as well, where plasmalemmal deformations occur only at synapses that are stimulated during fixation and do not occur at rest (75).

On the other hand, Heuser et al. (144) also reported

that the number of plasmalemmal deformations that appeared on stimulated frog terminals increased when the aldehyde fixative was diluted so that it would arrest transmitter secretion more slowly. Even though such signs of vesicle discharge may be only transient phenomena during life, they apparently persist and accumulate for some time during chemical fixation (144). This could explain why fused vesicles were more abundant in the muscles Couteaux & Pecot-Dechavassine (56) studied than Birks et al. (17) would have predicted on the basis of instantaneous rates of transmitter release. Also it illustrated that aldehyde fixation slows down and distorts the natural membrane perturbations that occur during transmitter secretion and may obscure different morphological stages of vesicle discharge. Perhaps methods eventually can be devised to observe such transient changes in synaptic structure without resort to fixation in aldehydes.

The distortion in the numbers of plasmalemmal deformations produced by using aldehyde fixatives was, however, useful because it allowed large enough numbers of these structures to be studied to see exactly what they are and where they occur, which is perhaps the most important finding to emerge from the many recent studies of synapses with the freeze-fracture technique. By using a fixative that produced continuous rows of plasmalemmal deformations in the neuromuscular junction and cutting serial sections through the active zones of these synapses, it became clear that, at least under these conditions, all the deformations were minute invaginations of the plasmalemma with exactly the diameter that would be expected if a synaptic vesicle were caught in the act of fusing with the plasmalemma (144). Thus the plasmalemma deformations at these locations were never places where synaptic vesicles simply contacted the plasmalemma or formed a junction with it (29). It was also apparent that the degree of collapse at which a fusing vesicle was captured might determine whether its top would be broken off during freeze-fracturing so that it would appear open, or whether the fracture would proceed around its whole perimeter so that it would appear closed.

From the freeze-fracture studies the important fact became clear that synaptic vesicles fuse with the plasmalemma at particular, well-defined regions on the presynaptic surface (Figs. 2, 3, and 5). The reason for this is not known. On the one hand, the presynaptic membrane could be specialized in these regions to permit synaptic vesicles to discharge periodically their contents. As discussed above, the first freeze-fracture studies of CNS synapses supported this possibility by implying that vesicle attachment sites were permanent differentiated structures in the presynaptic membrane, which simply opened and closed during transmitter release (223, 224, 263). However, this possibility became unlikely when it was found that the number of vesicle attachment sites increases during stimulation (226) and that in muscle and sym-



pathetic ganglion none are present, at least when synapses are completely resting (75, 144).

Another possibility is that the large intramembrane particles found in freeze-fracture replicas of the presynaptic membrane could be permanent specializations involved in vesicle discharge (77). Rows or clusters of such particles have been found to occur beside the vesicle fusion sites in a number of types of synapses, including synapses in the CNS (171) and in the frog sympathetic ganglion (75), as well as the neuromuscular junction (77, 144). These particles are present at rest, in the absence of plasmalemmal deformations, as well as during stimulation. However, although it is clear that, at the frog neuromuscular junction, vesicles do not fuse directly in top of these particles but beside them, it has not been possible to show that they occur at any fixed distance from them (144). Therefore, at the moment, it is hard to imagine how they could control interactions between vesicles and the plasma membrane, unless they are imagined to exert some control over the composition of intracellular fluids in the vicinity of the synaptic vesicles. Other than the proximity of these particles, there is nothing to see by the freeze-fracture technique that would distinguish the regions of the presynaptic membrane where vesicles will fuse with it.

On the other hand, vesicle discharge may be so highly localized simply because vesicles are concentrated near the presynaptic surface in particular zones to begin with. As seen above, in all the synapses studied so far, vesicles appear to cluster around the presynaptic densities. Whether this indicates that the dense material actively pulls vesicles into this region, or simply holds them there once they have diffused into the area, remains to be seen. This may be answered by further study of receptor synapses, where the presynaptic dense material extends deep into the cytoplasm and forms a dense ribbon that becomes covered with synaptic vesicles [Fig. 9; (37, 239, 257, 260, 261)]. In any case, the presynaptic density appears to be involved in maintaining a localized concentration of vesicles near the presynaptic membrane, in the exact regions where vesicle discharge is found (239). This special organization of the presynaptic cytoplasm could be sufficient to explain the distribution of vesicle discharge, without imagin-

ing there was anything special about the presynaptic membrane itself in these regions.

Certainly the vesicles that are associated with the presynaptic densities appear to be in a particularly favorable position to be discharged (Figs. 2 and 4). Thus they could contain an immediately available pool of transmitter quanta. If this were so, it would be reasonable to assume that the number of vesicles in the pool would affect how many discharge with each nerve impulse. This number would depend on how fast vesicles could be replaced after they discharged, relative to how fast they could discharge. In turn, the rate at which they were replaced should depend on how many vacated spots there were beside the presynaptic densities, regardless of whether vesicles arrived by simple Brownian movement or by being actively drawn into these regions. Thus, during repetitive stimulation, the number of "immediately available" vesicles would decline progressively and gradually reduce transmitter output, while the number of vacated spots along the presynaptic density would increase progressively and gradually raise the rate at which vesicles could be "mobilized" into favorable positions for discharge. When replacement or mobilization finally balanced discharge, the pool size should stabilize and transmitter output should reach a plateau.

The very rapid fatigue or depression of transmitter output that occurs at many synapses during the first few impulses of a tetanus is thought to indicate that the pool of immediately available quanta is extremely small and easily depleted (91, 287). However, a simple calculation of how many vesicles' line up

¹ The maximum number of 500-A vesicles that could line up along both sides of all the presynaptic densities on one frog end plate would be roughly 20,000. Well-oriented thin sections of these regions in end plates fixed at rest show that vesicles can indeed line up this closely (57). All these 20,000 vesicles are apparently able to discharge promptly; this can be inferred from freeze-fracture replicas of active zones in end plates fixed during very brief stimulation, which show that vesicle fusions rapidly appear all along the active zone, in such abundance that they closely border each other (144). The number of quanta that are discharged during the first few impulses can be as great as 300/impulse (157, 159, 183), but removing 300 quanta from 20,000 does not reduce the store enough to explain the approximately 20% decline in quantal release that occurs with each succeeding discharge during a brief tetanus (264).

FIG. 5. Freeze-fractured neuromuscular junction from an electrically stimulated frog sartorius muscle, viewed from outside the nerve terminal to observe the cytoplasmic leaflet of its split membrane. Ridges on the surface of the nerve terminal (*large arrows*) face folds (*F*) in the surface of the muscle. Small dimples in the surface of the terminal beside the ridges (*small arrows*) are thought to be loci where synaptic vesicles were fixed as they fused with the plasmalemma. Because very few such deformations occur in unstimulated terminals or in terminals stimulated in Ringer's solution containing magnesium to block transmitter release, vesicles are thought to fuse with the plasmalemma only during transmitter discharge. Platinum deposits *white*, shadows *dark*. $\times 50,000$.

along the presynaptic densities in a favorable position for discharge at the frog neuromuscular junction illustrates that there are far too many to become depleted by the first few stimuli. Thus, if all these vesicles represent the immediately available pool, the fraction of them that discharges with each succeeding impulse must also decline. This could result from some sort of inactivation of whatever triggers vesicle discharge, but it could also indicate that not all these favorably located vesicles have the same chance of discharging, so that some are more available than others. In this case, it will be necessary to look for more subtle differences in location or morphology among the vesicles that concentrate in these discharge regions.

RECYCLING OF SYNAPTIC VESICLES

A number of attempts have been made over the years to determine the physiological role of synaptic vesicles by experimentally altering their numbers. The reasoning has been that if vesicles are containers of transmitter quanta, then sufficiently prolonged and intense stimulation of transmitter release should produce depletion of vesicles, even if it does not produce persistent images of vesicle discharge.

De Robertis & Vas Ferreira (72) reported an early attempt to deplete synaptic vesicles in nerve terminals from rabbit adrenal glands by stimulating the splanchnic nerve for a few minutes before osmium fixation. Only by stimulating at 400 Hz were they able to deplete these terminals of synaptic vesicles; stimulating less intensely, at 100 Hz, paradoxically increased their vesicle counts (69, 72). Later, Birks et al. (17) reported that even quite drastic stimulation of the frog neuromuscular junction, by soaking it in either high potassium or hypertonic media, did not produce a convincing depletion of synaptic vesicles.

These early reports seemed to discourage further attempts, until Hubbard & Kwanbunbumpen (151) studied the effects of stimulating rat diaphragm neuromuscular junctions, again by soaking them in media containing high concentration of potassium (120 mM) or hypertonic sucrose. Such treatments produced a very modest depletion of vesicles, never more than 30%. Subsequently, Jones & Kwanbunbumpen (153) stimulated rat diaphragm neuromuscular junctions electrically at 10 Hz for 1.5 h, without producing any depletion of vesicles. However, both of these studies counted only the vesicles that lay close to the plasmalemma, or actually touched it, because they expected that this "specific" vesicle population should be the most affected by stimulation.

In fact, if they had considered the tendency for vesicles to cluster near the surface in the vicinity of the presynaptic specialization, they might have expected the opposite result. For example, Perri et al. (220) illustrated that stimulating rat sympathetic

ganglia at 20 Hz for 30 min reduced the numbers of vesicles that were located at a distance away from the presynaptic surface more than it affected the number of vesicles in the immediate vicinity of the presynaptic densities. This result reminded Perri and co-workers of Gray's idea that the presynaptic density may attract or trap synaptic vesicles.

Such a tendency for vesicles to cluster near the presynaptic surface may even be enhanced during stimulation. Quilliam & Tamarind (234) have reported that the number of vesicles located near presynaptic densities in rat sympathetic ganglia synapses, which they call the "local" vesicle population, actually increases above normal for a few minutes after stimulation. Also, Jones & Kwanbunbumpen (153) observed such a rebound "overshoot" in the number of vesicles near the presynaptic surface soon after stimulating rat neuromuscular junctions. Thus, if there were any pause between stimulation and fixation in the studies mentioned previously, and such a pause is almost inevitable when using aldehydes because they penetrate and fix tissues so slowly (144), then the mobilization of vesicles indicated by these experiments might well replace vesicles near the active zone that were discharged during the stimulations. For example, when sympathetic ganglia in the frog were stimulated throughout the whole period of fixation, depletion of the vesicles nearest the active zones was clearly seen and measured (75).

Although all these studies were probably complicated by the interaction of chemical fixatives with vesicle discharge and mobilization, they raise the possibility that stimulation alters the distribution of synaptic vesicles, and raise basic questions about which vesicles should be counted when looking for the morphological consequences of transmitter release. However, they did show that synaptic vesicles do not disappear as readily during stimulation as do secretory droplets in other types of cells. Of course this could be considered as evidence against the involvement of vesicles in transmitter discharge altogether, but it could also indicate simply that vesicles are conserved and used over again after they discharge transmitter.

Von Hungen et al. (281) found that the turnover of vesicles, or at least of some of the protein in their membrane, is far too slow to show that vesicles are destroyed each time they are discharged. Bittner & Kennedy (21) calculated how much membrane peripheral nerve terminals would need if they had to produce a new vesicle for each quantum discharged, and it seemed clearly beyond the realm of possibility. Dahlstrom & Haggendal (60) determined, from their measurements of the rate at which vesicles accumulate above axon ligatures, that the supply of new vesicles from the cell body to adrenergic neuromuscular junctions is far too slow to maintain the rate of

transmitter discharge and turnover these terminals display. All these studies concluded that vesicle membrane must be conserved after transmitter discharge and reused.

The conclusion that vesicles can discharge quanta over and over again also seemed inescapable after Heuser & Miledi (142) demonstrated that frog neuromuscular junctions stimulated relentlessly with lanthanum ions do not run out of vesicles until they have secreted millions of quanta, several times more than their original stock of vesicles. Ceccarelli et al. (42) reached the same conclusion after demonstrating that frog neuromuscular junctions stimulated electrically can release millions of quanta without any sign of vesicle depletion, as long as the stimulation is not too fast.

A number of studies have considered exactly how vesicle membrane might be conserved and used again. It depends, of course, on how vesicles discharge in the first place. If discharge involved nothing more than a transient perforation of adjacent vesicle and plasma membranes, the problem would simply be to close the perforation and restock the vesicle with transmitter. However, there is considerable morphological evidence that discharge involves a complete coalescence of vesicle and plasma membrane, or what de Duve (64) named "exocytosis." Indeed today, even without compelling morphological evidence, but simply on the basis of what is known about the fluid nature of biological membranes (112, 256), we could reason that complete coalescence probably must occur even if only small spots on vesicle and plasma membranes fuse, however briefly, during discharge.

The morphological evidence for coalescence of synaptic vesicles with the plasma membrane is similar to what has been found in other secretory cells (4, 155); namely, when stimulation does succeed in reducing the numbers of vesicles in synapses, it produces a concomitant expansion of the plasma membrane. Clark et al. (45) illustrated this in a most dramatic way, by applying black widow spider venom to frog neuromuscular junctions. This venom stimulated a massive burst of transmitter release and left the nerve terminals swollen and empty. Their measurements showed that the surface membrane of venom-treated synapses had expanded to about the extent one would expect if every vesicle coalesced with the plasmalemma during the treatment. Since that study, black widow spider venom has been found to stimulate other types of peripheral nerve terminals and leave them swollen and empty of vesicles as well (111, 135, 163, 205). Of course, this venom might produce an abnormal sort of vesicle exocytosis, but Ceccarelli et al. (42) showed that very low-frequency nerve stimulation of frog terminals, if continued for several hours, would also leave these terminals swollen and empty.

Although these results indicated that the final out-

come of persistent secretion is the coalescence of a large number of synaptic vesicles with the plasmalemma, there remained some doubt about whether this occurred during more physiological forms of stimulation. The results of stimulating synapses for very brief periods at high rates suggest that it does, because also under these conditions synaptic vesicles are depleted as the area of the plasmalemma increases. Pysh & Wiley (233) observed a 20% increase in the area of the plasmalemma of terminals in cat sympathetic ganglia stimulated for 10 min, and Heuser & Reese (143) observed a 20% average increase in area of the plasmalemma of frog motor nerve terminals stimulated for 1 min at 10 Hz. Such brief periods of stimulation did not produce frank swelling, so the plasmalemmal expansions resulted, in both cases, in the synaptic terminals becoming more wrinkled or convoluted. In both cases, the amount of membrane added to the surface was roughly the same as the amount of vesicle membrane that had disappeared during the stimulation resulting from a decline in the number of vesicles, which supported the notion that vesicles had coalesced with the plasmalemma. Also, the expansions were reversible, that is, they disappeared when the nerve endings were rested without stimulation.

With this sort of evidence that synaptic vesicle discharge involves complete coalescence with the plasmalemma, as exocytosis does in other secretory cells, the question of how vesicle membrane is reused becomes, first of all, a question of how vesicle membrane is retrieved from the plasmalemma after discharge. Palade (207) first considered this problem in terms of how pancreatic acinar cells recover discharged zymogen granule membrane from the plasmalemma. He thought it was most likely that membrane would be retrieved intact, by vesiculation of the plasmalemma. He had observed such a process in other cell types and had named it "micropinocytosis" (201). Palade proposed that what was needed to observe membrane retrieval by micropinocytosis was a natural marker, something that would be trapped in the vesicles as they formed, and then appear in the electron microscope.

Birks (15) recorded an attempt to study micropinocytosis at the frog neuromuscular junction this way, using the tracer thorium dioxide. Unfortunately most of this tracer must have become bound in the synaptic cleft, because very little entered the synaptic terminals, even after intense stimulation. However, Page noticed that ferritin appeared in an occasional vesicle at frog neuromuscular junctions after muscles had been soaked in ferritin to delineate their t-tubules (160); and Brightman (33) found ferritin in a few vesicles in CNS synapses while studying the distribution of this material in the brain.

The first experimental evidence that transmitter release is accompanied by a compensatory micropinocytosis was the finding that lanthanum ions not only

stimulated massive transmitter release from the frog neuromuscular junction, but at the same time stimulated the uptake of extracellular tracers (142). When the tracer horseradish peroxidase (HRP) was present during lanthanum ion stimulation, this glycoprotein appeared in a variety of membrane-bound compartments inside the terminals, including "coated" vesicles and flattened sacs which Heuser and Miledi called "cisternae." The relation these new compartments bore to synaptic vesicles was not elucidated until later studies, described below.

Holtzman et al. (147) also showed that electrical stimulation of lobster neuromuscular junctions provoked uptake of HRP into a large proportion of their synaptic vesicles; likewise, Heuser (139) and Ceccarelli et al. (43) showed that tetanizing frog neuromuscular junctions also stimulated uptake of HRP into their synaptic vesicles. More recently it was shown that a large proportion of the synaptic vesicles of some CNS synapses that became exposed to the tracer after intraventricular injection were loaded with HRP (271).

Heuser & Reese (143) have studied in some detail the exact sequence in which HRP is micropinocytosed into frog neuromuscular junctions during brief bursts of tetanic nerve stimulation. They reported that HRP first appeared in the coated vesicles and cisternae that proliferated in these synapses during stimulation, and not until later, after stimulation was stopped and the cisternae gradually resolved, did HRP appear in a large proportion of synaptic vesicles (Figs. 6 and 13). On this basis, they proposed that, at least at this synapse, synaptic vesicle membrane is retrieved from the plasmalemma by proliferation of coated vesicles and cisternae, not by a direct reverse of synaptic vesicle exocytosis, and then is recycled from these initial micropinocytotic compartments into new synaptic vesicles (143).

Gray (117) was the first to notice that certain of the vesicles in synapses are distinctively coated by some sort of cytoplasmic material; these he named "complex vesicles." Roth & Porter (249) presented the first evidence that such coated vesicles originate by micropinocytosis (118). They showed, in an insect oocyte, that micropinocytotic vesicles form at particular regions of the plasmalemma that possess a bristly cytoplasmic coating and proposed that the bristles could be involved in deforming the membrane into a sphere

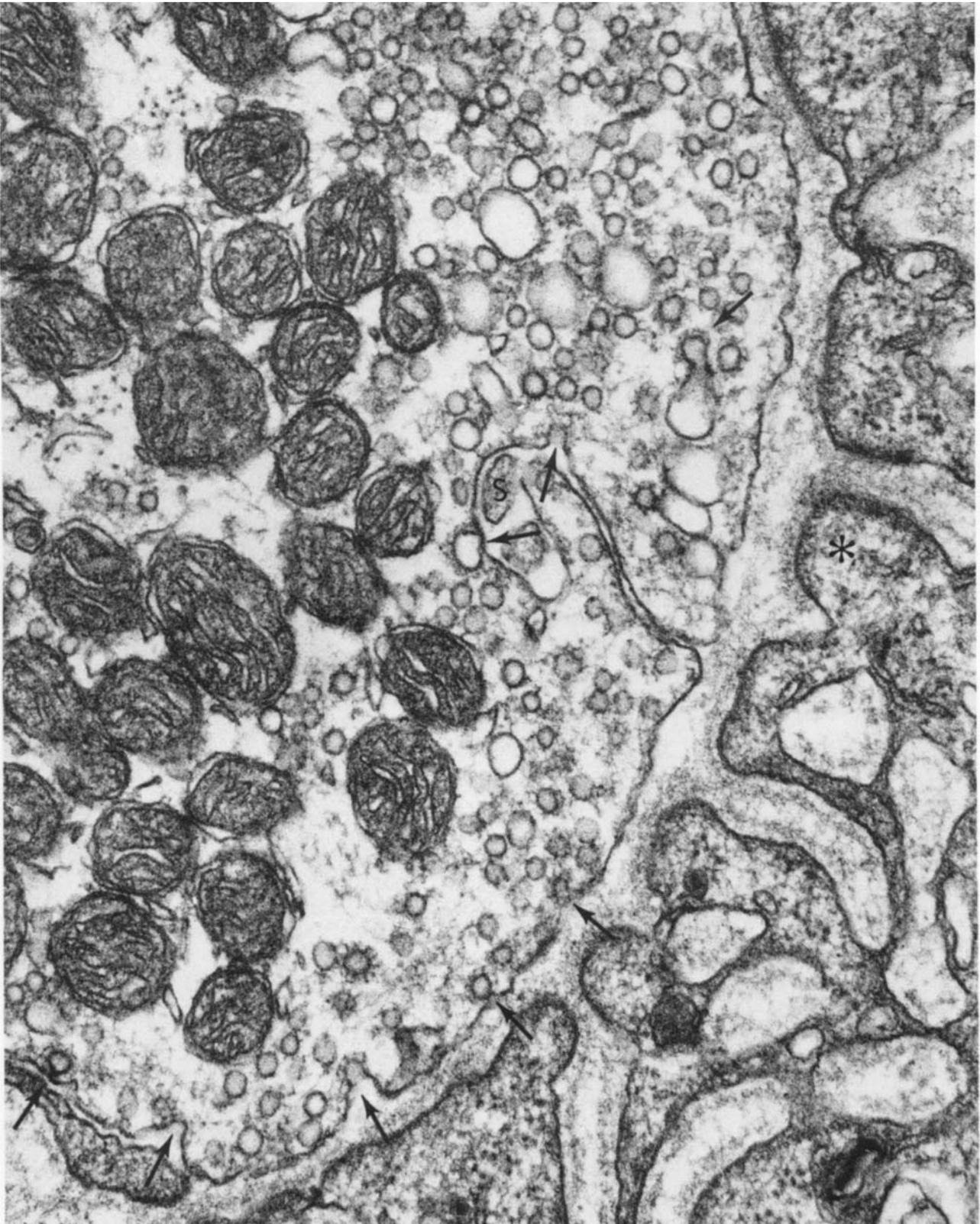
if, for example, their outer ends could repulse each other. Subsequently such bristly coats were found on micropinocytotic vesicles in all sorts of cells that engaged in protein uptake, including neurons (248), and this coincidence led naturally to the idea that the coat might confer a measure of selectivity for protein to the micropinocytotic vesicle (28). Now that evidence is gathering that nerve terminals can discharge proteins along with neurotransmitters, including enzymes involved in transmitter synthesis (197, 285, 288), or proteins that may help sequester the transmitters inside vesicles, it may be found that the proliferation of coated micropinocytotic vesicles is particularly involved in retrieving and conserving these essential proteins.

In addition, Douglas and co-workers (76, 198) have proposed that the formation of coated vesicles in posterior pituitary endings is the means for retrieving the membrane of neurosecretory granules that have fused with the plasmalemma in order to discharge their contents. They have proposed a similar role for coated vesicles in retrieving discharged chromaffin granule membrane in adrenal medullary cells, because in these tissues stimulation of secretion causes coated vesicles to proliferate and take up extracellular tracers (127, 128).

Coated micropinocytotic vesicles have been found in a variety of CNS synapses. Andres found them in synapses in the olfactory bulb, as well as in mossy fiber terminals in the cerebellum, where Gray had first seen them and called them "complex vesicles" (see Fig. 11). Andres (7) thought they might be involved in retrieving transmitter substances liberated during vesicle discharge. Westrum (287) found them in synapses in the olfactory cortex and proposed that they might also be a means to retrieve synaptic vesicle membrane and form new synaptic vesicles. Sites of coated vesicle formation are also recognizable in freeze-fracture replicas [Figs. 7 and 8; (144, 171, 226)]. Recently, Gray & Willis (124) illustrated that coated vesicles could be found in every sort of synapse where they are sought (Fig. 9).

Kanaseki & Kadota (156) showed that the brain was a particularly rich source of coated vesicles for biochemical analyses and added an important new piece of information about their structure by examining isolated coated vesicles with negative stains. This revealed that the "bristly" appearance of the coat in

FIG. 6. Cross section of a neuromuscular junction in a rat diaphragm whose natural activity had been maintained until the moment of fixation. Coated vesicles appear to have been caught in the act of formation at several regions of the plasmalemma (*arrows*), including the invaginated regions that surround a Schwann process (*S*). Other coated vesicles are free in the axoplasm or coalesce with larger vacuoles or cisternae that are dispersed among the synaptic vesicles (*top arrow*). The dense postsynaptic specialization is particularly prominent at the tops of certain folds, where the muscle membrane is cut obliquely (*asterisk*). $\times 100,000$.



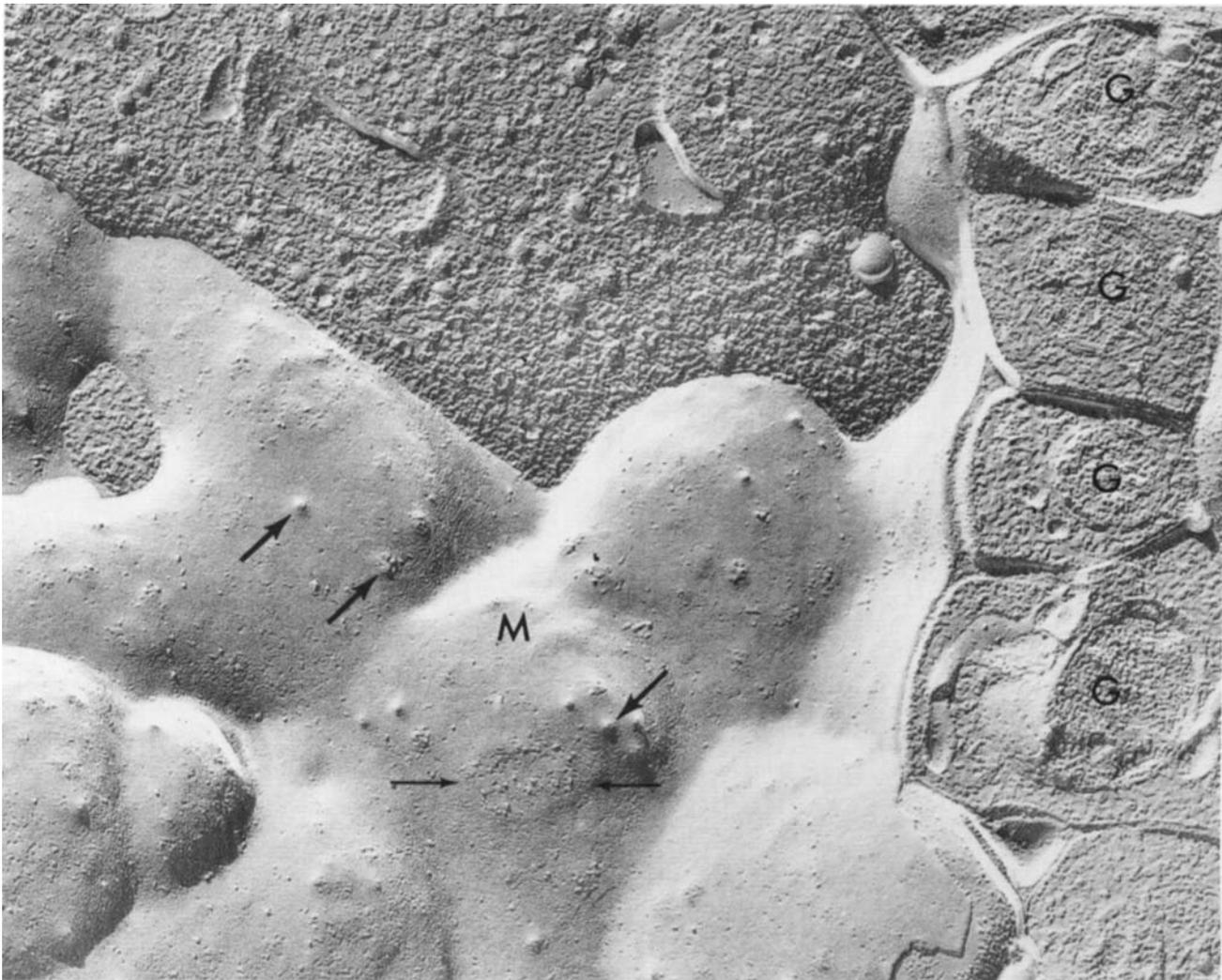


FIG. 7. Freeze-fractured cerebellar glomerulus. A palisade of granule cell dendrites (*G*) bulges against a mossy fiber terminal whose outer leaflet is exposed (*M*, below). The fracture then turns across the cytoplasm of the mossy terminal, exposing synaptic vesicles. The small deformations (*upper arrows*) on the external leaflet of its fractured plasmalemma are situated where coated vesicles are seen to pinch off in thin sections. The small pits at active zones (*between opposed arrows*) are imprints of fields of large particles which characterize the cytoplasmic half of the presynaptic membrane at most CNS synapses. Platinum deposits are *white*, shadows *black*. $\times 80,000$.

thin sections is actually an image overlap phenomenon, a flat projection of a delicate "basket" composed of thin cytoplasmic filaments that surround the coated vesicle in three dimensions, linked together into hexagons and pentagons. Gray & Willis (124) have since resolved this filamentous basket around coated vesicles in thin sections by electron-microscopic stereoscopy. Kanaseki and Kadota proposed that these filaments might deform the membrane into a sphere in a manner somewhat different from that Roth and Porter had imagined, if they were able to rearrange from a flat hexagonal pattern of connections into a dome-shaped pattern of hexagons and pentagons.

Recently, Gray, (119, 121) has reported that, in

material prepared by his methods, coated vesicles often appear to be surrounded by unusually empty-looking zones that are devoid of the usual complement of fine cytoplasmic filaments. He proposed that the coat may be nothing more than an artifactual condensation of these fine cytoplasmic filaments as a result of poor fixation. This does not explain why the coat always has such a delicate and precise polygonal organization, or why it forms only around micropinocytotic vesicles. Also it does not explain the results obtained by Kanaseki and Kadota with tissue fractionation, unless the same artifact occurred at the moment tissues are ruptured during homogenization. It seems more likely that the "clear zones" Gray finds around coated vesicles are a real variation in the

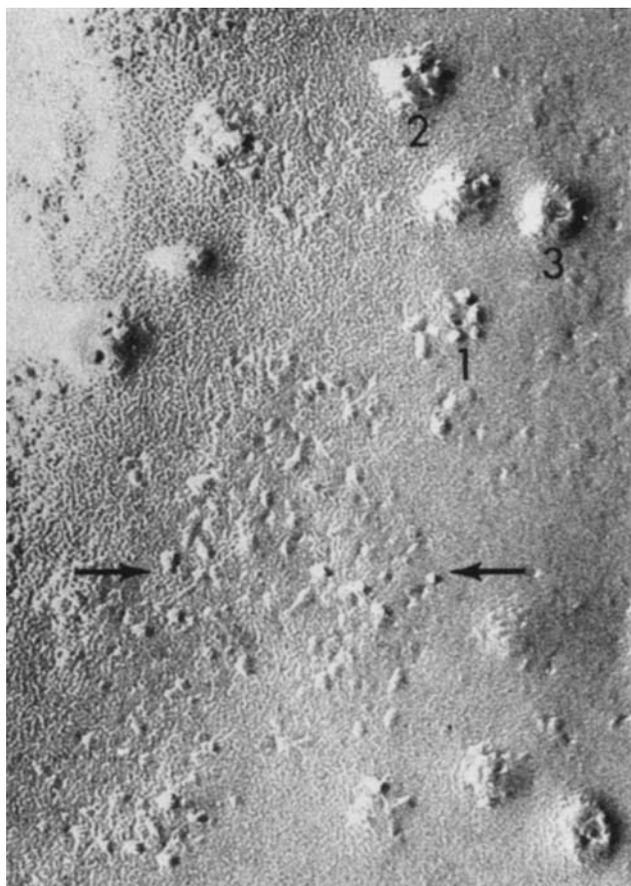


FIG. 8. Higher magnification of plasmalemmal deformations in the external leaflet of the fractured presynaptic membrane of a mossy fiber in a cerebellar glomerulus. Such deformations are usually found just beside an active zone of a synapse, which is demarcated here by the *opposed arrows*. In thin sections, coated vesicles appear to pinch off from the regions where these plasmalemmal deformations occur, so these deformations are regarded as sequential stages in the formation of coated vesicles. The sequence of stages could be 1: large particles gather into clusters in the presynaptic plasmalemma, 2: regions with particle clusters become deformed or curved inward toward the axoplasm (thin sections through these deformed regions have a characteristic cytoplasmic coat), 3: the particle-rich region had presumably formed a nearly complete vesicle which fractured off with the axoplasm, leaving a crater at its last point of contact with the surface. Platinum deposits *dark*, shadows *light*. $\times 200,000$.

filamentous ectoplasm of the synapse and indicate something about how the baskets form around micropinocytotic vesicles during life.

Coated vesicles were first described in neuromuscular junctions by Andres and Düring (8, 80) who concluded that they might be involved in retrieving materials discharged from the terminals. Later, Nickel et al. (200) and Miledi & Slater (191) confirmed these findings. Zacks & Saito (293) injected HRP into rat skeletal muscle *in vivo* and illustrated that it was taken up into the motor nerve terminals exclusively by coated vesicles. Thus the demonstration that nerve stimulation increased the rate of HRP uptake by coated vesicles simply illustrated an accel-

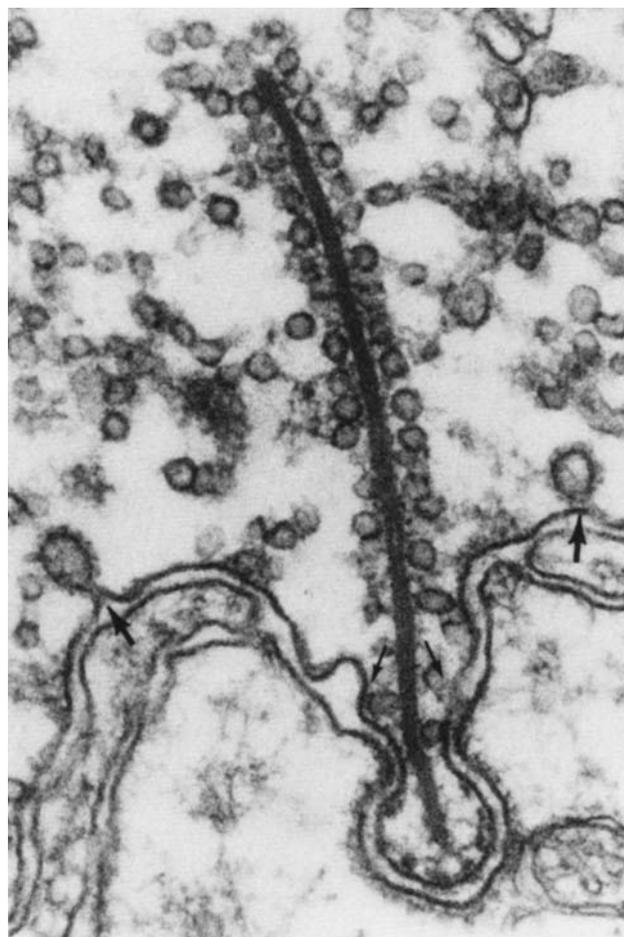


FIG. 9. Synapse of an electroreceptor cell on its afferent sensory axon, from the skate. Synaptic vesicles cluster along the presynaptic ribbon, and at its base appear to fuse with the presynaptic plasmalemma (*small arrows*). Coated pits (*large arrows*) are thought to be a stage in the formation of coated vesicles. $\times 65,000$.

eration of the usual sort of micropinocytosis that occurs at the neuromuscular junction [(143); Fig. 6].

Nevertheless, even though coated vesicles proliferate and take up extracellular tracers during stimulation, they may not be the only way in which vesicle membrane is retrieved from the plasmalemma. It has been suggested that synaptic vesicles are able to close off and return directly to the axoplasm after discharge. However, this path has never been demonstrated. Ceccarelli et al. (42) published an example of an open vesicle at an active zone that contained HRP, which they proposed was a vesicle forming by a direct reversal of exocytosis; but the particular terminal they showed had already been stimulated slowly in HRP for 2 h, and a number of vesicles already contained tracer that they had presumably acquired earlier. In this situation there was no way to know whether the vesicle in question was discharging tracer it has contained inside of the terminal or picking up tracer from the extracellular space. When the

very first wave of HRP uptake was examined, at a time when very few synaptic vesicles contained tracer, it was apparent that this uptake does not occur into synaptic vesicles near the active zones but only into coated vesicles lying away from the active zones (143).

Nevertheless, in order to prove that coated vesicles are the exclusive path for retrieving synaptic vesicle membrane, or components of it necessary to form new synaptic vesicles, a way must be found to block coated vesicle formation without disturbing exocytosis. If coated vesicles are necessary for recovery of synaptic vesicles, a synapse treated this way should accumulate no tracers and promptly run out of synaptic vesicles. Two recent studies are promising leads in this respect; Atwood, Lang, and Moran (9, 174) showed that crayfish synapses treated with the metabolic inhibitor dinitrophenol became depleted of vesicles rapidly during brief stimulation, while normally they are extremely resistant to fatigue and show no vesicle depletion; Clark et al. (45) demonstrated that frog neuromuscular junctions treated with black widow spider venom run out of vesicles after only one round of discharge, that is, without showing any indication that vesicles can be reused. Thus, both the venom and the metabolic inhibitor may block coated vesicle formation.

However, if such mixing of vesicle and plasma membranes occurs prior to recovery of vesicle membrane, some questions would be raised about how these membranes could maintain their chemical individuality. Exactly how distinct they are chemically is not at all clear at the moment. Some earlier biochemical separations of vesicle and plasma membranes reported distinct differences, both in their lipid and in their protein constituents (86, 149). However, more recent efforts have stressed the similarity of these two membranes (129, 195, 196). Nevertheless, if we extrapolate from the studies on other secretory cells where cleaner separations of secretory granules and plasma membranes can be achieved (188), there should be some chemical differences. We must ask, as the authors of these studies have asked, how this chemical individuality is maintained.

Attention focuses on the formation of coated vesicles and whether they might be the means to retrieve vesicle membrane and exclude plasma membrane. Ukena, Berlin, and co-workers (12, 274) have shown that endocytosis in leukocytes and macrophages can be highly specific and can internalize nearly half of the plasma membrane of these cells without disturbing an active transport process that occurs through their surfaces. As mentioned before, coated vesicles also form the "transitional elements" that link endoplasmic reticulum with Golgi areas in secretory cells (208), and coated vesicles can form primary lysosomes that link the Golgi area with phagocytotic compartments in other epithelial cells (110). In both cases coated vesicles probably confer a degree of specificity on the membrane redistribution that they mediate.

VESICLE TURNOVER AND TRANSMITTER METABOLISM

It may be informative to consider how the turnover of synaptic vesicles we have been discussing could be linked to transmitter metabolism. Cholinergic synapses have been studied the most in this regard, even though their vesicles always appear "empty" in the electron microscope. Acetylcholine does not react with aldehydes or osmium and washes out of tissues during fixation, so that it cannot be observed autoradiographically or histochemically.

The store of transmitter in cholinergic synapses is not depleted by prolonged nerve stimulation; this was recognized in the first demonstrations of acetylcholine secretion at the neuromuscular junction by Dale et al. (62) and at the sympathetic ganglion by Brown & Feldberg (34). Years later, Birks & MacIntosh (19) studied the sympathetic ganglion in more detail and confirmed that acetylcholine is normally synthesized fast enough to balance secretion, but they found the balance could be tipped by a small dose of hemicholinium-3. Acetylcholine stores could be nearly exhausted by stimulation in the presence of this drug. Hemicholinium-3 has since been shown to stop acetylcholine synthesis indirectly, by blocking choline entry into the synapse (46, 47, 74, 255, 292).

Several investigators have looked at sympathetic ganglia from cats after their acetylcholine stores have been exhausted by stimulation in hemicholinium-3. Green (125) saw no depletion of synaptic vesicles, but Parducz et al. (214) reported that synaptic vesicle numbers were drastically reduced and concluded that synthesis of new vesicles depends on synthesis of acetylcholine. However, a number of later studies illustrated that preganglionic terminals can be transiently depleted of vesicles by the sort of stimulation used by Parducz and co-workers, even when hemicholinium-3 is not present and acetylcholine stores are not depleted (16, 220, 232). At the moment, the bulk of evidence is against the idea that vesicle formation depends on acetylcholine synthesis in these synapses.

The effect of hemicholinium-3 is not entirely clear at the neuromuscular junction either. Elmqvist & Quastel (90) showed that prolonged stimulation in the presence of hemicholinium-3 led to a progressive reduction in the size of each acetylcholine quantum but did not reduce the number of quanta that discharged with each nerve impulse. This suggested that the number of acetylcholine packets in the terminal did not diminish, but the diminishing store of acetylcholine became reequilibrated equally among all of them. Katz (158) explained that in terms of the vesicle hypothesis, this would be expected if vesicles actively accumulate acetylcholine from the cytoplasm but remain permeable to it, so that they could exchange acetylcholine with empty vesicles recovered from the plasmalemma after discharge. It was somewhat surprising then, when Jones & Kwanbunbumpen (153) reported that a rat dia-

phragm stimulated in hemicholinium-3 showed a slight depletion of vesicles. Other investigators could not confirm this, either in mammalian or frog neuromuscular junctions (143, 152).

Jones & Kwanbunbumpen (153) also reported that the vesicles which remain after stimulating the rat diaphragm in hemicholinium-3 were 40% smaller than normal, and proposed that this could reflect the reduction in quantal amplitude discovered by Elmqvist & Quastel (90). Korneliussen (165) further considered the possibility that a reduced content of transmitter could alter the osmotic properties of vesicles, so that they might shrink or collapse during certain types of hypertonic fixation. However, neither Heuser & Reese (143) nor Ceccarelli et al. (43) observed any reduction in vesicle size in frog neuromuscular junctions stimulated in hemicholinium-3, and Ceccarelli et al. (43) have pointed out that, even in the absence of hemicholinium-3, quantal amplitude declines during prolonged stimulation of frog neuromuscular junctions, without any indication that vesicles become smaller.

Nevertheless there are other reasons for suspecting that the contents of synaptic vesicles affect their size and shape. Determining whether populations of synaptic vesicles are round or flattened (272), or large or small (178), has become a useful method for typing synapses in the CNS, even though the flattening of vesicles is clearly due to osmotic effects of fixatives or subsequent solutions applied to the tissue during processing (27, 277). At many locations in the nervous system, round vesicles predominate in synapses that exert an excitatory action, whereas flattened, ellipsoidal vesicles predominate in synapses with an inhibitory action (211, 231, 273). This correlation presumably reflects the fact that excitation and inhibition in the CNS are usually mediated by different transmitters, which are characteristically stored in different types of packages. It is not surprising, in view of the variety of transmitters thought to be present in the brain, that intermediate types of vesicle populations are found in some regions (95, 96).

VESICLES AND TRANSMITTER IN ADRENERGIC SYNAPSES

In adrenergic synapses, the relation between turnover of synaptic vesicles and transmitter metabolism is in some ways easier to determine, since vesicles that contain norepinephrine can be recognized in the electron microscope by their dense core. Pellegrino de Iraldi and de Robertis (216, 243) were the first to report that some of the nerve terminals in adrenergically innervated tissues contained synaptic vesicles with dense cores. Wolfe et al. (290) illustrated by electron-microscopic autoradiography that radioactive norepinephrine applied to these tissues is taken up almost exclusively by the nerve terminals that contain such dense-cored vesicles. Subsequently, Pel-

egrino de Iraldi & de Robertis (217) and Richardson (244) showed that depleting these tissues of norepinephrine, by treating them with reserpine, makes the dense cores disappear from a majority of these vesicles. Euler and co-workers (93, 94) had shown that reserpine blocks norepinephrine accumulation into intracellular storage granules, thus allowing it to leak slowly out and to become metabolized by cytoplasmic monoamine oxidase. Van Orden et al. (279) later showed that restocking only the cytoplasm of such terminals with norepinephrine, by blocking monoamine oxidase while reserpine was still present, did not make the dense granules reappear. Therefore the dense cores seemed to result from norepinephrine stored in the vesicles (Fig. 10).

The cores did not seem to result from staining of a carrier protein inside such vesicles, if one exists similar to the chromogranins in adrenal medullary granules, because it would not be expected to leak out during reserpine treatment. Later support for this conclusion came from the illustration of Hokfelt & Jonsson (146) that electron-microscopic fixatives are definitely capable of oxidizing norepinephrine into an electron-dense precipitate and the discovery of Tranzer & Thoenen (270) that certain false transmitters, which are more strongly oxidized by electron-microscopic fixatives, produce even darker cores when they accumulate in these vesicles. Thus current evidence is strong that the dense core represents a store of norepinephrine.

Normally, stimulating adrenergic synapses does not deplete their store of transmitter, just as it does not deplete cholinergic synapses, unless transmitter synthesis is blocked (22). Van Orden et al. (280) found that stimulation did not reduce the number of dense-cored vesicles in adrenergic terminals unless norepinephrine synthesis was blocked at the same time. However, even when their treatments reduced the number of dense-cored vesicles by over 80%, they did not reduce the total number of vesicles; instead a larger proportion of vesicles looked empty. This was good evidence that adrenergic vesicles persist even when they are empty, which is the same conclusion that was reached for cholinergic vesicles on less direct grounds. Assuming that norepinephrine discharge involves exocytosis of dense-cored vesicles, the results of Van Orden and co-workers would indicate that adrenergic vesicles are retrieved and conserved even if they cannot refill with transmitter.

The morphological evidence for exocytosis of dense-cored vesicles is, however, less compelling in some ways than for other sorts of synaptic vesicles. Burnstock & Holman (38) have shown that norepinephrine is discharged in small pulses or multimolecular bursts, and Weinshilbaum et al. (285) have shown that intravesicular proteins are discharged along with norepinephrine; few examples of vesicle-sized invaginations in the plasmalemmas of adrenergic nerve terminals have been published, however (99, 100, 102). Presynaptic densities and vesicle clusters

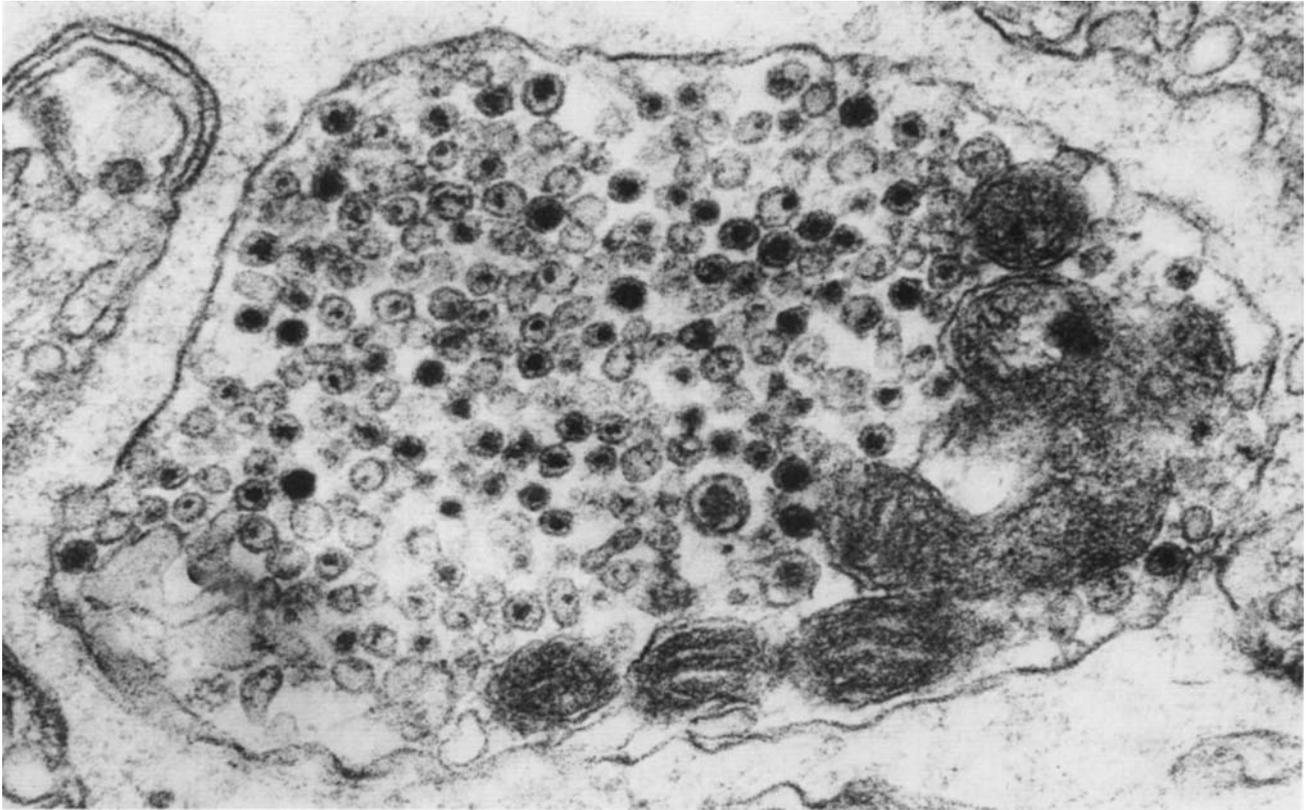


FIG. 10. Adrenergic varicosity in the rat vas deferens. A large proportion of the synaptic vesicles that cluster in such varicosities contain electron-dense cores of various sizes and shapes. It has so far not been possible to define regions in such varicosities where the presynaptic membrane is specialized into discrete active zones. $\times 110,000$. [From Basbaum (11).]

seem to be absent in at least some types of adrenergic synapses (113). At the moment, the best morphological evidence for exocytosis are two reports that black widow spider venom discharges norepinephrine from adrenergic nerve terminals and leaves them swollen and empty of vesicles (111, 135, 228), but as discussed before when considering this result in cholinergic terminals, the marked degree of swelling raises the possibility that this venom makes vesicles coalesce with the plasmalemma in an abnormal manner. No signs of vesicle turnover, such as uptake of extracellular tracers into dense-cored vesicles, have been reported so far. Coated vesicles can be found in adrenergic synapses, but they are not abundant.

These may all be indications that adrenergic terminals secrete rather slowly compared to cholinergic terminals. Their natural frequency of firing may be much less than, for example, that of the neuromuscular junction (1, 105, 106), and the amount of transmitter released per impulse may be at least an order of magnitude less (61, 107). Nevertheless we cannot yet dismiss the possibility that adrenergic synapses discharge transmitter in a fundamentally different way from that in which transmitter is released from cholinergic synapses (60).

ULTIMATE ORIGIN AND FATE OF SYNAPTIC VESICLES

Regardless of how vesicles are conserved and used over again locally, there must be a supply of new vesicle components from synthetic centers in the cell body. Palay (210) recognized that most axons contain narrow, angular tubes or canaliculi of endoplasmic reticulum, and he proposed this could be a supply of ready-made membrane for new vesicles. Robertson (245) found that this form of axonal endoplasmic reticulum extends all the way into the synaptic terminals in skeletal muscle and comes into close proximity with synaptic vesicles. Later, Birks (15) noticed the same phenomenon and proposed that the endoplasmic reticulum in terminals could be called "synaptic tubules." Recently, Droz et al. (78) have utilized high-voltage electron microscopy of thick sections to obtain three-dimensional images of the close association between axonal endoplasmic reticulum and synaptic vesicles, which suggest how the endoplasmic reticulum could be a source of new synaptic vesicles (237). Needed now are convincing images of new synaptic vesicles budding from the axonal endoplasmic reticulum. Also it has been suggested that synaptic

vesicles bud from endoplasmic reticulum in certain synapses from an invertebrate (54, 138, 148).

Pellegrino de Iraldi & de Robertis (218) proposed an alternative source for new synaptic vesicle components, based on their observations of vesicle accumulation above axonal constrictions in rat sciatic nerve. They examined rather thick sections and thought they could discern dilated microtubules that were continuous with varicose tubes of membrane. On this basis they proposed that vesicles might originate from microtubules (218). This is unlikely because microtubules are an assembly of fibrous proteins (269), whereas vesicles are composed of membranes that do not contain any microtubular protein (154). Pellegrino de Iraldi and de Robertis may have simply witnessed a close spatial association of microtubules with axonal endoplasmic reticulum, which could reflect the involvement of microtubules in axonal transport of new membrane (253).

Smith et al. (258) showed an exceptionally close association of synaptic vesicles with axonal microtubules in the giant axons of the lamprey spinal cord which dramatically illustrates how microtubules could transport vesicles toward the synaptic terminals. Recently, Gray (120) has utilized a new fixation method to illustrate that a few microtubules extend all the way into the synaptic terminal zones, where he thought they might conduct vesicles directly onto the active zones.

The eventual fate of synaptic vesicle membrane is likewise not known. From the work by Kristensson & Olsson (167), we can appreciate today that a variety of proteins can be pinocytosed by the nerve terminal and transported back along the axon, in a retrograde direction, to the cell body, where they are sequestered in what appear to be large digestive vacuoles (175). As pointed out earlier, nerve stimulation greatly augments pinocytosis of protein at the synapse, and much of this protein ends up in synaptic vesicles because this pinocytosis is the first step in the pathway of vesicle recycling (143). Thus it is not unreasonable to imagine that the protein which ends up in digestive vacuoles in the cell body delineates an alternate path by which a portion of the retrieved vesicle membrane, perhaps the part which is "worn out," is not made into a new generation of vesicles but is removed from the synapse and digested in the cell body. Examination of peroxidase uptake by cultured spinal cord neurons suggests that much of the protein taken up at synapses ends up in dense bodies in cell somas (266).

Evidence that the retrograde transport of proteins is closely coupled to synaptic vesicle recycling comes from Litchy's illustration (181) that tetanic nerve stimulation greatly increased the amount of protein tracer that was transported in a retrograde direction up frog motor axons. Since this did not occur when transmitter discharge was blocked with magnesium ions during the stimulation, it may have reflected an acceleration of vesicle recycling.

DEVELOPMENT AND DEGENERATION OF SYNAPTIC TERMINALS

It is of interest in considering the structure of the active zone to follow the development of this complex structure, or more specifically, to see how the morphology of a growing nerve tip changes when it contacts another neuron, or effector cell, and begins to secrete transmitter. Cajal (39) was the first to recognize that the advancing neurite is capped by an expansion which he called the "growth cone" (289). Pomerat et al. (229) used microcinematography to illustrate that this region of the neurite is constantly moving, extending fine exploratory processes called filopodia this way and that, promptly retracting some and following others, advancing over rough terrain and halting occasionally at some obstacle or neighboring cell. Yamada et al. (291) studied such expansions in the electron microscope and found that nothing is visible in their finest processes except a delicate meshwork of filamentous material, while back in their main body occur a variety of membranous organelles.

Bunge (35) investigated how these organelles are involved in the growth of the axon and the eventual appearance of synaptic vesicles. Two forms of intracellular membrane are particularly prominent: broad patches of tangled, angular tubes and discrete clumps of larger, smooth vacuoles. It is not known which of these represents the source of new plasmalemma needed for axon growth or for extension of new filopodia. Bunge (35) found that the angular tubes occasionally appear to be continuous with the plasmalemma, and extracellular tracers can enter them even after fixation, which confirms that they open directly onto the surface and therefore could be a source of new surface membrane. However, Pfenniger & Bunge (225) have found that the clumps of vacuoles often occur in small mounds just beneath the plasmalemma and are composed of membrane that displays few intramembranous particles in freeze-fracture, like the plasmalemma of growing regions; so they too might be a source of new membrane. On the other hand, growth cones must also retrieve plasmalemma from the surface when they retract or pinocytose extracellular materials. Birks, Mackay, and Weldon (20) have illustrated that many of the other membranous organelles found in growth cones, including the coated vesicles, multivesicular bodies, and dense bodies, will pick up extracellular tracers.

Growth cones have not been reported to contain clusters of vesicles of uniform size like synaptic vesicles. Bunge et al. (36) reviewed the evidence that these specialized organelles cannot be recognized until there are rudimentary presynaptic densities for them to cluster around; these first appear along narrow neurites, not at their growing tips. However, it has not been ruled out that growth cones contain synaptic vesicles or can occasionally secrete a quan-

tum of transmitter before they form a contact. In fact, growth cones of adrenergic neurons contain a particularly large number of dense-cored vesicles the size of synaptic vesicles (44).

The maturation of synaptic contacts from a rudimentary presynaptic density with a small halo of synaptic vesicles has not been completely studied in the electron microscope. In regenerating peripheral neuromuscular synapses where maturation can be timed and studied physiologically with some degree of accuracy, it appears to involve simply a progressive growth and elaboration of the same structures (115, 145, 161, 162, 164, 179, 267). Physiologically the synapse releases progressively more quanta and becomes more resistant to fatigue (66, 103), while morphologically the synapse develops progressively larger or more numerous presynaptic densities and more synaptic vesicles. It never completely loses the membranous organelles that characterize the growth cone because, as we have seen, tortuous tubes of endoplasmic reticulum, coated vesicles, and larger vacuoles or multivesicular bodies are also found in adult synapses where they appear to have a role in the turnover of vesicles and plasmalemma. Moreover, Hashimoto, Palay, and Sotelo (137, 262) have pointed out that some neuronal processes which look very much like growth cones can be found in the brains of adult animals, and suggest that neuronal remodelling or synaptic growth continues in the adult.

Consideration of the degenerative changes that occur when nerve terminals are cut off from their cell bodies contributes some additional correlations between structure and function of the synaptic terminal. A vast literature has formed about methods to stain and localize degenerating terminals, which are used to make lesions and trace pathways in the CNS. Most such studies have concentrated on the later stages of degeneration, when dying terminals become pyknotic and filled with argyrophilic filaments or osmiophilic material that makes them differentially stainable. The ultrastructural basis of these late degenerative changes was well described by Gray & Guillery (122).

Perhaps more relevant to the mechanism of transmitter secretion are the earliest changes that occur during degeneration. These begin often with rather subtle changes in mitochondria and synaptic vesicles. Mitochondria typically become swollen and pale (185), and so long as this can be carefully distinguished from the mitochondrial swelling that results from slow fixation or tissue anoxia, it appears to be a rather specific change in shape like that occurring in isolated mitochondria when calcium is added to their environment (126, 130, 131, 185). In some cases the swollen mitochondria in degenerating terminals even contain small electron-dense deposits like those found in isolated mitochondria that have accumulated calcium from their environment (215). Heuser

et al. (140) have shown that bathing nerve terminals in isotonic calcium chloride rapidly produces this mitochondrial change. The same sort of mitochondrial swelling can also be produced by tetanic nerve stimulation (143, 182, 283), but in this case the mitochondria slowly recover their normal shape during rest, and it is believed that the excess calcium accumulated during the stimulation is slowly cleared from the axoplasm (23).

At about the time this mitochondrial change is occurring, synaptic vesicles usually disappear from degenerating terminals, leaving them empty and swollen looking (67, 68, 185). However, this vesicle change is not as consistent. In some synapses, not all vesicles disappear, and the remaining ones clump tightly together or swell (18, 59, 191, 242). These vesicle changes are also greatly accelerated by bathing nerve terminals in isotonic calcium chloride (140).

In the peripheral synapses that have been investigated, physiological signs of transmitter secretion stop at about the time vesicles disappear (18, 151). Often this block is preceded by an agonal burst of spontaneous transmitter secretion, which may occur as calcium floods the terminals, and therefore presumably correlates with the early mitochondrial changes. Furthermore an interesting coincidence has been found in the situations where the few remaining vesicles either clump together or swell; the agonal transmitter secretion includes a number of unusually large quanta, which could come from discharge of these altered vesicles (275, 276).

What compromises the synapse's ability to resist calcium influx, if that is in fact what initiates degeneration, remains to be determined (140). Perhaps it is simply the most sensitive indication of a general deterioration spreading down the axon from the cut to the terminal, or perhaps it is specifically deranged by interruption of supply of some essential materials from the cell body.

GLIAL AND SCHWANN CELLS NEXT TO SYNAPTIC TERMINALS

Presynaptic terminals are often covered by glial cells, or Schwann cells, except at their immediate contact with the postsynaptic cells. Often, thin glial processes completely embrace presynaptic terminals and intrude into the synaptic cleft, but they never invade the active zones, which is another indication that these regions adhere tightly.

It has been known for some years that, when presynaptic terminals degenerate, these investing glial cells become phagocytic and engulf the remains of the terminal (48, 259). However, it has only recently been appreciated that glial cells can expand or contract to cover more or less of the terminals. For example, tetanic nerve stimulation somehow provokes Schwann cells at the neuromuscular junction to ex-

pand and press many thin pseudopodia into the nerve terminals (43, 143). It is difficult to know whether Schwann cells actively sculpt these shape changes or simply expand to maintain a cover on the presynaptic terminals as their plasmalemma expands during stimulation. The Schwann cells are capable of maintaining a complete covering even while nerve terminals are grossly swollen by stimulating agents such as black widow spider venom (45) or cardiac glycosides (14), although they fail to do so, and even appear to contract, if high concentrations of magnesium are applied during such stimulation (J. E. Heuser, unpublished observations).

Thus the special anatomic relationships of glia and Schwann cells to synapses suggest a special, but as yet undefined, role for glia in some aspect of synaptic transmission. Whether these anatomic relationships imply an exchange of certain materials between glia and synapses (203), a role in isolating nerve terminals (168), or a role in the maintenance of contact between the pre- and postsynaptic elements of the synapse remains to be determined.

STRUCTURE OF THE POSTSYNAPTIC ACTIVE ZONE

The postsynaptic membrane "senses" the presence of a chemical neurotransmitter by means of receptors that somehow modulate its permeability to inorganic ions. The structural basis of this permeability change remains beyond the reach of current electron-microscopic techniques, which require that the membrane be fixed and stained before viewing. However, certain structural specializations have been identified in postsynaptic membranes that have indicated where the receptors, and thus the postsynaptic active zones, may be located.

The first postsynaptic specialization to be found was described by Palade (206) and Palay (209) as a localized "thickening" of the postsynaptic membrane. Palay (210) illustrated that such thickenings lie directly opposite the presynaptic densities in CNS synapses, and he considered them to be a postsynaptic component of the "synaptic complex" (Fig. 1). By staining with phosphotungstic acid, Gray (116) was able to see that the postsynaptic membrane looked thicker in these regions because it was coated by a fuzzy layer of dense, granular material that gradually faded into the underlying cytoplasm without a clear boundary. This material appeared more filamentous than granular to de Robertis (70), so he called it a "subs synaptic web." Bloom & Aghajanian (25, 26) showed that this dense postsynaptic material can be stained selectively with phosphotungstic acid without staining the rest of the postsynaptic membrane.

Deeper cytoplasmic structures are associated with the postsynaptic dense material in certain synapses. Taxi (265) found a second dense stratum beneath the

postsynaptic densities, separated from them by wispy filamentous material, at synapses on frog sympathetic neurons. Pappas & Waxman (213) have since found similar "subjunctional dense bodies," as they called them, dangling from the postsynaptic densities in some types of synapses in the mammalian CNS.

The dense fuzz under postsynaptic membranes varies in thickness in different synapses, and to some extent with different staining methods, but Gray (116) noticed that in the cerebral cortex it is considerably thicker at the postsynaptic regions of dendritic spines than it is at postsynaptic sites on neuronal cell bodies. He proposed that two broad types of synaptic complexes could be distinguished in the cerebral cortex: a type I complex, characterized by a thick postsynaptic density that covers a relatively large area and by a relatively broad synaptic cleft that contains a prominent intermediate dense line; and a type II complex, characterized by a thinner postsynaptic density covering a relatively smaller area and by a narrower synaptic cleft without a prominent dense line. Thus, Gray (116) classified the synaptic complexes he observed on dendritic spines as type I and those he observed on neuronal cell bodies or mainstream dendrites as type II (Fig. 11). However, in many regions of the CNS, synapses have since been found that do not fit all the criteria for either type I or type II (122). In subcortical regions of the brain particularly, many synapses are difficult to classify on the basis of the structure of the synaptic complex (283). Moreover the selective distribution of the two types on cell bodies and dendrites is not absolute, because type I synapses are also found on cell bodies, for example on stellate cells in the cerebral cortex (49).

Colonnier (49) reexamined synaptic structure in the cerebral cortex and concluded that the width of the synaptic cleft, and especially the thickness of the postsynaptic density, are the only consistent differences among various sorts of synaptic junctions, and the only sure ways to discriminate between them in the cortex. He stressed this by proposing that type I junctions, whose postsynaptic densities are thicker than their presynaptic densities, could be called "asymmetric," while type II junctions, whose postsynaptic densities are relatively thinner, could be called "symmetrical." Unfortunately the term symmetrical tends to obscure the fact that type II synapses are undoubtedly just as functionally polarized as type I synapses, even though their pre- and postsynaptic densities are roughly of the same thickness. Nevertheless this terminology has been used subsequently in a number of studies which have confirmed that most synapses clearly fall into one of these categories although intermediate ones have been found (49, 122, 211, 212, 240, 278, 283).

In several instances where different types of synapses are located on different parts of a neuron, so

that their electrophysiological behavior can be studied independently, type I synapses have been found to be excitatory and type II to be inhibitory (6, 82, 211, 236). However, in light of the variety of chemical transmitters found in the CNS and the variety of effects that each can exert in different regions, it seems unlikely that this correlation would be absolute. Nevertheless this trend for the structure of the postsynaptic dense material to conform to the type of synaptic action has raised the possibility that these specialized regions are directly involved in synaptic transmission.

There is other evidence that the receptor molecules are especially concentrated at the dense "thickened" regions of the postsynaptic membrane. This evidence includes, first, recognition that the postsynaptic membrane *itself* looks different in the regions coated with dense material, both in thin sections and freeze-fracture replicas (Figs. 2, 11, and 12), and second, demonstration that a snake toxin which binds irreversibly to cholinergic receptors at the neuromuscular junction can be localized to the regions of the postsynaptic membrane that are underlaid with dense material. These points are discussed in more detail in the succeeding paragraphs.

The postsynaptic membrane that has been most completely studied to date is the muscle sarcolemma at the frog neuromuscular junction. As described previously, synaptic vesicles appear to discharge at this synapse from a number of elongated presynaptic active zones which lie just opposite the mouths of the postsynaptic folds. Nearly all the muscle membrane beneath this synapse is coated with fuzzy dense material, except for the deepest recesses of the postsynaptic folds. Birks et al. (17) first appreciated this by means of the phosphotungstic acid stain, and Couteaux & Pecot-Dechavassine (55) confirmed it with uranyl acetate staining, to be sure that phosphotungstic acid had not failed to gain access to the deeper parts of the folds (Figs. 4 and 6).

Couteaux and Pecot-Dechavassine pointed out that

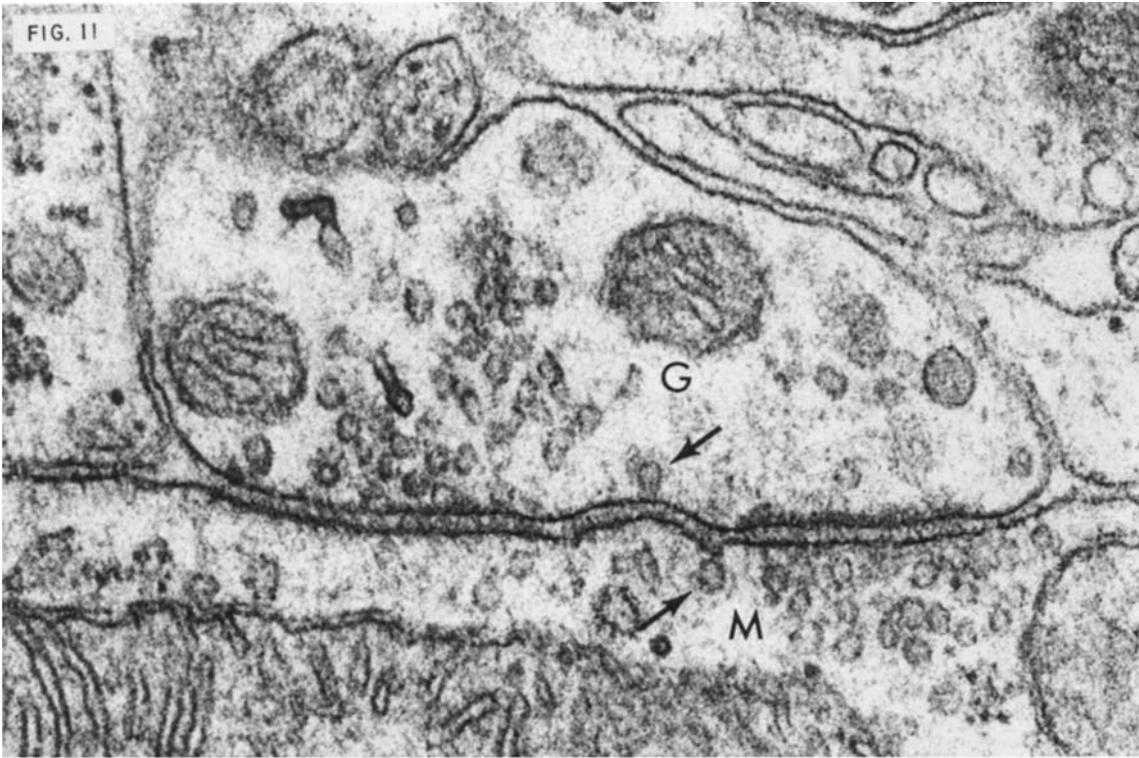
the cytoplasmic material composing the postsynaptic density in this junction is irregularly partitioned into separate plaques each of which appears to rigidify a small region of the postsynaptic membrane, roughly 0.1 μm in diameter, which retains its gently bulging contour regardless of the extent of contraction of the muscle and convolution of the postsynapse. They also noted that these slightly convex plaques of postsynaptic dense material are linked by delicate whisps to bundles of filaments that course through the sarcoplasm between the folds, analogous perhaps to the "subjunctional bodies" found at other synapses (213). Therefore, at the frog neuromuscular junction, the postsynaptic densities appear to connect with an elaborate cytoskeleton that underlies the complex folded configuration of the postsynaptic membrane (Figs. 4 and 6).

Rosenbluth (246) has produced extremely thin sections through frog neuromuscular junctions which illustrate that the postsynaptic membrane itself appears thicker in these dense regions, mainly because its outer leaflet looks more distinct and granular. He proposed that the granules he saw could be the cholinergic receptor molecules.

The internal structure of the postsynaptic membrane that is revealed by freeze-fracture is also different in these dense regions. Ever since Branton (30) recognized that freeze-fracturing splits biological membranes along their inner lipid core, the minute deformations or "particles" that are found scattered over fractured membranes have been scrutinized with growing interest. Evidence is accumulating, primarily from work on erythrocytes, that these particles are perturbations in the lipid core of the membrane produced by proteins which are partly embedded in the membrane, including some surface receptor molecules (31, 32). Since the concentration and distribution of these particles revealed by freeze-fracturing have in some cases been related to the functional state of the membrane (31), there has been growing interest in what this approach would reveal

FIG. 11. Reciprocal synapse between granule cell and mitral cell dendrites in the glomerulus of the olfactory bulb. Synaptic vesicles in the granule cell dendrite (*G*) cluster near a type II junction which lacks a prominent coat of cytoplasmic dense material on the postsynaptic membrane. Vesicles in the mitral dendrite (*M*) cluster near a type I junction which is characterized by a prominent cytoplasmic coat beneath the postsynaptic membrane at the active zone. Coated vesicles (*arrows*) are caught in the process of pinching off from both the mitral and granule cell membranes. At reciprocal synapses adjacent active zones are aimed in opposite directions. $\times 100,000$.

FIG. 12. Freeze-fractured reciprocal synapse from the same region of the olfactory bulb as that shown in Fig. 11. The external leaflet of a fractured granule cell plasmalemma is in full view (*G*) and contains a cluster of particles where this terminal is postsynaptic to a mitral dendrite (*M*, at *right*). The plasmalemmal deformations scattered about on this surface (*arrow*) are thought to be sites at which coated or synaptic vesicles are fused with the plasmalemma. A similar plasmalemmal deformation, viewed from the opposite side, is present on the cytoplasmic leaflet of the mitral cell membrane (*arrow at right*). Synaptic vesicles are visible in the fractured axoplasm of the granule cell terminal (*asterisk*). Platinum deposits *white*, shadows *dark*. $\times 80,000$.



in postsynaptic membranes.

Initially, Sandri et al. (251) found distant clusters of particles in the postsynaptic membranes of certain CNS synapses (Fig. 12). Subsequently, Dreyer et al. (77) found clusters of particles in the postsynaptic membrane of the frog neuromuscular junction, beneath the nerve terminal and also in the postsynaptic folds. Heuser et al. (144) noticed that these particle clusters are located on gentle bulges or convexities of the postsynaptic membrane that conform to the size and distribution of the postsynaptic dense plaques which Couteaux & Pecot-Dechavassine (55) identified in thin sections of this synapse (Fig. 13). No particle clusters were found in the depths of the folds. Thus these clusters of intramembranous particles are another way in which the postsynaptic membrane is structurally differentiated in the dense, thickened regions.

All the freeze-fracture studies of the frog neuromuscular junction agree that the particles cluster predominantly on the cytoplasmic leaflet of the fractured postsynaptic membrane. At other excitatory cholinergic synapses, intramembranous particles assume a similar distribution. At the rat neuromuscular junction, Rash & Ellisman (238) have shown that particles cluster on the inner half of the postsynaptic sarcolemma. In frog sympathetic ganglia, Dickinson & Reese (75) have found clusters of particles on the inner half of neuronal membranes beneath synaptic terminals, and in *Torpedo* electric organ the broad postsynaptic surface of each electroplax cell is covered with intramembranous particles which are found on the inner half of the fractured membrane (199, 247). In all these instances, the particles are spaced about 100 Å apart, so that they reach a density approaching 10,000/μm², which is similar to the concentration of dense particles Rosenbluth (246) found in the outer layer of thin-sectioned membranes. This figure is approximately the density of cholinergic receptor molecules that has been estimated from the amounts of labeled toxins or drugs that bind to these membranes (133, 250). The outer leaflet of these membranes is also structurally distinct. Orzi et al.

(202) have shown that the outer leaflet of *Torpedo* postsynaptic membrane is composed almost entirely of tiny particles tightly packed in linear arrays. Similar tiny particles can occasionally be resolved on the outer half of the postsynaptic membrane at frog neuromuscular junctions as well but do not appear to be as numerous or tightly packed (144).

An additional feature of *Torpedo* postsynaptic membranes has been revealed by deep etching, which results in subliming ice away from around freeze-fractured membranes to expose their actual outer surfaces, as well as surfaces revealed by fracturing the membrane. On what appeared to be the true outer surface of the postsynaptic membranes, Cartaud et al. (40) found small rosettes of tiny bumps packed closely together. These rosettes were nearly the same size and shape as the oligomers of receptor protein that Changeux and co-workers (41) isolated from *Torpedo* membranes and observed by negative staining. Cartaud and associates pointed out that, at the margin between an exposed outer surface and a fractured region of the membrane, the intramembranous particles which sit on the cytoplasmic leaflet of the fractured membrane appeared to be tall enough to extend all the way through the outer leaflet. Some even appear to be crowned on top by a rosette of tiny bumps. However, they found many more rosettes on the outer surface than intramembranous particles on the freeze-fracture face, possibly because a receptor oligomer on the outer surface produces an intramembranous deformation beneath it only when it is in a particular functional state. However, there is no information available yet as to whether depolarization or the application of transmitter agonists or antagonists affects the structure or density of intramembranous particles in postsynaptic membranes. Thus, for the moment, there is no direct evidence that intramembranous particles are parts of the postsynaptic receptors or their associated ion channels within the membrane.

Alternatively, particles could be involved in anchoring the postsynaptic membrane to the presynaptic terminal. De Robertis and co-workers (70) were the

FIG. 13. Frog neuromuscular junction frozen without fixation during a recovery period following intense stimulation. In this view of the external leaflet of the plasmalemma of the nerve terminal (*lower half*) are a number of crater-shaped deformations which are situated in regions where coated vesicles are known to form, including regions of the plasmalemma elevated by the Schwann processes (*S*). At the *top*, the nerve terminal is torn away completely to expose the active zone of the muscle membrane, which is characterized by the high concentrations of large particles that adhere to its cytoplasmic leaflet during fracturing. These particles form an almost continuous mat beneath the nerve terminal and occur in patches of high density immediately beside the terminal. No patches of particles are present in the depths of the postsynaptic folds (*F*) where sarcolemmal caveolae (*arrow at left*), like those over the rest of the surface of the muscle (*arrow at right*), are found. Platinum deposits *dark*, shadows *light*. ×75,000.



first to notice that where the postsynaptic membrane is underlaid by dense material, the synaptic cleft is also bridged by myriad fine filaments (Figs. 1 and 11). They imagined that these filaments were direct extensions of the dense "subs synaptic web" in the postsynaptic cytoplasm. If this were so, intramembranous particles could be sites where such anchoring filaments traverse the postsynaptic membrane. Similar sorts of intramembranous particles have been found clustered in attachment plaques or desmosomes in other types of epithelia, and in some cases have been interpreted as broken-off filaments that traverse the membrane to link the adjacent cells (88). However, important variations have been noted in the distribution of intramembranous particles in different sorts of attachment plaques, which preclude any simple correlation with postsynaptic intramembranous particles (187).

Furthermore there is now histochemical evidence that the receptors at neuromuscular junctions are also confined to the postsynaptic areas underlaid by dense material. Several investigators have allowed labeled α -bungarotoxin to bind irreversibly to muscles and block neuromuscular transmission and then have sought to localize the sites where the toxin is bound. Lee et al. (177) first performed light-microscopic autoradiography to show that radio-iodinated α -bungarotoxin binds to rat diaphragms in the region of the neuromuscular junctions. Anderson & Cohen (5) used fluorescence microscopy to illustrate that fluorescently tagged α -bungarotoxin binds just beneath the nerve terminals at frog neuromuscular junctions, and also binds in the postsynaptic folds to some extent. Fertuck & Salpeter (101) carried this approach to a higher degree of resolution by performing electron-microscopic autoradiography of rat muscles labeled with intensely radioactive α -bungarotoxin. Their quantitative analyses illustrated that α -bungarotoxin binding sites are located predominantly in regions where the postsynaptic membrane is underlaid by dense material, and one of their published autoradiographs clearly showed that very few silver grains develop over deeper portions of the postsynaptic folds, where the postsynaptic membrane is not coated by a dense fuzz. Daniels & Vogel (63) have achieved still higher resolution of α -bungarotoxin binding sites by an electron-microscopic immunohistochemical method which has confirmed that receptors occur predominantly in the region of the postsynaptic density (180).

Additional evidence that the clusters of intramembranous particles in the thickened regions of the postsynaptic membrane are associated with the receptors is that, in other kinds of synapses where acetylcholine is not the transmitter, the arrangement of intramembranous particles is quite different. In fact, the first freeze-fracture studies of synapses reported that particles clustered on the external leaflet of the postsynaptic membrane, rather than on the

cytoplasmic leaflet as they do at the cholinergic synapses discussed above [Fig. 2; (251)]. More recently the freeze-fracture technique has been used to identify variations in the postsynaptic membranes of several identifiable types of synapses in the CNS (171, 173). Several types of synapses in the cerebellum and olfactory bulb that are known to be inhibitory were compared with other types of synapses in the same regions that are excitatory. This approach revealed a consistent difference; all the excitatory synapses bore distinct clusters of unusually large particles on the external leaflet of their postsynaptic membranes, while none of the inhibitory synapses possessed any recognizable particle clusters (171, 173). Thus the synapses with particles in the outer leaflets of their membranes, which Sandri and associates (251) found, probably belonged to the excitatory class. The postsynaptic membranes of the inhibitory synapses that were studied looked very much like nonsynaptic membranes, with particles of various sizes scattered randomly over both of their freeze-fracture faces. This paucity of intramembranous particles may be related to the scarcity of the postsynaptic densities at this symmetrical or type II synapse and could indicate that receptors are more diffusely distributed at inhibitory synapses.

The clusters of postsynaptic particles in excitatory cerebellar synapses appear to be relatively stable arrangements. Experimental treatments that alter the distribution of the individual particles which are scattered elsewhere in neuronal membranes do not appear to disrupt the postsynaptic particle clusters (141, 144). In terms of the fluid-mosaic model of biological membranes (112, 256), which is currently gaining wide support, the ability of individual particles to move under certain conditions would suggest that they represent molecules that float in the neuronal membrane and can diffuse laterally because its lipid core is fluid. Presumably the clusters of postsynaptic particles cannot move because they are linked to structures either inside or outside the postsynaptic membrane. As we have seen, they could be anchored to the dense fuzz that underlies the membrane in these regions, or alternatively they could be linked to the intersynaptic filaments that course through the synaptic cleft in these regions.

The stability of the receptors and the factors controlling their location in the postsynaptic membrane have been studied in some detail at the neuromuscular junction. Miledi and others (73, 189, 268) have shown that embryonic and denervated muscles are sensitive to acetylcholine over their entire surface, although in culture, according to Fischbach & Cohen (104), embryonic muscles apparently have scattered patches of higher sensitivity. Such muscles somehow stimulate motor nerves to grow and synapse upon them (10, 84, 85). In turn, the nerves somehow stimulate the muscle to accumulate and restrict receptors to the region of synapsis (190). The factors that could

mediate this interaction are described elsewhere in this volume (see the chapter by Rosenthal in this *Handbook*).

Observation of bungarotoxin binding sites with the electron microscope in developing or denervated motor end plates will probably provide useful information about where receptors are made and how they are inserted into the postsynaptic membrane. Also comparison of changes in the distribution of toxin binding sites on the muscle membrane with the fine-structural appearance of the postsynaptic membrane in thin sections and freeze-fracture replicas may provide further information on whether the dense cytoplasmic material underneath the membrane is involved in anchoring receptors and restricting them to the synaptic region.

There is some evidence that the specialized dense regions of the postsynaptic membrane originate from vesicles coated with fuzzy material that fuse with the plasmalemma (3). This has been seen particularly clearly in developing sympathetic ganglion cells in tissue culture. In regions where these neurons are contacted by growing axons, Rees et al. (241) found a dense cytoplasmic coat on the postsynaptic membrane and a number of vesicles with fuzzy coats in the adjacent cytoplasm. Some of these fuzzy vesicles were connected with the postsynaptic membrane. Since Rees et al. (241) found that this sort of vesicle did not take up extracellular tracers from the culture medium, it was concluded that they were joining the surface, not pinching off from it. However, the fuzzy material which these vesicles appear to deliver to the postsynaptic membrane looks similar to the filamentous coat that typically surrounds micropinocytotic vesicles as they pinch off from a surface. Thus the relationship of these fuzzy vesicles in developing synapses to the coated vesicles which Waxman & Pappas (284) and others have found pinching off from the postsynaptic regions of adult synapses remains to be determined.

Little is known about the sequence of growth and development of the postsynaptic dense specialization. Apparently it can begin to form in the absence of a presynaptic terminal (136, 172, 179). Lentz (179) has reported that by the time he could find motor nerves in proximity to embryonic muscles in the newt, the nearby muscle was already differentiated by rudimentary dense plaques. These appear to be roughly the same size as the dense plaques in adult muscle

but formed only the slightest convexities on an otherwise smooth postsynaptic surface. Couteaux (53) has shown that the muscle surface does not begin to expand and fold until the nerve has been present and functional for some time. He explained that these folds grow progressively deeper during development until they reach their adult configuration, which varies greatly in different types of muscle. In red muscle fibers whose neuromuscular junctions fire steadily at relatively low frequencies, the postsynaptic folds are relatively sparse and shallow; in larger, white muscle fibers whose neuromuscular junctions fire sporadically at high frequencies, the postsynaptic folds are plentiful and deep (79, 114, 166, 204); and in muscles from patients with the myasthenic syndrome, whose neuromuscular junctions apparently transmit well only at high frequencies (89, 170), postsynaptic folds are greatly overgrown (92, 251).

Couteaux and Taxi (52, 58) have shown that acetylcholinesterase occurs throughout these folds; apparently it is attached to the basement membrane that extends into their deepest recesses (13, 133). Perhaps the deeper folds of white muscle fibers provide relatively more of this esterase, to hydrolyze the transmitter and shut off synaptic currents faster, in order to allow these junctions to operate at higher frequencies. Alternatively, Eccles & Jaeger (33) have proposed that the folds are simply a source of extracellular ions for supporting the endplate currents, which must be larger in order to fire large white muscle fibers.

In contrast to the folds, the postsynaptic density grows during development until it reaches a similar extent in all muscles. We have seen that, in the adult, dense material coats nearly all the postsynaptic membrane that is directly opposite the nerve terminal but extends only a short distance into each fold. This is particularly apparent in mammalian muscles with deep folds, where the major portion of them is not coated (97). Thus the total area of the postsynaptic density depends on how large a contact the presynaptic terminal forms, but depends much less on how deeply the postsynapse is folded. Since receptors appear to be restricted to these dense regions, the number of receptors may thus be admirably matched to the secretory capabilities of the presynaptic terminal, which Kuno (169) has shown to depend on the size of the junction as well.

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