EXTRACELLULAR SYNAPTIC VESICLES IN THE MOUSE HEART

MARY A. GRILLO. From the Departments of Physiology and Rehabilitation Medicine,
New York University Medical Center, New York 10016

INTRODUCTION

This paper reports the chance discovery of membrane-limited profiles having the morphology of synaptic vesicles in the extracellular space bordering upon nerve endings in the mouse atrium. Although not previously described and completely unexpected, such vesicles were found to be a characteristic feature of this tissue, and they were observed after fixation by a variety of different methods. Because they may bear on a number of problems of neuroanatomical interest, such as the fate of synaptic vesicles and the anatomical basis of the quantal release of neurotransmitter substances, a brief description of these extracellular vesicles seems warranted.

MATERIALS AND METHODS

This investigation was carried out on 10 Swiss albino mice of both sexes, weighing 30–50 g and ranging in age from 3 to 18 months. The mice were divided into three groups: (a) five were immunosympathectomized at birth through the administration of bovine anti-nerve-growth-factor serum[1] (0.05 ml per 1.5 g body weight injected daily for 5–8 days beginning immediately after birth); (b) one was injected at birth with normal, instead of immune, bovine serum; (c) four were untreated. The treated and untreated controls were of the same age and, in some cases, the same litter as the first group. Except for a marked ptosis

---

[1] Obtained from Abbott Laboratories, Scientific Divisions, North Chicago, Ill.

and enophthalmos, the experimental animals were grossly indistinguishable from the controls.

The cardiac tissue was obtained primarily from the anterior wall of the right atrium and was fixed either by immersion or by a modification of the perfusion technique previously described (12). Half the animals (three experimental and two controls) were rapidly cooled in an ice-water bath to a rectal temperature of 15°–20°C immediately before fixation. The tissues were fixed in the following solutions: (a) 1–1.3% OsO_4 buffered to pH 7.2–7.4 with Veronal-acetate, phosphate, or a balanced salt solution (10, 15); (b) 2% glutaraldehyde in Veronal-acetate buffer (pH 7.4) containing 0.54% $CaCl_2$ and filtered before use. Postfixation of the blocks was carried out in Veronal-acetate—buffered OsO_4. (c) 1.65% $NaMnO_4$ in Veronal-acetate buffer (pH 7.4). The tissues were dehydrated in methanol and embedded in Epon. Thin sections mounted on naked or carbon-coated grids were stained with uranyl acetate alone or were doubly stained with uranyl acetate followed by lead citrate, and examined in a Philips-300 electron microscope.

RESULTS

Interposed between the axolemma and the basal lamina of naked axons in the mouse atrium are seen, on occasion, circular and oval profiles measuring $\sim$500 A in diameter (range 400–900 A). They are bounded by a single, smooth, limiting membrane, $\sim$75 A thick, of the trilaminar unit membrane type (Fig. 3). In a majority of cases the internum of the profile appears homogeneous and exhibits only slight variations in electron

opacity, comparable in range to the variations shown by the agranular vesicles enclosed within the axon. Apparent exceptions to this statement were of two kinds. In the first instance (Fig. 7), the profile appears darker than the agranular vesicles, but its texture is more or less homogeneous. In the second instance (Fig. 5), the profile is also darker but this increased density is due to the presence of a discrete, ovoid-shaped condensation, $\sim$100 A across, placed near the center of the profile; similar images within the associated axon are identifiable as synaptic vesicles of the small granular type. This type of vesicle is generally thought to be associated with monoamine storage (3, 8); it was not found in the cardiac nerves of immunosympathectomized mice.

In addition to the 500-A profiles, infrequent examples of profiles measuring $\sim$1000 A in diameter were also seen in the extracellular space around nerve endings. These invariably contained a core of moderately electron-opaque material, finely granular in texture and $\sim$700 A in size (Figs. 8–10). Their resemblance to the large granular vesicles found in a variety of nerve fibers (Figs. 1, 6, 7, 8) is striking.

The axon membrane and the paramembranous axoplasm opposite the profiles are variable in appearance. The membrane may be smooth (Figs. 2 and 3) or it may show an inpocketing which appears coated or furry on its axoplasmic surface (Fig. 7). Rarely, the cytoplasm is devoid of formed organelles; occasionally a mitochondrion is present; often there is a scattering of synaptic vesicles (Fig. 3). Of special interest is the not infrequent occurrence of dense patches (Figs. 1 and 4). These consist of roughly conical masses of finely filamentous and granular material apposed to the plasma membrane and closely associated with aggregates of synaptic vesicles. Unlike the presynaptic projections commonly seen at synaptic contacts between nerve cells (1, 7), the dense patches show little or no evidence of being organized in a hexagonal pattern, although this possibility cannot be excluded without serial sections. They are somewhat similar to the densities seen occasionally at close neuromuscular contacts in the rat colon (16) and the rabbit iris (14), and they closely resemble the dense zones found regularly at skeletal neuromuscular junctions (2). They differ, however, in that they occur wherever congregations of synaptic vesicles are found, irrespective of the location of the axon and its proximity to an effector cell. (In contrast to the frog ventricle [17], close neuro-

FIGURE 1 Two vesicles (arrows) in the periaxonal cleft which separates the plasma membrane (*pm*) of a sheathless axon from its surrounding basal lamina (*b*). Opposite the extracellular vesicles, the paramembranous axoplasm exhibits a patch of electron-opaque material which is closely associated with an aggregate of synaptic vesicles. Agranular endoplasmic reticulum, *er*; granular vesicle, *g*. Immunosympathectomized mouse. Fixed in 1% OsO_4 in McEwen's saline. $\times$ 61,000.

FIGURE 2 Arrows indicate two groups of extracellular vesicles in the periaxonal cleft surrounding a pair of naked axons which are enclosed in a common basal lamina. Immunosympathectomized mouse. Fixed in 1% OsO_4 in McEwen's saline. $\times$ 55,000.

FIGURE 3 Trilaminate structure of the limiting membranes of vesicles lying within and outside an axon. Plasma membrane, *pm*. Immunosympathectomized mouse. Fixed in 1% OsO_4 in McEwen's saline. $\times$ 132,000.

FIGURE 4 Dense patches (*dp*) and their associated synaptic vesicles at high magnification. Opposite each patch is an extracellular vesicle. Normal mouse. Fixed in 1% OsO_4 in McEwen's saline. $\times$ 110,000.

FIGURE 5 Extracellular vesicle (arrow) located in a widening of the periaxonal cleft and containing a small density. Similar dense-centered vesicles are visible in the associated axon. Cardiac muscle cell, *m*. Normal mouse. Fixed in 1.65% $NaMnO_4$ in Veronal-acetate buffer. $\times$ 84,000.

FIGURE 6 A string of synaptic vesicles (*v*) bulging into a bay-like recess (*r*) formed by an inpocketing of the plasma membrane (*p*) of a sheathed axon. The entrance to the recess is marked by an arrow. Schwann cell cytoplasm, *s*; basal lamina *b*. Normal mouse. Fixed in 1% OsO_4 in Veronal-acetate buffer with added $CaCl_2$ (0.5%) and sucrose (1%). $\times$ 84,000.
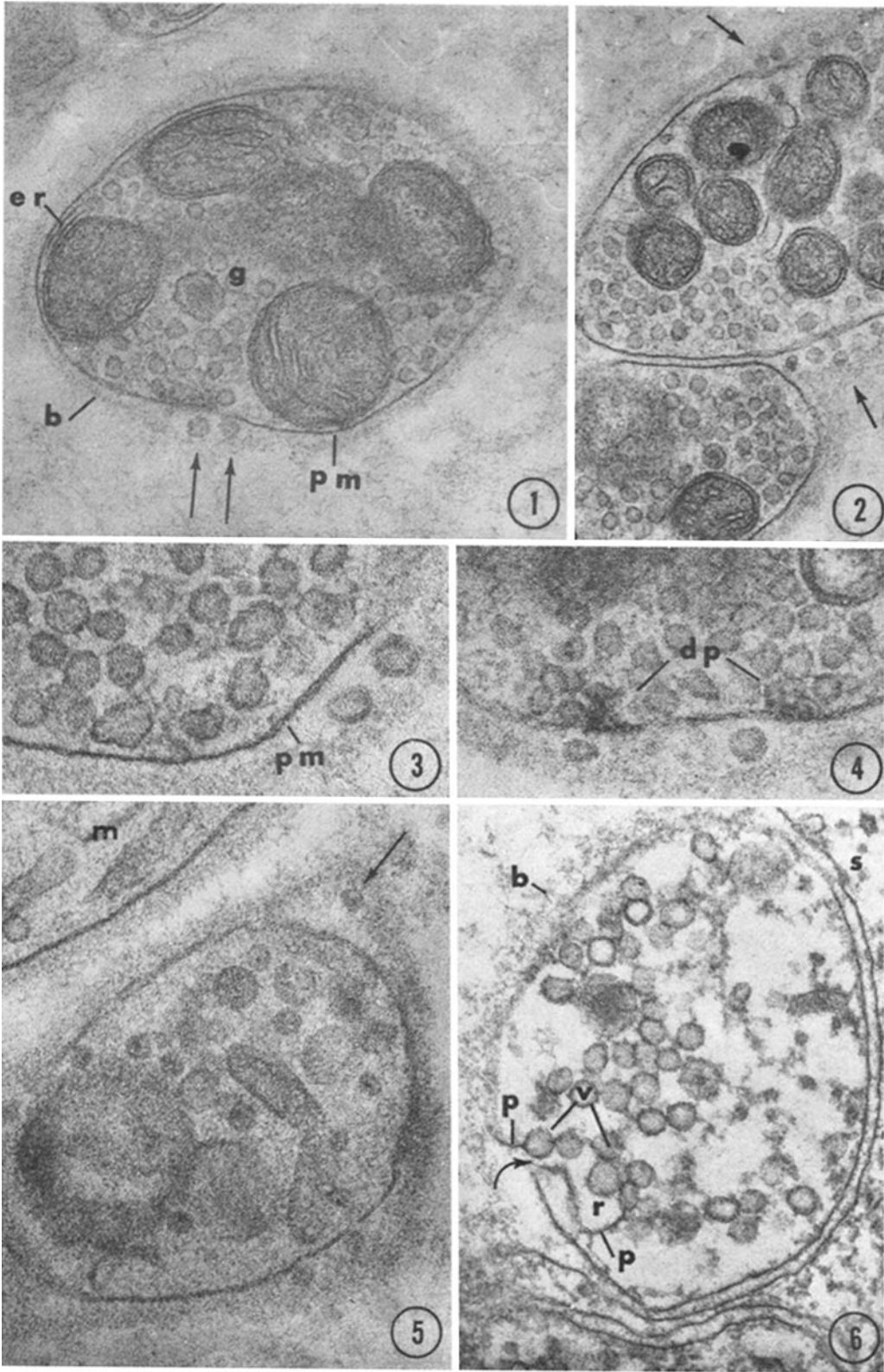
muscular contacts with a separation of 200 A are rare in the mouse atrium and were observed only twice.)

While some profiles lie very close to the axolemma (Figs. 1–4) and may even be fused with it (Fig. 9?), others are distant and reside in bays created by irregular outpocketings of the basal lamina (Figs. 5 and 10). It is evident from this that it should be possible to obtain sections in which the profiles are surrounded completely by a basal lamina and in no apparent association with an axon. Fig. 8 represents, I believe, one such section; the profile in this case measures $\sim$1000 A and, as expected, it contains a droplet of opaque material. In a converse situation (Fig. 7), the profiles lie in a recess formed by a deep indentation of the axon membrane. It follows from this that (even though such occurrences would be rare) there is a chance of finding sections in which the profiles appear in a membrane-limited space surrounded by axoplasm— resembling, possibly, a multivesicular body. Although I have not, in fact, found such a section, Fig. 6 approaches it. However, in this section, as well as in an adjacent one, profiles unattached to the axon membrane are not evident; instead, there is a string of synaptic vesicles bulging into a bay-like area as if in various stages of being extruded. Although the bulging surface seems to be limited by a single membrane which serves both as an axolemma and as a wall

for the protruding vesicles, this appearance may be spurious due to the thickness of the section and the incomplete staining of the membrane components.

As seen in nonserial sections, the extracellular profiles, when present, usually number from 1 to 5 per axon, although up to 13 have been encountered. When multiple, they show a tendency to occur in groups (Figs. 2 and 7). Although in many instances the profiles face the nearest visible muscle cell (Figs. 5 and 8), exceptions are numerous (Fig. 10), especially among the naked axons located in the loose connective tissue of the epi- and endocardium. With one exception, all the profiles encountered thus far have been associated with the vesiculated rather than the predominately microtubule-containing portions of the axon. Although, in order to minmiize the chance of confusing the profiles with sections through Schwann cell processes, attention has been focused on finding the profiles around naked axons, indications are that they also occur near axons with an incomplete Schwann sheath (e.g., Fig. 9). In these cases, they are related to the naked side of the axon, which is also the side where synaptic vesicles are typically concentrated and where dense patches occur.

Although no attempt was made to quantify the observations, several comments can be made about the frequency of their occurrence. Firstly, profiles were observed near sheathless axons

---

FIGURE 7 Two groups of vesicles (arrows) in the extracellular space bordering on a vesiculated axon. The axon membrane opposite the group on the right is both recessed and pitted. The pits (p) have a corona of filamentous material on their cytoplasmic surfaces. Immunosympathectomized mouse. Fixed in 1% OsO4 in McEwen's saline. × 61,000.

FIGURE 8 Membrane-limited extracellular vesicle (arrow) containing a core of electron-opaque material and surrounded by a basal lamina. The identification of the mass of electron-opaque material (*) lying near the cardiac muscle cell (m) is uncertain; it resembles the dense contents of the extracellular vesicle and the atrial granules, and it is also similar to portions of the basal lamina. Axon, A. Immunosympathectomized mouse. Fixed in 1% OsO4 in McEwen's saline. × 69,000.

FIGURE 9 Granule-containing vesicle (arrow) in the periaxonal cleft of a partially sheathed axon. This vesicle differs in both size and content from the granular vesicles visible in the associated adrenergic axon. Schwann cell cytoplasm, s. Normal mouse. Fixed in 1% OsO4 in Veronal-acetate buffer containing 5 mg CaCl2 per ml. × 66,000.

FIGURE 10 A group of vesicles (arrow) in a dilated portion of the periaxonal cleft of a naked axon. One of the extracellular vesicles is considerably larger than the others, and it is granulated. A portion of a cardiac muscle fiber is visible in the lower right corner. Immunosympathectomized mouse. Fixed in 1.3% OsO4 in Rosenbluth's saline. × 57,000.
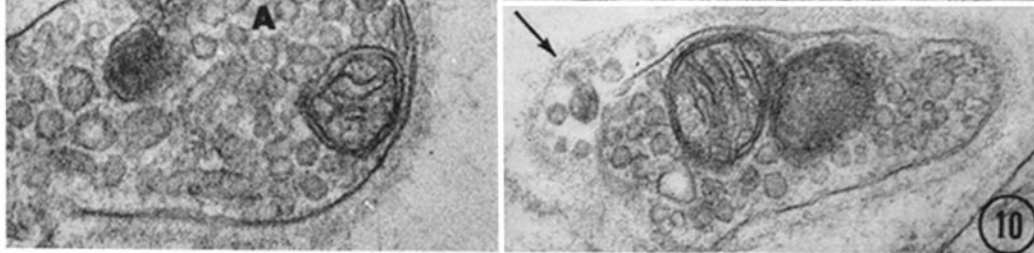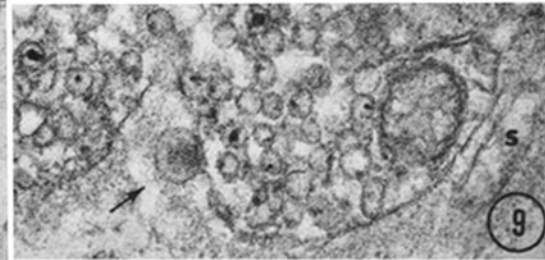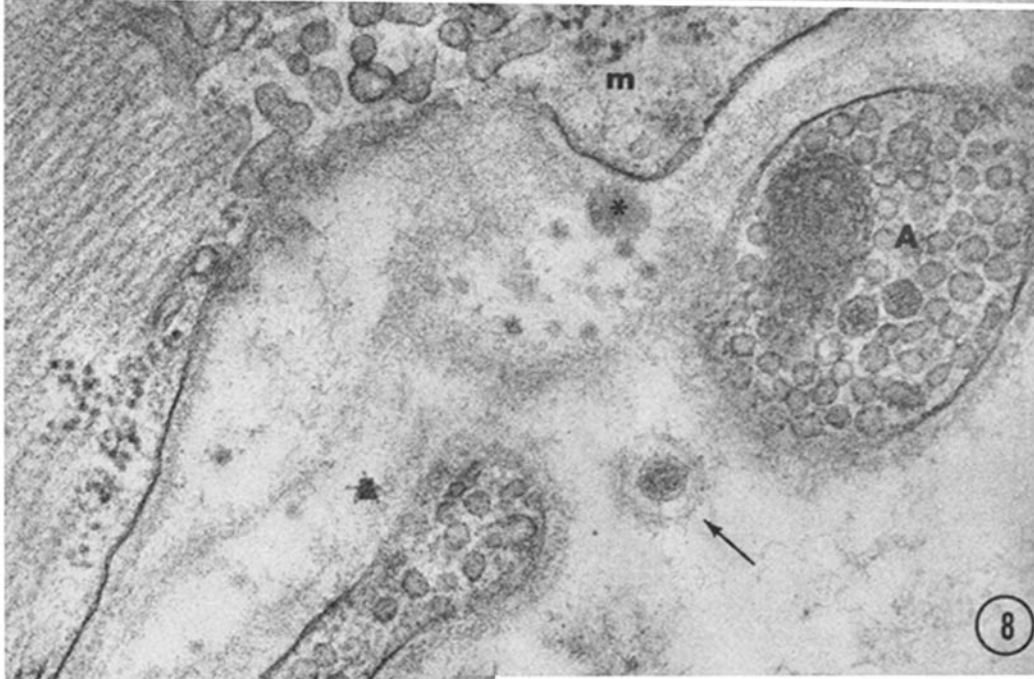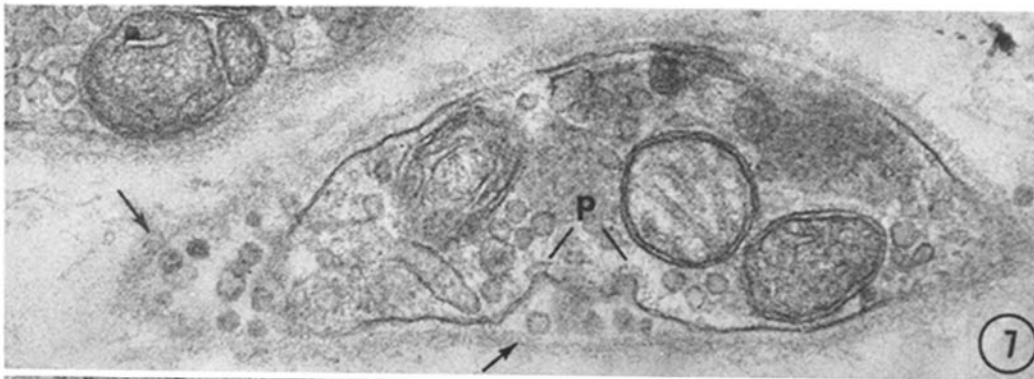
in all the animals studied. Secondly, at least two or three examples were encountered on each grid, but the profiles were noticeably more numerous in the atria fixed by perfusion with a slightly hypertonic solution of osmium tetroxide (1% OsO_4 in McEwen's saline) where 25–30 profiles were often encountered on each grid.

DISCUSSION

The simplest interpretation which can be given to the observations described in this paper is that the extracellular profiles found near nerve endings in the mouse atrium constitute extruded synaptic vesicles. Were they the filiform processes of Schwann cells or aberrant collagen fibers, one would expect to see elliptic and rod-shaped profiles in some planes of section; thus far, whenever such profiles have been seen, they have always been associated with identifiable masses of Schwann cell cytoplasm and have not been found next to "naked" axons. Furthermore, unlike collagen, the extracellular profiles are limited by a trilaminar membrane. But perhaps the most valuable clue to the true identity of the profiles is the presence within some of them (particularly the large profiles) of a core of electron-opaque material which renders them indistinguishable from certain granular synaptic vesicles found in autonomic fibers. Although it is conceivable that the specific granules of the atrial muscle cells might look the same in sections not passing through their largest diameter, and that these granules could gain access to the periaxonal space at close neuromuscular junctions and at other sites where the axon's basal lamina is deficient or missing to produce the images observed, this explanation seems unlikely in view of the evidence that the atrial granules are released by a process of exocytosis (11).

As is evident even from the illustrations (e.g., Figs. 7, 8, and 10), all the tissues used in this study showed known and easily recognizable artifacts. These artifacts differ in nature, however, from one preparation to another and cannot be correlated readily with the incidence of the extracellular profiles. When profiles were first observed, it was suspected that they might represent synaptic vesicles that had spilled out into the extracellular space through membranes ruptured artifactually in another plane of section, or, alternatively, that they might be an illusion resulting either from an inability to visualize exist-

ing axon membranes due to the obliquity of section, or from a disintegration of pre-existing membranes during processing for electron microscopy. To gain some insight into the relevance of broken membranes, the incidence of profiles in one specimen which showed a multitude of interrupted membranes was compared with another in which the membranes appeared intact; no difference was found. On the basis of the present evidence, it is therefore not possible to determine whether these extracellular vesicles have been extruded artifactually or, alternatively, released as a part of a physiological process.

In discussions of the physiological role of synaptic vesicles in the quantal release of neurotransmitter substances, it has been generally assumed that the vesicles discharge their contents directly into the synaptic cleft by a process of reversed micropinocytosis. Although the evidence for this view has always been circumstantial, not until recently have alternative mechanisms even been considered. The recent demonstration of differences in chemical composition between external presynaptic membranes and synaptic vesicles isolated from the central nervous system (18), and the failure to demonstrate increased numbers of "discharging vesicles" with greatly increased rates of quantal release even though the number of synaptic vesicles was significantly reduced (9), have provided the impetus for the proposal of two new hypotheses. These state: (a) the vesicles discharge their contents into the cleft indirectly via the axoplasmic matrix (13); and (b) the vesicles are part of a complex interconnecting system of tubules which communicates through, but does not actually fuse with, the axon membrane in the process of transmitter release (18). The present findings suggest still another possibility—namely, that the discharge is brought about by an extrusion of entire vesicles, not merely their contents, into the extracellular space. This mechanism, if confirmed, would represent a unique hybrid of merocrine and apocrine secretory processes. The frequent, although not constant, association of the extracellular vesicles with special dense patches on the axon membrane suggests that the process of extrusion may initiate at these sites, but no direct evidence for this view has been obtained.

If vesicle extrusion is indeed related to quantal release, is it specific to the mouse heart or does it occur more generally? If more general, is it a

special feature of those tissues where close neuro-effector junctions are infrequent or absent and where the transmitter action characteristically has a long latency and duration (4, 5, 6), or does it occur at synapses everywhere? Perhaps the relatively large number of naked axons in the mouse atrium has favored the detection of a physiological process of general occurrence. But it is also conceivable that this same property might make these axons exceptionally fragile and unduly sensitive to adverse environmental conditions, such as the conditions which prevail during fixation. Experiments similar to those carried out by Hubbard and Kwanbunbumpen (9) on the diaphragm-phrenic nerve preparation should help to clarify what role, if any, the extracellular synaptic vesicles play in transmission.

REFERENCES

1. AKERT, K., H. MOOR, K. PFENNINGER, and C. SANDRI. 1969. Contributions of new impregnation methods and freeze etching to the problems of synaptic fine structure. *In* Progress in Brain Research, Vol. 31, Mechanisms of Synaptic Transmission. K. Akert and P. G. Waser, editors. Elsevier Publishing Co., Amsterdam. 223.

2. BIRKS, R., H. E. HUXLEY, and B. KATZ. 1960. The fine structure of the neuromuscular junction of the frog. *J. Physiol. (London).* 150:134.

3. BLOOM, F. E. 1970. The fine structural localization of biogenic monoamines in nervous tissue. *Int. Rev. Neurobiol.* In press.

4. BURNSTOCK, G., and M. E. HOLMAN. 1966. Junction potentials at adrenergic synapses. *Pharmacol. Rev.* 18:481

5. BURNSTOCK, G., and N. C. R. MERRILLEES. 1964. Structural and experimental studies on autonomic nerve endings in smooth muscle. *In* Pharmacology of Smooth Muscle. Proceedings of the 2nd International Pharmacology Meeting, Prague. E. Bulbring, editor. The Macmillan Co., New York. 6:1.

6. ECCLES, J. C. 1964. The Physiology of Synapses. Academic Press Inc., New York.

7. GRAY, E. G., and R. W. GUILLERY. 1966. Synaptic morphology in the normal and degenerating nervous system. *Int. Rev. Cytol.* 19:111.

8. GRILLO, M. A. 1966. Electron microscopy of sympathetic tissue. *Pharmacol. Rev.* 18:387.

9. HUBBARD, J. I., and S. KWANBUNBUMPEN. 1968. Evidence for the vesicle hypothesis. *J. Physiol. (London).* 194:407.

10. McEWEN, L. M. 1956. The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine, hexamethonium and ouabain. *J. Physiol. (London).* 131:678.

11. NELSON, J. M. 1967. An electron microscopic study of the effects of the nerve growth factor and its antiserum on the atrial muscle cells of the mouse. Thesis. Western Reserve University School of Medicine.

12. PALAY, S. L., S. M. McGEE-RUSSELL, S. GORDON, JR., and M. A. GRILLO. 1962. Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide. *J. Cell Biol.* 12:385.

13. PETERS, A., S. L. PALAY, and H. de F. WEBSTER. 1970. The Fine Structure of the Nervous System. Harper and Row, Publishers, New York.

14. RICHARDSON, K. C. 1964. The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscle. *Amer. J. Anat.* 114:173.

15. ROSENBLUTH, J. 1966. Redundant myelin sheaths and other ultrastructural features of the toad cerebellum. *J. Cell Biol.* 28:73.

16. THAEMERT, J. C. 1963. The ultrastructure and disposition of vesiculated nerve processes in smooth muscle. *J. Cell Biol.* 16:361.

17. THAEMERT, J. C. 1966. Ultrastructure of cardiac muscle and nerve contiguities. *J. Cell Biol.* 29:156.

18. WHITTAKER, V. P. 1966. Some properties of synaptic membranes isolated from the nervous system. *Ann. N. Y. Acad. Sci.* 137:982.