

ON THE ISOLATION OF NERVE ENDINGS AND SYNAPTIC VESICLES

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Since the first description of the "synaptic vesicles" (1, 2) the suggestion was made that they could be associated with acetylcholine and other chemical synaptic mediators. Later work gave further information about the possible composition of synaptic vesicles and their role in synaptic functions. It was shown that they degenerate very early in the nerve terminal after section of the axon (3), a finding that can be correlated with the decrease in transmitter content of the synapse (4, 5).

It was also found that the synaptic vesicles of some of the retina synapses were reduced in size with disuse (6) and changed with dark adaptation and stimulation with light (7). Finally, striking changes in the number of synaptic vesicles were found within the cholinergic nerve endings of the adrenal medulla after stimulation of the splanchnic nerve with supramaximal pulses of different frequencies (8). These experiments led to the conclusion that within the synaptic ending a balance exists between the formation and release of synaptic vesicles, and that this balance depends on the frequency of stimulation (9). At the same time the concept of "synaptic vesicles" was associated with the "quantized" release of acetylcholine at the neuromuscular junction (10), and it became widely used in the physiological literature on synaptic transmission (see 11).

From the biochemical viewpoint, Feldberg (12) gave some of the first evidence that acetylcholine is associated with a protein containing a particulate component which was generally identified with mitochondria. Recently, it was found that the largest proportion of acetylcholine is bound to the so called mitochondrial fraction (13, 14). However, upon further sedimentation of this fraction on a gradient, Whittaker (13) could demonstrate that most of it is associated with subcellular structures different from mitochondria. He concluded that most of the particle-bound acetylcholine and 5-hydroxytryptamine is localized in a vesicular layer which was interpreted as representing the synaptic vesicles.

The fact that synaptic vesicles have a mean size

of about 400 A (8) makes it difficult to conceive that it could sediment as such in a fraction of the size of mitochondria. The published electron micrographs of Whittaker (13) were not clear enough to bring out this point, and the mean particle diameter found was larger (650 A) than that of synaptic vesicles.

Our laboratory has been engaged in the last year in a similar effort, trying to use several fractionation methods and a close study of each fraction by electron microscopy. While a detailed account of the techniques used and of the results obtained on the submicroscopic analysis of the fractions is being prepared for publication, we would like to present some observations that bear on the work of Whittaker (13) and that may clarify the apparent contradiction between the size of synaptic vesicles and the fraction in which acetylcholine is generally found.

As material we used the most superficial layers of the brain cortex of the rat and the dog in which the synaptic endings are very abundant. Using a modification of the method of Berger (15) for isolation of mitochondria, we found that together with the isolated mitochondria there are numerous intact endings filled with synaptic vesicles (Figs. 1 and 2). Some of these endings show small mitochondria within. The surface membrane of the ending is intact and it may even show its synaptic portion, characterized by higher density and attachment to the postsynaptic component. Essentially similar results were found by repeating the isolation with the method used by Whittaker (13) for the mitochondrial fraction, which also shows numerous intact endings filled with synaptic vesicles. These observations were interpreted as indicating that homogenization was not strong enough to produce the disruption of most of synaptic terminals. Using a tighter teflon homogenizer and longer periods, a mitochondrial fraction showing fewer intact nerve endings was obtained.

In this case, after isolation of mitochondria, a microsomal fraction was sedimented which is devoid of intact endings. It consists of a complex

mixture of vesicular and membranous elements together with clusters of ribosomes (Fig. 3). This microsomal fraction was centrifuged further in a continuous density gradient of sucrose and polyvinylpyrrolidone (PVP). This treatment gave 3 fractions (A, B, and C) in suspension, and one pellet made almost exclusively of membranes with ribosomes attached.

Of the three fractions, A is made of empty vesicles, membranes, and fine tubules and is morphologically similar to the cholinesterase-rich fraction described by Toschi (16) in brain tissue. Fraction B is formed mainly by vesicular and tubular elements, many of which have a dense content, and whose size average is slightly higher (510 Å) than that of the synaptic vesicles (Fig. 4). Fraction C is similar to B, but made of larger vesicular elements. Studies are now being carried out to assay the acetylcholine content of all of these submicrosomal fractions.

These results, together with those of Whittaker (13), indicate the difficulties encountered in isolating synaptic vesicles from the numerous and complex subcellular components found in nervous tissues. It seems that the further "purification" of intact synaptic terminals may provide a good starting point for the isolation of "pure" synaptic vesicles. When this is done the synaptic vesicles will be probably found in one of the submicrosomal fractions. In our results, submicrosomal fraction B is the one that contains dense vesicles whose size and morphology is more similar to the synaptic vesicles.

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FIGURE 1

Electronmicrograph of a "mitochondrial fraction" from the outer cortex of the rat brain, showing, in addition to free mitochondria (*mi*), numerous nerve endings filled with synaptic vesicles (*sv*). The arrows mark a synaptic ending with attached subsynaptic membrane. Nervous tissue was homogenized in sucrose m 0.25 with a teflon pestle for 2 minutes at 900 R.P.M., centrifuged at 800 *g* for sedimentation of nuclei and tissue residues, and then at 4700 *g* for 15 minutes for sedimentation of the mitochondrial fraction. The pellet was fixed in 1 per cent OsO₄ in periston Bayer. × 40,000.

SUMMARY

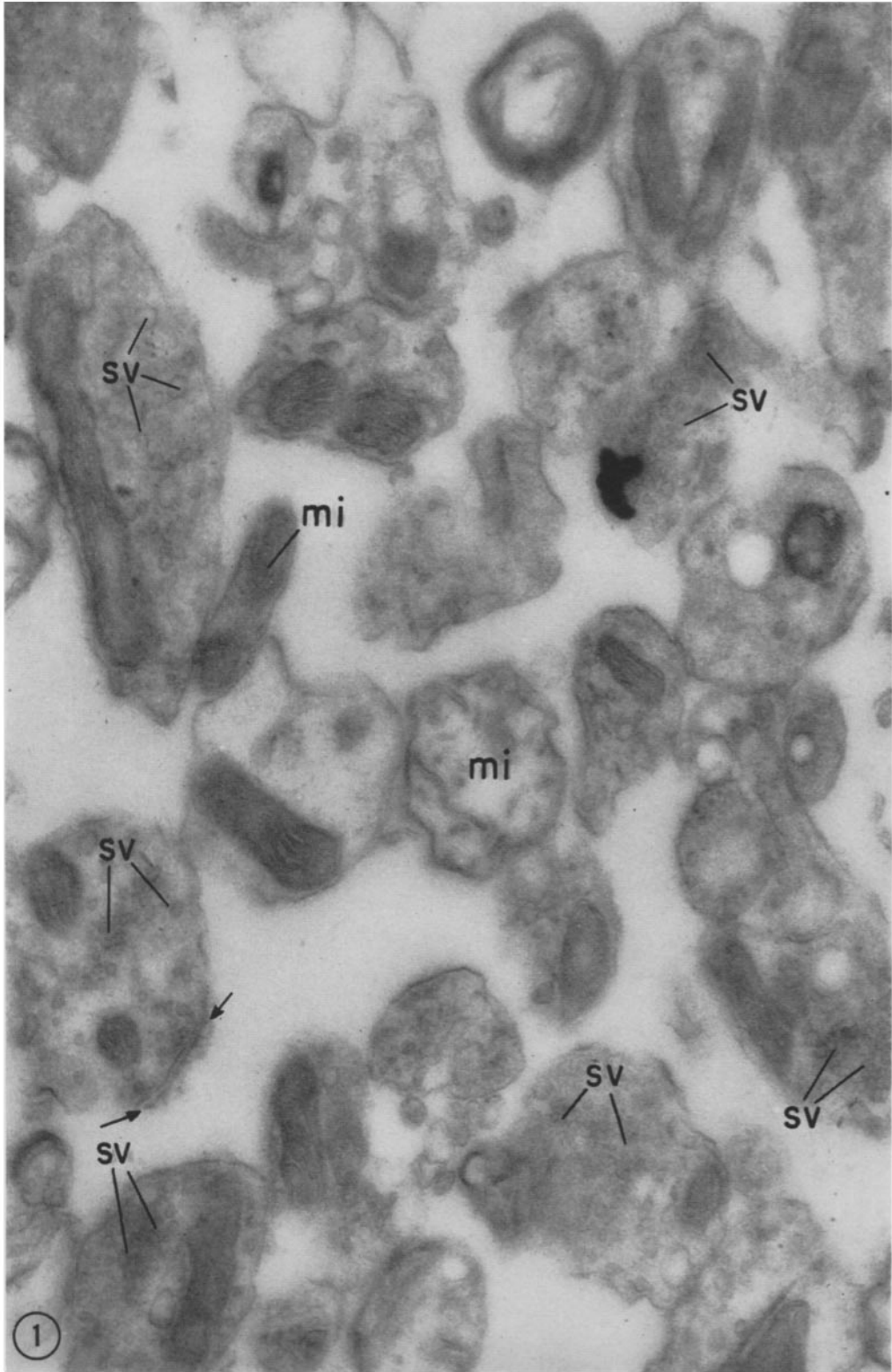
The outer layers of the brain cortex were homogenized and the mitochondrial and microsomal fractions were observed under the electron microscope. The mitochondrial fractions prepared with a modification of Berger's method and with that of Whittaker (13) showed numerous intact nerve endings filled with synaptic vesicles. This fact may explain the finding (13, 14) that acetylcholine is in a fraction that sediments together with mitochondria. Upon more intense and prolonged homogenization fewer intact endings are observed in the mitochondrial fraction. Sedimentation of the microsomal fraction in a continuous gradient gives four microsomal subfractions. Of these, fraction B contains vesicles of the size and morphology of synaptic vesicles.

Addendum. After this paper was submitted for publication (May, 1960), Whittaker and Gray (August, 1960) published a short note describing results similar to those presented here, regarding the presence of numerous nervous endings in the mitochondria fraction of the CNS (18).

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FIGURE 2

Electronmicrograph of a "mitochondrial fraction" prepared in a similar way as Fig. 1. Three nervous endings filled with synaptic vesicles (*sv*) and two mitochondria (*mi*) are observed. At the lower left, one ending shows the attached postsynaptic membrane (*pm*) below which there is a filamentous material. $\times 62,000$.

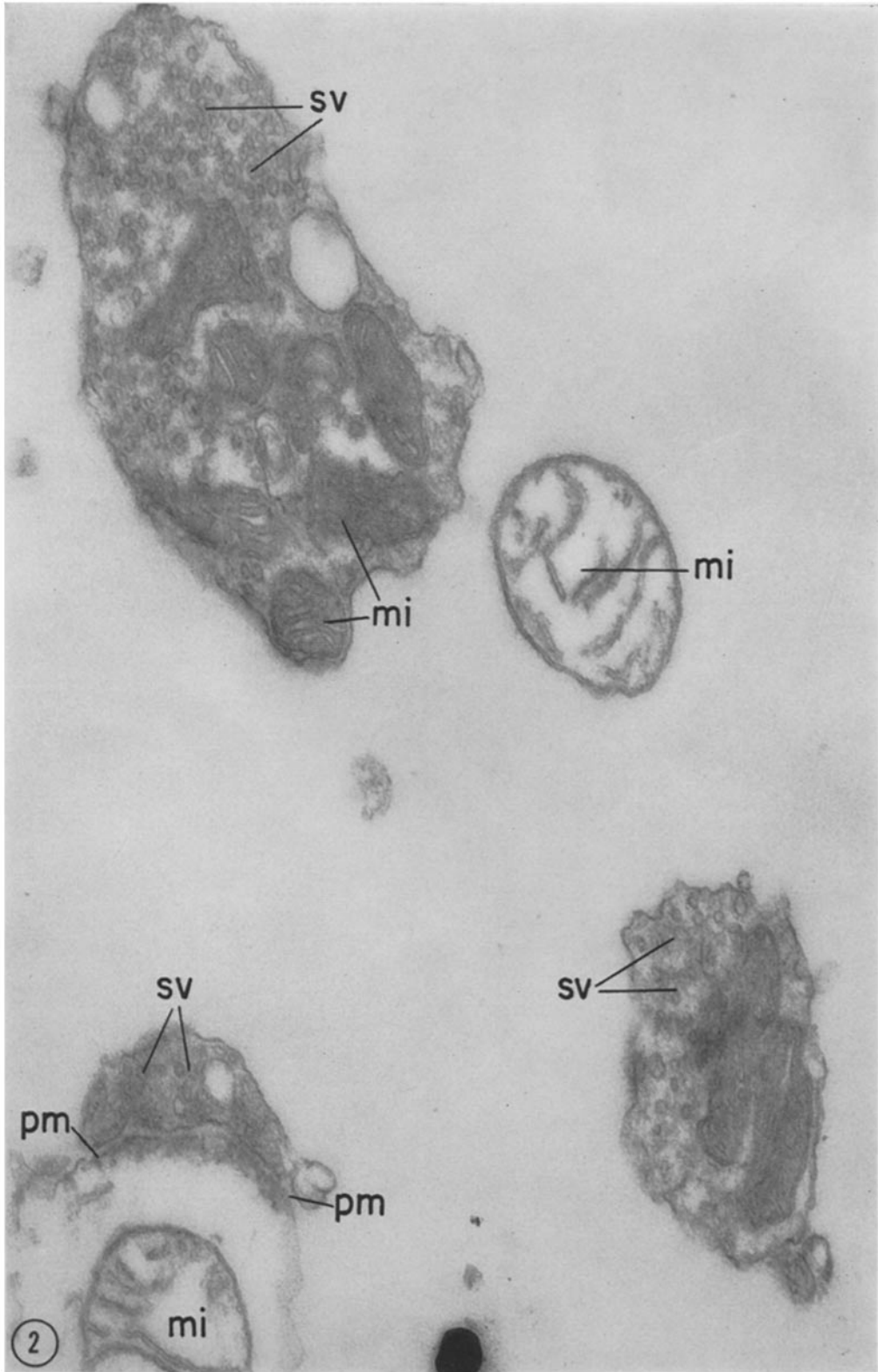


FIGURE 3

Electronmicrograph of a section of a "microsomal fraction" from the outer cortex of the dog brain. The tissue was homogenized in sucrose 0.25 plus 7.3 per cent PVP at pH 7.6 with a tight teflon pestle for 7 minutes, and then centrifuged 10 minutes at 1500 *g* for nuclei, 20 minutes at 20,000 *g* for mitochondria, and at 60,000 *g* during 30 minutes for microsomes. As seen in the figure, this pellet contains large and small vesicles (*v*), membranes (*m*), and clusters of ribosomes (*r*). $\times 36,000$.

FIGURE 4

Electronmicrograph of a similar material as Fig. 3 after resuspension of the microsomes and centrifugation in a continuous gradient of PVP, according to Novikoff (17), for 60 minutes at 2000 *g*. This material is composed of tubules and small dense vesicles (*v*) and corresponds to subfraction B, the mean size of the vesicles being 510 A. $\times 32,000$.

