SELECTIVE DEMONSTRATION OF A TYPE OF SYNAPTIC VESICLE BY PHOSPHOTUNGSTIC ACID STAINING

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Fine structural analyses of nerve terminals in both the central and in the peripheral nervous systems have demonstrated the presence of synaptic vesicles, which were postulated to be the storage sites of neurotransmitters. Several different types of synaptic vesicles have been described (see ref. 6). Electron-lucent vesices, 400–500 A in diameter, constitute the most commonly occurring type in central and in peripheral synapses. Because they are consistently found in cholinergic nerve terminals, these vesicles were postulated to constitute the site of acetylcholine storage, an assumption confirmed by isolation studies (8). Granulated vesicles, 300–500 A in diameter, with an electron-opaque core are characteristic of peripheral adrenergic terminals (7). Combined pharmacological and electron microscopical studies have shown that these vesicles contain norepinephrine (NE) (19, 20) and also, in nerve endings of rat pineal gland, 5-hydroxytryptamine (5-HT) (16). The presence of another type of vesicle, a larger one (800–1000 A in diameter) which possesses an internal content of variable density, has been also observed. These large granulated vesicles (LGV) occur in terminals which also contain electron-lucent or granulated vesicles, e.g. postganglionic sympathetic terminals, endings in the sphincter pupillae, preganglionic cholinergic fibers to sympathetic ganglia, preganglionic terminals supplying the adrenal medulla, parasympathetic ganglia, endings and neuronal perikarya of the central nervous system (CNS) of areas rich or poor in monoamines in both vertebrates and invertebrates, stumps of injured peripheral nerves or central tracts, etc. (for bibliography see ref. 12). There is not yet general agreement concerning the significance of the LGV.

When phosphotungstic acid is added to the absolute ethanol (E-PTA) during the dehydration of glutaraldehyde-fixed tissue, the dense cores of the LGV in the CNS seem to be specifically stained (1). In order to determine the relationship between the LGV in peripheral nerve endings and those of CNS terminals, the reactivity of the former to E-PTA was studied. The possible participation of biogenic amines in the reaction was also analyzed.

MATERIAL AND METHODS

Pineal glands and vas deferens from normal adult rats, and from rats receiving 10 mg/kg of reserpine (Serpasol, Ciba) intraperitoneally 6 hr before decapitation, were fixed in 3% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, for 4 hr at 4°C. After washing in 0.3 M sucrose in the same buffer, some blocks were postfixed for 2 hr in 1.5% buffered osmium tetroxide, and others in 2.5% potassium dichromate in 0.2 M acetate buffer pH 4.1 as previously described (14). The remaining blocks were directly dehydrated in ethanol after glutaraldehyde fixation and were immersed for 1 hr in 1% phosphotungstic acid in absolute ethanol (E-PTA) essentially as described by Bloom and Aghajanian (1, 3). The superior cervical ganglion, median eminence and suprachiasmatic nucleus of normal rats were similarly fixed and stained with osmium tetroxide and with E-PTA. Embedding was done in Epon 812. Thin sections of material which had been processed with glutaraldehyde-osmium tetroxide were stained with lead citrate, and the rest were examined without further staining under a Siemens Elmiskop I electron microscope.

OBSERVATIONS

In adrenergic nerve endings lying in the perivascular space of the pineal gland, two different types
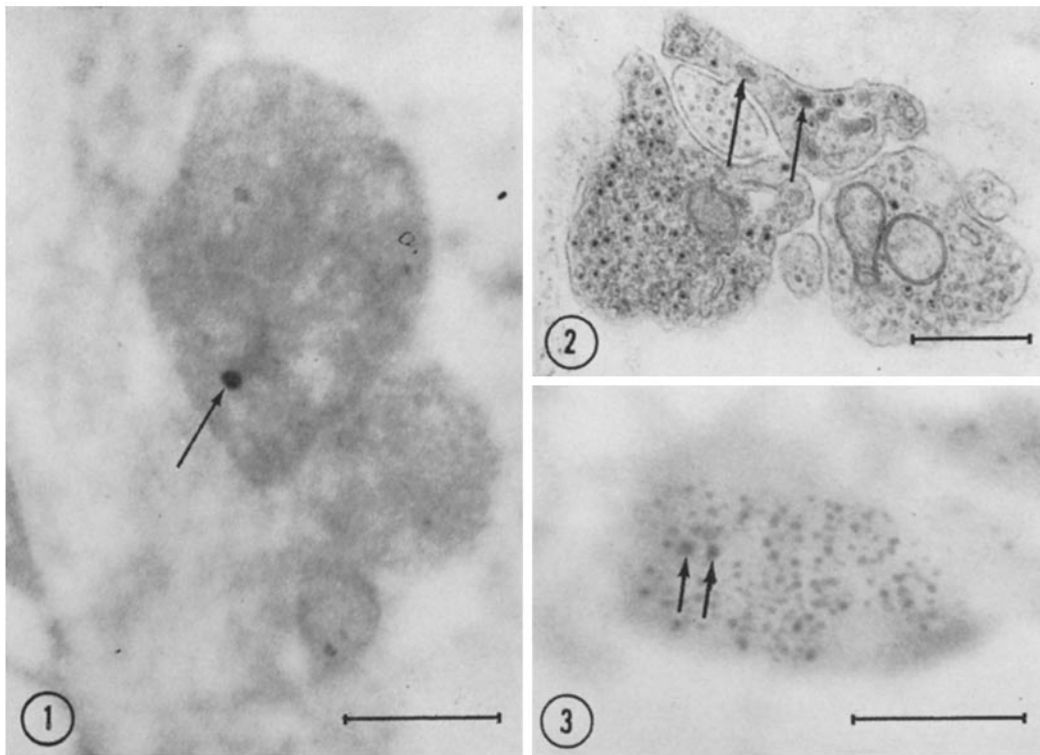
Figures 1 to 9. The magnification line corresponds to 0.5 μ in all figures.

FIGURE 1 The E-PTA positive reaction is observed in a dense sphere (arrow) in an adrenergic nerve ending of the perivascular space of a normal pineal gland. Glutaraldehyde-E-PTA. $\times$ 39,000.

FIGURE 2 Sympathetic nerve endings in the pineal gland exhibiting both small and large (arrows) granulated vesicles. Glutaraldehyde-OsO$_4$. $\times$ 31,000.

FIGURE 3 Clusters of granules reacting with dichromate that correspond to a pineal nerve ending. Reactive sites, corresponding to the cores of both small and large granulated vesicles (arrows), are seen. Glutaraldehyde-dichromate. $\times$ 46,000.

of granulated vesicles may be observed after glutaraldehyde-osmium tetroxide fixation (Fig. 2): numerous small vesicles (SGV) about 400 A in diameter and a few larger vesicles (LGV) 800–1000 A in diameter. The storage of monoamines in the cores of both types of vesicles is demonstrated by the reaction given with the glutaraldehyde-dichromate procedure (Fig. 3). In nerve endings of pineal glands fixed in glutaraldehyde and stained with E-PTA, reactive sites were observed which corresponded in size and in frequency to the cores of the LGV (Fig. 1). No limiting membrane was observed surrounding the reactive core. Therefore, absolute correlation between the cores revealed by E-PTA and those demonstrated by

conventional techniques could not be made. However, with these limitations in mind and because of the similarities listed above, the reactive sites demonstrated by E-PTA were assumed to correspond to the cores of the LGV. Reserpine administration caused disappearance of the cores of the SGV in glutaraldehyde-osmium tetroxide-fixed tissue, while the cores of the LGV remained unaltered (Fig. 5). The absence of reaction with glutaraldehyde-dichromate (Fig 6) reflected the degree of monoamine depletion and confirmed that both types of granulated vesicles participate in monoamine storage. Nevertheless, the E-PTA reactive material observed in nerves of normal pineal glands showed no change following reser-
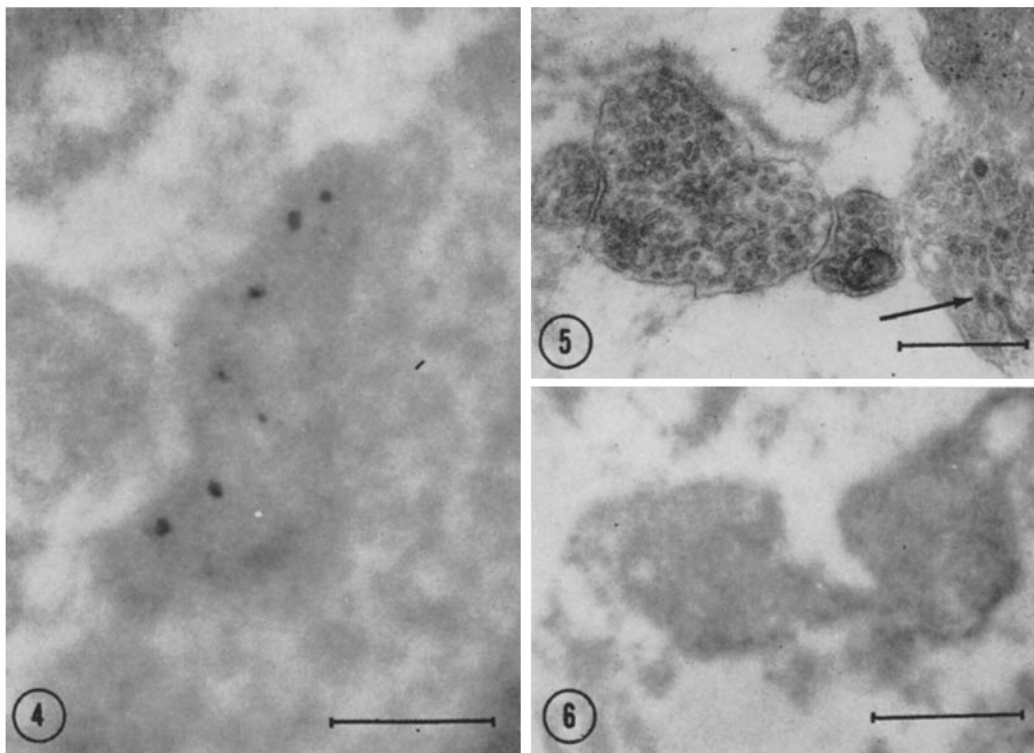
FIGURE 4   A pineal nerve ending of a rat given reserpine. Note that the circular densities appearing in the ending after E-PTA exposure are unaffected by the treatment. Glutaraldehyde-E-PTA. × 43,000.

FIGURE 5   The dense cores of the small vesicles have disappeared from these endings in a rat receiving reserpine. A large granulated vesicle is seen (arrow). Glutaraldehyde-OsO₄ . × 35,000.

FIGURE 6   After reserpine administration no reaction is observed in the nerve endings treated with dichromate. Glutaraldehyde-dichromate. × 40,000.

pine (Fig. 4). Similar observations were made in the nerves of the vas deferens.

The nerve processes lying between cells of the superior cervical ganglion and identified as belonging to preganglionic cholinergic fibers showed the presence of numerous small, clear vesicles and some LGV (Fig. 7 *a*). After exposure to E-PTA, the cores of what were probably LGV could be recognized (Fig. 7 *b*).

Two different types of central synapses were studied with the E-PTA staining: those of the outer zone of the median eminence and those of the suprachiasmatic nucleus, in which fluorescence histochemical methods have demonstrated dopamine and 5-HT, respectively (10). In both types of synapses the vesicular population is composed of clear vesicles and LGV (Figs. 8 *a* and 9 *a*). The

E-PTA stain discloses in both terminals spherical or annular densities (Figs. 8 *b* and 9 *b*) matching in size, location, and frequency the cores of LGV observed with conventional techniques.

In accordance with previous studies of E-PTA staining in the CNS (1, 3), the material present at synaptic junctions as well as at nuclei, nucleoli, and erythrocytic membranes was found to stain with E-PTA.

DISCUSSION

Previous studies have shown that in peripheral adrenergic terminals the cores of the small granulated vesicles (SGV) disappear after monoamine depletion (18, 19) while those of the large granulated vesicles (LGV) remain unaltered (4, 5, 9, 13). These results, obtained with osmium tetroxide or
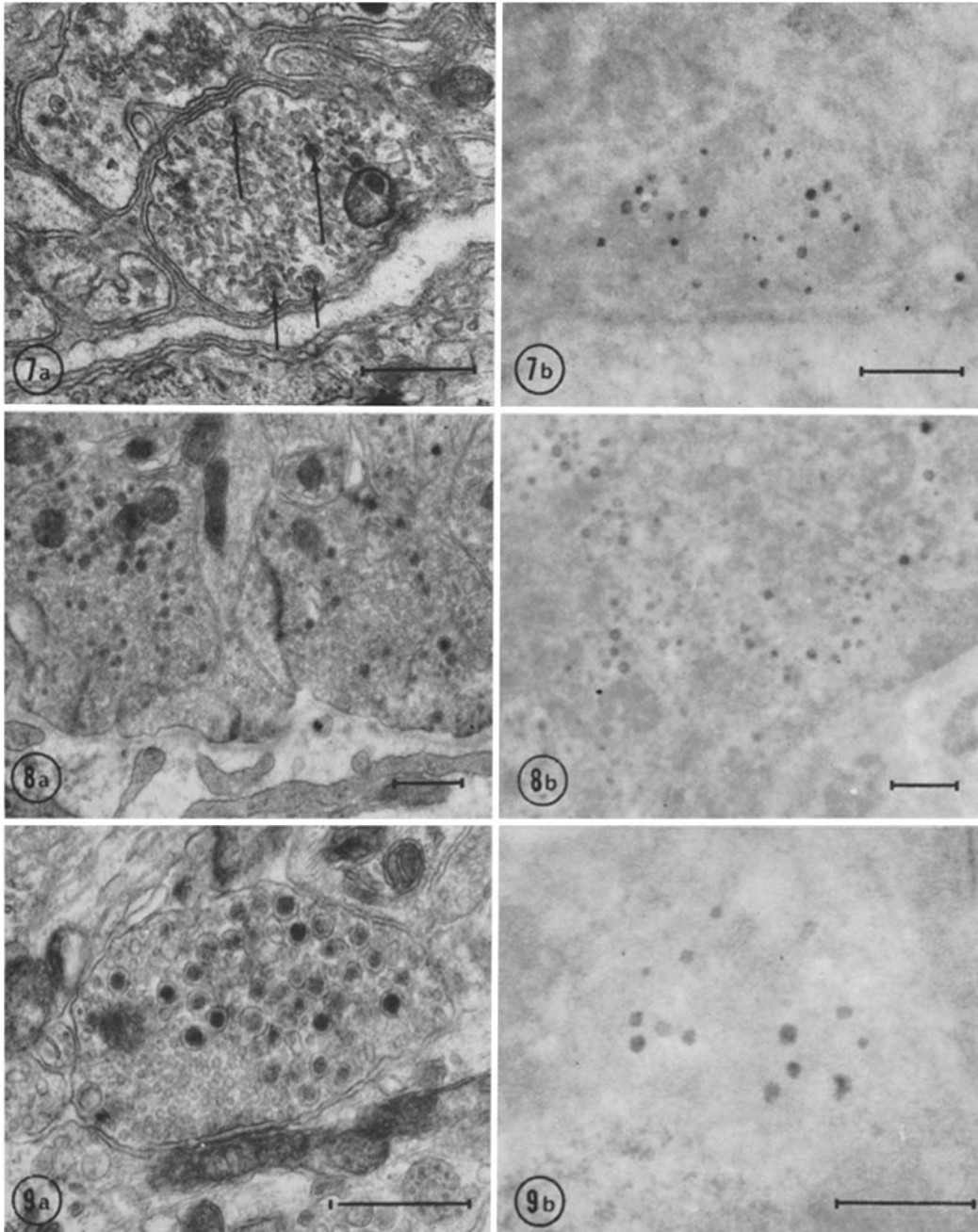
FIGURE 7 *a* A nerve process in the superior cervical ganglion is shown containing a mixed population of clear vesicles and LGV (arrows). Glutaraldehyde-OsO₄ . × 30,000. In Fig. 7 *b*, the sites staining with E-PTA are seen and correspond to the cores of the LGV. Glutaraldehyde − E-PTA. × 30,000.

FIGURE 8 *a* External layer of the median eminence in which nerve terminals, containing clear vesicles and LGV, are observed. Glutaraldehyde-OsO₄ . × 19,000. In Fig. 8 *b*, the reactive sites appearing after E-PTA are apparent and roughly correspond in size and distribution to the LGV observed in Fig. 8a. Glutaraldehyde-E-PTA. × 19,000.

FIGURE 9 *a* A neural process in the suprachiasmatic nucleus showing small electron-lucent vesicles and LGV. Glutaraldehyde-OsO₄ . × 37,000. In Fig. 9 *b*, E-PTA staining of a similar structure shows reactive spherical sites that probably correspond to the cores of the LGV. Glutaraldehyde-E-PTA. × 37,000.

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glutaraldehyde-osmium tetroxide fixation, were interpreted as an indication that LGV normally do not store monoamines. However, the first direct evidence that the LGV store NE and 5-HT in the adrenergic nerves of the pineal gland and NE in the vas deferens was provided by the use of selective cytochemical techniques for monoamines (16). In depletion experiments it was found that the LGV reacted in the same manner as the small granulated vesicles (22) and also behaved similarly with respect to amine storage and uptake (17). That the E-PTA staining is independent of the content of monoamines is demonstrated by the fact that only the LGV display a positive reaction in endings in which amines are also stored in SGV. Moreover, the E-PTA staining of LGV in peripheral adrenergic endings persists after reserpine treatment which, as shown by the cytochemical reaction, depletes NE and 5-HT present in those vesicles. These findings support the hypothesis that the cores of LGV in peripheral nerves contain at least two electron-opaque components (22), i.e., the biogenic amine revealed by specific cytochemical reactions, and the E-PTA positive substance, which is resistant to reserpine treatment and probably corresponds to the osmiophilic core observed in those vesicles after amine depletion.

The localization of amine stores in central adrenergic synapses is still uncertain because of the technical difficulties posed by the handling of brain tissue for ultrastructural and cytochemical analysis. In these synapses, LGV occur associated with small electron-lucent vesicles (21) and, although several studies suggested that they constitute the storage site of monoamines, the evidence regarding their change in number or electron opacity with pharmacological treatments has been controversial (2). The use of conventional techniques for electron microscopy may interfere with the detection of these changes, as was the case of LGV in peripheral nerves (22). Several ultrastructural and cytochemical characteristics are shared by both central and peripheral monoaminergic endings, i.e., the demonstration of monoamines by the glutaraldehyde-dichromate procedure in LGV of hypothalamic (24) as well as of peripheral (16) adrenergic endings; the demonstration of SGV characteristic of peripheral adrenergic nerves in various central adrenergic terminals by perman-

ganate fixation (14); and finally, the present finding that E-PTA characterizes the cores of the LGV in peripheral as well as in central adrenergic endings. These facts lend support to the hypothesis that there is a similar morphological and cytochemical organization in both central and peripheral monoaminergic endings.

The chemical nature of the substances reacting with E-PTA is largely unknown. The results of enzymatic extractions suggest that a basic protein may be responsible for this reaction (3), which seems to be characteristic of cell sites involved in ionic calcium concentration (11). The matrix of the monoamine-storing LGV in adrenergic endings could represent a bound enzyme involved in amine metabolism (23). However, these vesicles are common to endings storing different neurotransmitters as shown by the presence of E-PTA reactive sites in the cholinergic endings of the superior cervical ganglion. On the other hand, the possibility must be considered that the LGV might take part in general processes occurring in presynaptic terminals other than those specifically related to transmitter substances. Thus, in this case, the presence of monoamines in the LGV of adrenergic endings could be explained as being a result of some physicochemical interaction between the amine molecule and another component of the LGV, as has been postulated for the incorporation of biogenic amines into the glycolipoprotein matrix of lysosomes (18). Clearly, further studies are necessary to determine whether the LGV of different endings have a similar physiological significance or whether they represent a heterogeneous population of presynaptic organelles having in common only their morphological aspect and the property of staining with E-PTA.

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