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D. CATECHOLAMINE STORAGE PARTICLES IN THE CENTRAL NERVOUS SYSTEM

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LOCALIZATION OF CATECHOLAMINES IN SYNAPTOSOMES

Noradrenaline (NE). Although the role of catecholamines in the central nervous system was discussed at the first Symposium on Catecholamines in 1958, our knowledge of the subcellular storage particles containing these amines was at that time largely confined to the chromaffin granules of the adrenal medulla (2, 10, 13) and the smaller particles from splenic nerves (6). The study of the storage sites of biogenic amines in the central nervous system began with an investigation concerning acetylcholine and the enzyme which synthesizes it, choline acetyltransferase (choline acetylase). Acetylcholine is still the central transmitter about which most is known, so that this review will necessarily be concerned to some extent with the storage in brain of amines other than the catecholamines.

Hebb and Whittaker (11) found that 70 to 75 % of the total acetylcholine content of brain tissue remained bound to particulate material after homogenization in 0.32 M sucrose. By a combination of differential and density-gradient centrifugation, they were able to separate from mitochondria and other known subcellular organelles a fraction which accounted for most of the particulate acetylcholine and choline acetyltransferase present in the original homogenate. The acetylcholine was immune to attack by cholinesterase and the transferase was in an "occluded" state, that is, procedures calculated to break down lipoprotein membrane barriers had to be applied before full enzyme activity was obtained. The fraction was identified by Gray and Whittaker (8, 9, see also 34) as consisting largely of pinched-off presynaptic nerve terminals. These findings have been confirmed in a large number of other laboratories (for a review, see 35). When brain tissue is homogenized under controlled conditions (36) the presynaptic nerve terminals are pinched off or torn away from their attachments to form discrete particles which retain the fine structure and transmitter content of the original nerve endings. To emphasize the combination of particulate nature and synaptic origin of these particles the name synaptosome has been adopted for them (37).

At an early stage in the research it was realized that the acetylcholine-containing fraction might well be rich in other putative central transmitters, and the presence of bound 5-hydroxytryptamine (5-HT) in the fraction was established (1, 32, 33). This finding was confirmed later by another method (23a). Chruściel (4), working with dog hypothalamus, was the first to show that NE was also localized in this fraction. His finding was confirmed and extended in experiments with rat forebrain (23), rat brain stem (18) (fig. 1) and guinea-pig diencephalon and midbrain (23). Similar results were obtained with a somewhat different fractionation procedure (26).

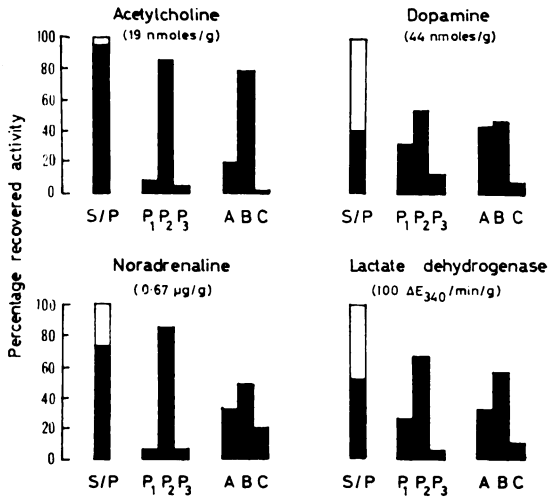

FIG. 1. Distribution of acetylcholine, dopamine and lactate dehydrogenase in subcellular fractions of dog caudate nucleus (17) and of NE in fractions of rat brain stem (18). The first block (*S/P*) in each series gives the distribution of the compound between particle-bound (black) and free supernatant (white) forms; the second group of blocks gives the distribution of the compound between crude nuclear (P_1), crude mitochondrial (P_2) and microsomal (P_3) fractions; and the third group the distribution between three subfractions of P_2, myelin (*A*), synaptosomes (*B*) and mitochondria (*C*).

The equilibrium density of synaptosomes in a sucrose density gradient is equivalent to that of 0.8 to 1.0 M sucrose; they come to rest in a region of the gradient above mitochondria (equilibrium density equivalent to 1.4 to 1.5 M sucrose). Thus, as previously pointed out (26, 34, 40), the particles associated with bound acetylcholine and NE are, on the one hand, much lighter than those concerned with amine storage in cells other than neurones (*e.g.*, mast cell granules, chromaffin granules, 5-HT-containing granules of the duodenum) all of which sediment below mitochondria; and on the other hand, they are larger and heavier than the particles isolated from adrenergically innervated peripheral tissues (26), which yield few or no synaptosomes on homogenization (fig. 4).

The development of a fluorescent histochemical method to demonstrate catecholamines and 5-HT (3) has provided evidence of another kind for their localization in nerve tissue. Nerve endings showing the fluorescent reaction are pinched off by homogenization and are concentrated in the synaptosome fraction (19).

Dopamine. This amine is present in relatively high concentrations in the caudate nucleus, perhaps as a transmitter. The release of dopamine from the organ has been reported to be increased by stimulating the nucleus centromedianus of the thalamus (21). The extremely low concentration of NE in this region makes it unlikely that dopamine is present simply as a precursor of NE.

The fluorescent histochemical method shows diffuse, green fluorescence, which is thought to be due to an abundance of very fine dopamine-containing fibres.

Under optimum conditions, varicosities similar to those present in other catecholamine-containing fibres are seen, but many terminals are probably submicroscopic (7).

The results of subcellular fractionation (fig. 1) are somewhat equivocal (17). Only about 40 % of the dopamine remains bound to sedimentable material, and only about 25 % of this is recoverable in the synaptosome fraction. The remainder is present in the crude nuclear fraction, the myelin fraction and the microsomal fraction. The distribution pattern resembles that of lactate dehydrogenase (16), the marker of soluble cytoplasmic elements, rather than that of acetylcholine.

This finding, however, is not conclusive evidence against a localization of dopamine in nerve endings, or a transmitter role for this amine. The yield of synaptosomes from a given type of nerve ending depends on its geometry and mechanical properties. The club-like structure of most nerve endings and their mechanical strength relative to surrounding tissue elements evidently favour the "pinching-off" process which occurs during homogenization. It may be otherwise with the varicosities which constitute the regions of synaptic contact for catecholamine-containing neurones. There is much evidence, from fluorescence and enzyme studies, that transmitters are distributed, in varying amounts, throughout the length of the neurones which contain them. The proportion of transmitter recovered in the synaptosome fraction will depend, not only on the efficiency with which presynaptic terminals are converted to synaptosomes, but also on the extent to which the cell bodies and axons of the neurones are represented in the tissue sample undergoing homogenization (fig. 2). In general, the amount of bound transmitter will be T_EV_E when T_E is the concentration of transmitter in volume V_E of surviving nerve endings, and the amount of "free" (high-speed supernatant) transmitter will be T_BV_B where T_B is the concentration of transmitter in volume V_B of cell-body or axon or both. Clearly T_BV_B may be greater than T_EV_E even when $T_B < T_E$ provided V_B is sufficiently greater than V_E. This condition is likely to be met if any considerable number of transmitter-containing cell bodies or axons or both are present in the sample; and if the endings are

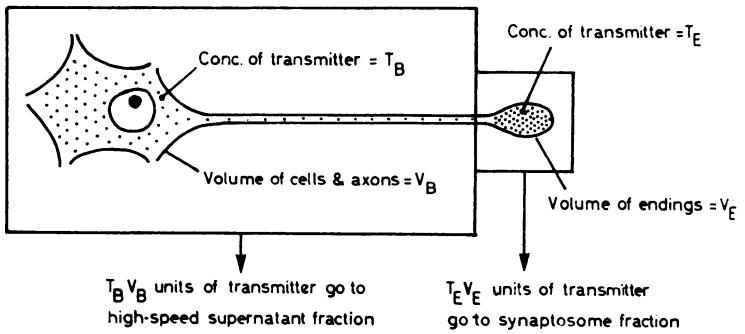

FIG. 2. Diagram to illustrate dependence of proportion of free and bound transmitter on the relative volumes of cell bodies and endings included in the homogenate and the relative concentration of transmitter in different parts of the neurone. T_BV_B may be greater than T_EV_E even when $T_B < T_E$ provided $V_B \gg V_E$.

mainly elsewhere almost all the transmitter could be in the free form after homogenizing.

With acetylcholine, this situation is in fact encountered in the spinal cord, in which cholinergic neurones are represented mainly by the large cell bodies of motoneurones, and in which the only known cholinergic endings are those of the recurrent collaterals to Renshaw cells. In this part of the central nervous system about 90 % of the acetylcholine is found in the free form (12). Shute and Lewis (30) have demonstrated histochemically that the caudate nucleus in the rat receives a dense cholinergic innervation from the striatal radiation from the ventral midbrain tegmentum. Thus most cholinergic neurones in the caudate nucleus are probably represented only by their axon terminations; this would account for the high proportion of bound acetylcholine in this fraction.

If this explanation for the rather low percentage of bound dopamine is correct, it is still necessary to account for the rather diffuse distribution of bound dopamine among the various fractions. This may simply mean that the synaptosomes derived from dopamine-containing neurones have a much greater range of sizes than those from cholinergic neurones, a likely possibility if the synaptosomes are derived from varicosities, some of which are large enough to be visible in the light microscope and some submicroscopic in size (7). Electron microscopic examination of the nuclear (P_1), myelin (A) and microsomal (P_3) fractions revealed the presence of incompletely disrupted tissue fragments in P_1 and of synaptosomes in all three fractions. In addition there were many small vesicles in P_3, some of which might have come from nerve endings. However, subfractionation of P_3 did not show a specific localization of dopamine in a light fraction in which the smaller vesicles were concentrated.

In summary, although the synaptosome fraction from dog caudate nucleus is rich in dopamine, the localization of the amine in this fraction is not so clearly defined as that of acetylcholine. However, owing to the greater heterogeneity of the other fractions compared to those prepared from guinea-pig forebrain, a localization of dopamine within nerve endings is by no means excluded.

LOCALIZATION OF CATECHOLAMINES IN SUBFRACTIONS OF DISRUPTED SYNAPTOSOMES

Technique. Whittaker *et al.* (37) have devised a density gradient procedure whereby synaptosomes, disrupted by suspension in water, can be separated into a series of fractions containing, respectively, soluble constituents of the synaptosome cytoplasm (fraction O), synaptic vesicles (fraction D), external synaptosome membranes (fractions E, F, G), intact or incompletely disrupted synaptosomes (fraction H) and intraterminal mitochondria (fraction I). Microsomes and myelin, if present as contaminants, are recovered mainly in fraction E. The fraction of synaptic vesicles, especially if prepared from cortical synaptosomes, is very homogeneous, contamination by larger particles being less than 3 % (38). Preparations from caudate nucleus or diencephalon and midbrain are less homogeneous and the yield of vesicles is lower.

On disruption in water, about half of the bound acetylcholine is released and,

in the absence of an anticholinesterase, destroyed. This is the "labile" fraction of bound acetylcholine (33). The remainder (the stable fraction) is lost much more slowly. When a water-treated preparation, prepared in the absence of anticholinesterase (and thus containing only the "stable" bound acetylcholine) is separated on the density gradient, the "stable" bound acetylcholine is found to be bimodally distributed: about 30 % (whole forebrain) or 50 % (cerebral cortex) is recovered in the synaptic vesicle fraction (*D*) and the remainder mainly in the fraction containing intact and partially disrupted synaptosomes (*H*), which enclose, of course, many synaptic vesicles. The labile fraction of bound acetylcholine, if stabilized after release by the presence of an anticholinesterase, is recovered mainly in fraction *O* (soluble cytoplasmic constituents). Experiments with preparations containing only the "stable" bound acetylcholine but in which an anticholinesterase had been added to the gradient showed that considerable amounts of acetylcholine are released during centrifugation, at least from cerebral cortical vesicles (38).

Noradrenaline. Much less work has been done with catecholamines and what has been done has been hampered by the lack of a sufficiently sensitive assay method and by the stability of free amine in the presence of particulate material. When a crude synaptosome fraction from guinea-pig diencephalon and midbrain is suspended in water, NE is released, again to the extent of about 50 % of the total bound amine, but unlike the labile bound fraction of acetylcholine, this is not destroyed in the absence of an enzyme inhibitor. In order to determine the distribution of the "stable" fraction of the bound amine in the density gradient it is necessary to centrifuge each density gradient fraction and to determine the amine content of the particulate material in each fraction. As will be seen from figure 3, significant amounts of "stable" bound amine are associated with microvesicular material present in the upper part of the gradient and there is some evidence of a bimodal distribution, though not as sharp as that seen with acetylcholine. The amount of "stable" bound amine bound to microvesicular material has probably been underestimated because of leakage of NE from the vesicles and incomplete sedimentation of vesicular material. Even in the lower part of the gradient, where sedimentation of particulate material would be expected to be complete, 40 to 50% of the total amine remains in the supernatant. This free amine cannot have arisen by diffusion from fraction *O* since solutes of small molecular weight in control experiments did not diffuse appreciably beyond fraction *E*. It must therefore be attributed to incomplete sedimentation of particulate material, to leakage of bound amine during separation, or to both.

Electron micrographs of the microvesicular material in fractions *D* and *E* from diencephalon and midbrain show that it is much less homogeneous than the corresponding fraction from cortex or forebrain. However, there are considerable numbers of synaptic vesicles present, and also larger granular vesicles of the kind that have been observed in peripheral adrenergic neurones. Two other groups (20, 29) have also demonstrated an association of NE with microvesicular material derived from rat brain stem and hypothalamus respectively.

Dopamine. With a crude synaptosome preparation from dog caudate nucleus

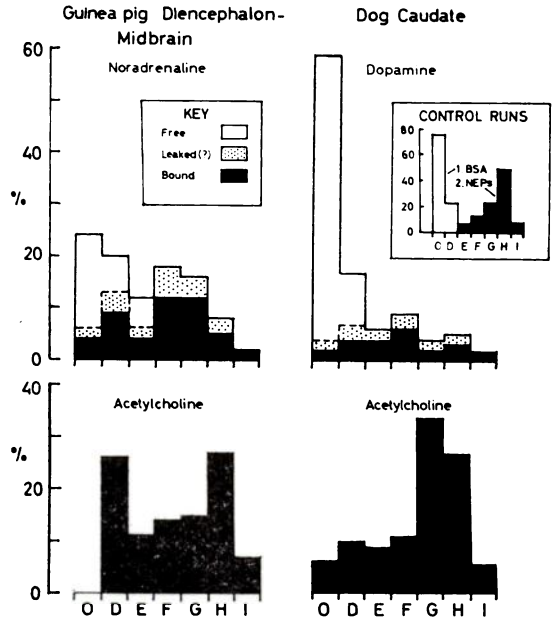

FIG. 3. Distribution of acetylcholine, NE and dopamine in density-gradient fractions of disrupted synaptosomes (17, 24). The diagram in the insert is a composite of two control experiments, one (white blocks) with bovine serum albumin (BSA) showing the limited extent to which this soluble substance diffuses out of the top (*O*) fraction during a run, the other (black blocks) with undisrupted synaptosomes (NEPs) of guinea-pig forebrain showing a unimodal distribution of acetylcholine with a peak in fraction *H* (density equivalent to 1.0 to 1.2 M sucrose). The presence of appreciable amounts of bound NE and acetylcholine in the upper part of the density gradient in association with microvesicular material and the release of most of the dopamine into the free form will be noted in the experiments with disrupted synaptosomes from guinea-pig diencephalon and midbrain and dog caudate nucleus respectively. For nomenclature of fractions see text. An estimate has been made (stippled blocks) for the probable leakage of particulate amine in fractions *O* and *D*.

(24) and under conditions which release relatively little acetylcholine and few vesicles, almost all the dopamine is released as the free amine (fig. 3). Its distribution down the density gradient can be accounted for by diffusion from fraction *O* except for a small residual peak of bound material lower down the gradient perhaps accounted for by unbroken synaptosomes in that fraction.

It is concluded from the foregoing that acetylcholine exists in synaptosomes in two forms, a readily releasable (labile bound) form possibly pre-existing in a free state in the nerve ending cytoplasm or, if bound, only very loosely so, and a more stable bound form, within synaptic vesicles. Recent studies (38) suggest that this acetylcholine may be present as an approximately isotonic solution within the vesicle and that each cholinergic vesicle may contain about 2000 molecules. The mechanism of uptake is being clarified by tracer studies. Choline acetyltransferase is localized exclusively in the neuronal cytoplasm (37) and the soluble cytoplasmic (labile bound) acetylcholine may well represent a pool of freshly synthesized acetylcholine awating storage in synaptic vesicles.

Although the evidence from subcellular fractionation is less complete in the case of NE, a similar storage mechanism seems indicated. For dopamine, definite evidence of localization in microvesicular storage granules within nerve terminals is so far lacking.

THE SIGNIFICANCE OF DENSE-CORED VESICLES

In electron micrographs of adrenergically innervated peripheral tissues, regions of neurites are regularly seen which contain accumulations of vesicles of two types (22, 27, and earlier references cited therein). These have been referred to as granular (or dense-cored) and agranular respectively. The granular type tends to be somewhat larger than the agranular and is characterized by a core of electron-dense material when the tissue is fixed and stained with heavy metals (fig. 4). The agranular type appears to be identical with the synaptic vesicles seen in central and somatic nerve endings.

The regions of the neurites containing dense-cored vesicles correspond to the varicosities seen in the flurescent histochemical method. In cardiac and smooth muscle there appear to be no end organs comparable with the motor end plate of voluntary muscle and the varicosities may represent multiple points of synaptic contact. Nerve endings containing similar granular vesicles are also found in the pineal gland and other regions of the brain having a high monoamine content, *e.g.*, the hypothalamus (25, 28). The fluorescence method confirms the presence in these regions of nerve fibres containing monoamines and displaying varicosities, similar in general appearance to peripheral adrenergic nerves (7, 25a).

The granular vesicles have been considered to be the actual sites of NE storage within the terminals and the evidence in favour of this view may be summarized as follows: 1) NE is a reducing agent and its presence in high local concentration in storage granules would be expected to induce an intense local deposition of heavy metal stains. A similar intense reaction is shown by the chromaffin granules of the adrenal medullary cells which are known to be the storage sites of catecholamine in these cells (for discussion see 28). 2) Autoradiography of tissues induced to take up labelled NE shows radioactivity associated with endings containing granular vesicles (39). 3) The granular vesicles are depleted (5) (pineal, vas deferens) by drugs such as reserpine and guanethidine known to deplete tissues of NE (31) and this effect is antagonized by monoamine oxidase (MAO) inhibitors which inhibit the pharmacological action of these drugs (5). 4) Variations in the number of granular vesicles parallel variations in NE content in different parts of the hypothalamus (14).

Difficulties for the theory are: 1) Only about 30 to 50 % of the vesicles in endings are of the granular kind. This raises the question, "What is the function of the agranular vesicles and what do they contain?" 2) MAO inhibitors do not increase the proportion of granular vesicles although they greatly raise tissue levels of NE (5). 3) Evidence from subcellular fractionation studies comparable to that available for the adrenal medullary granules or for the localization of acetylcholine in agranular vesicles is lacking, in that, so far, attempts to obtain pure preparations of dense-cored vesicles have not been successful. Microvesicular preparations from brain or peripheral tissues rich in NE contain granular vesicles

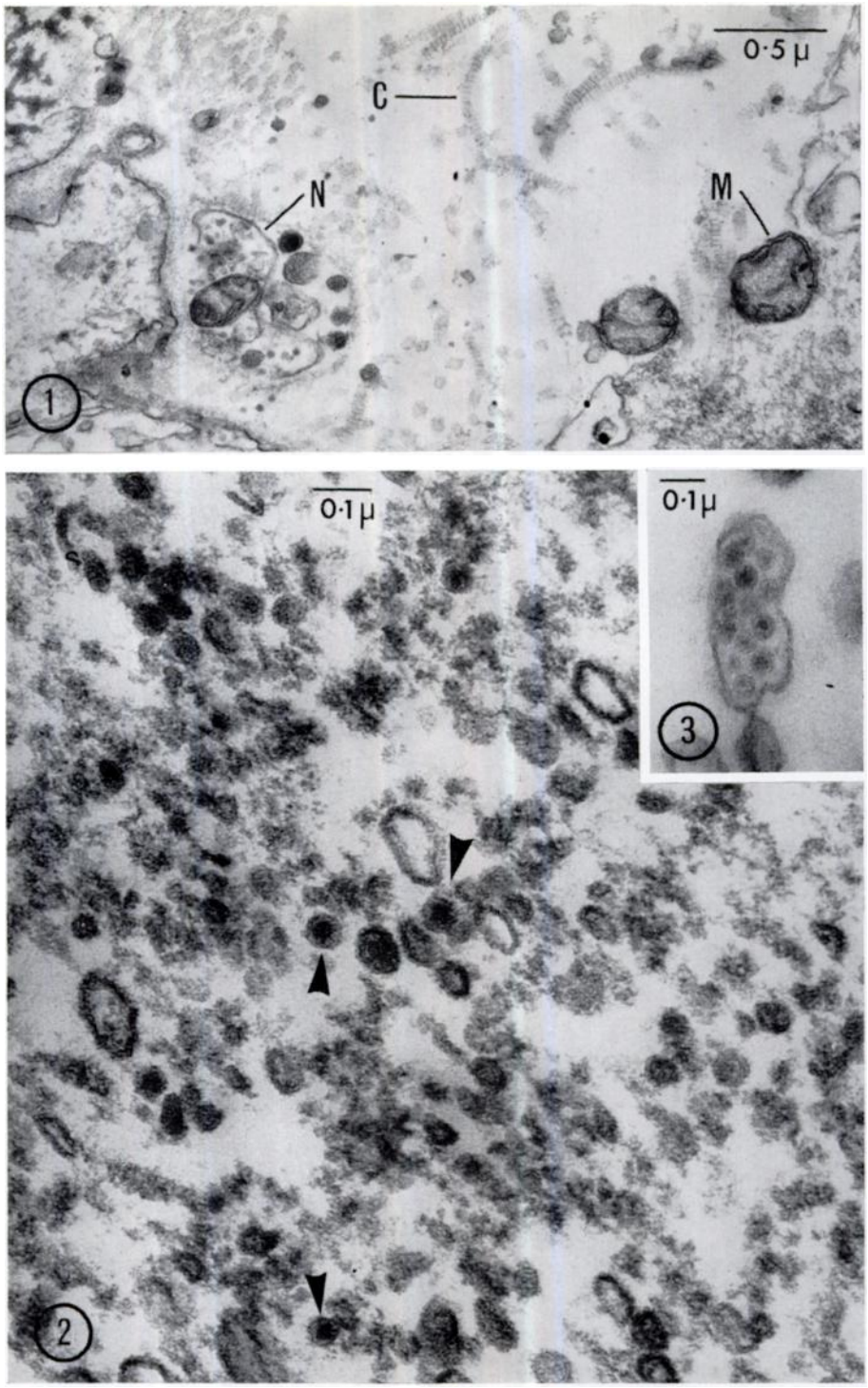

FIG. 4. Subcellular fractions of rat vas deferens. The organ was powdered in liquid nitrogen before homogenizing and separating into fractions P_1, P_2, P_3 and S_3. Fraction P_1 (plate 1) contained about 40% of the NE of the original tissue and many tissue fragments with well preserved nerve endings containing dense cores (N), mitochondria (M) and collagen fibres (C). Very few synaptosomes (plate 3) were formed. Most seemed to break up, releasing their vesicles, which were recovered in the P_3 and S_3 fractions. About 10% of the NE was recovered in the high speed pellet, P_3, which contained many free dense-cored vesicles (plate 2, arrows); about 40% was recovered in the high-speed supernatant, which also contained numerous agranular vesicles. (Electron micrographs by F. Clementi.)

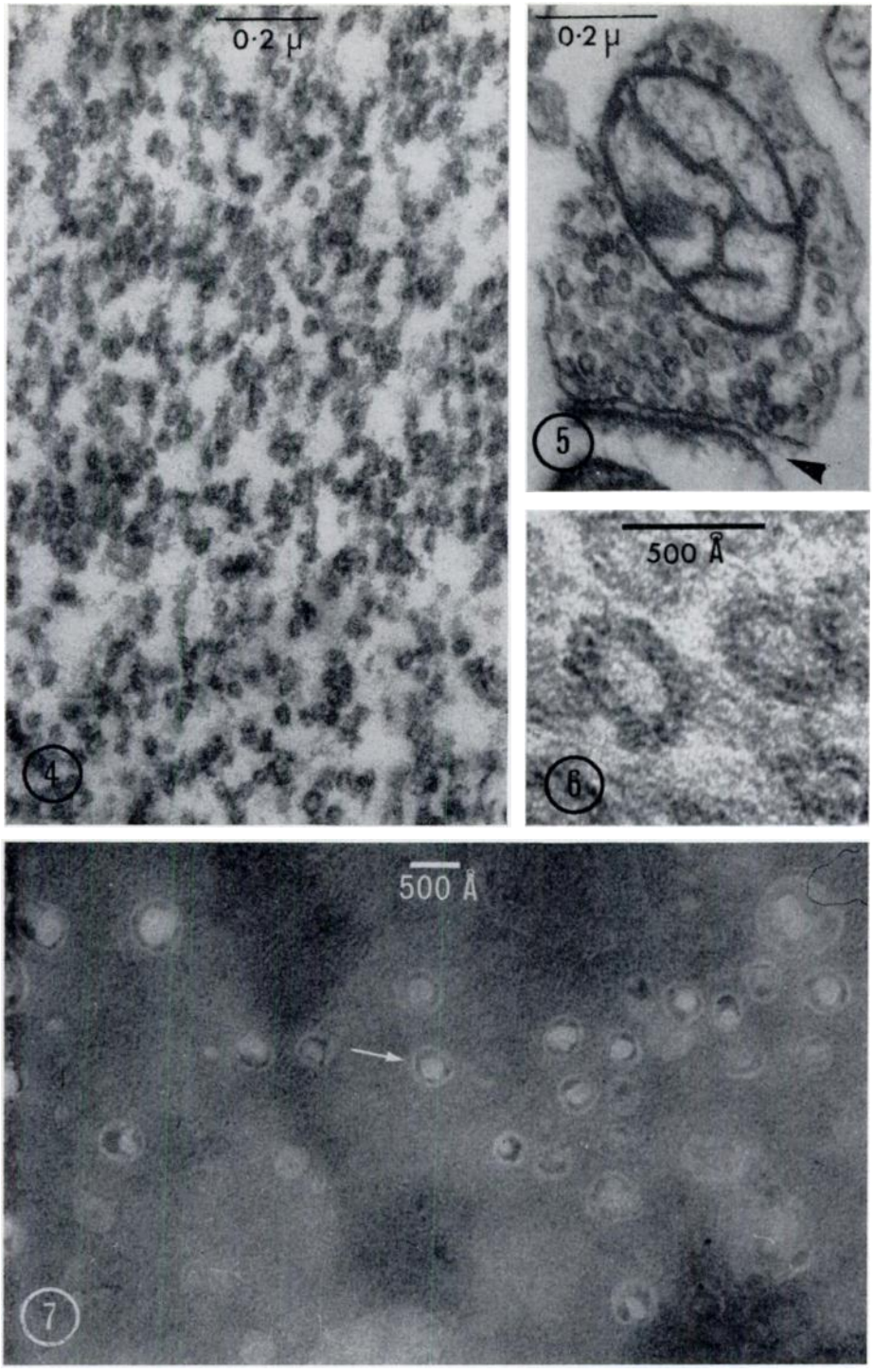

FIG. 5. Fraction *D* from disrupted cerebral cortical synaptosomes is a very homogeneous preparation of synaptic vesicles (plate 4), which appear agranular in positive staining and closely resemble the vesicles seen inside synaptosomes (plates 5 and 6) or intact nerve endings. Note the unit membrane bounding the vesicles (plate 6) and the filaments in the synaptic cleft (plate 5, arrow). By contrast, treatment with gold chloride and negative staining reveals a core (arrow) within these agranular vesicles (plate 7). Plates 4–6 were obtained by Dr. M. N. Sheridan and plate 7 by Dr. M. Israël.

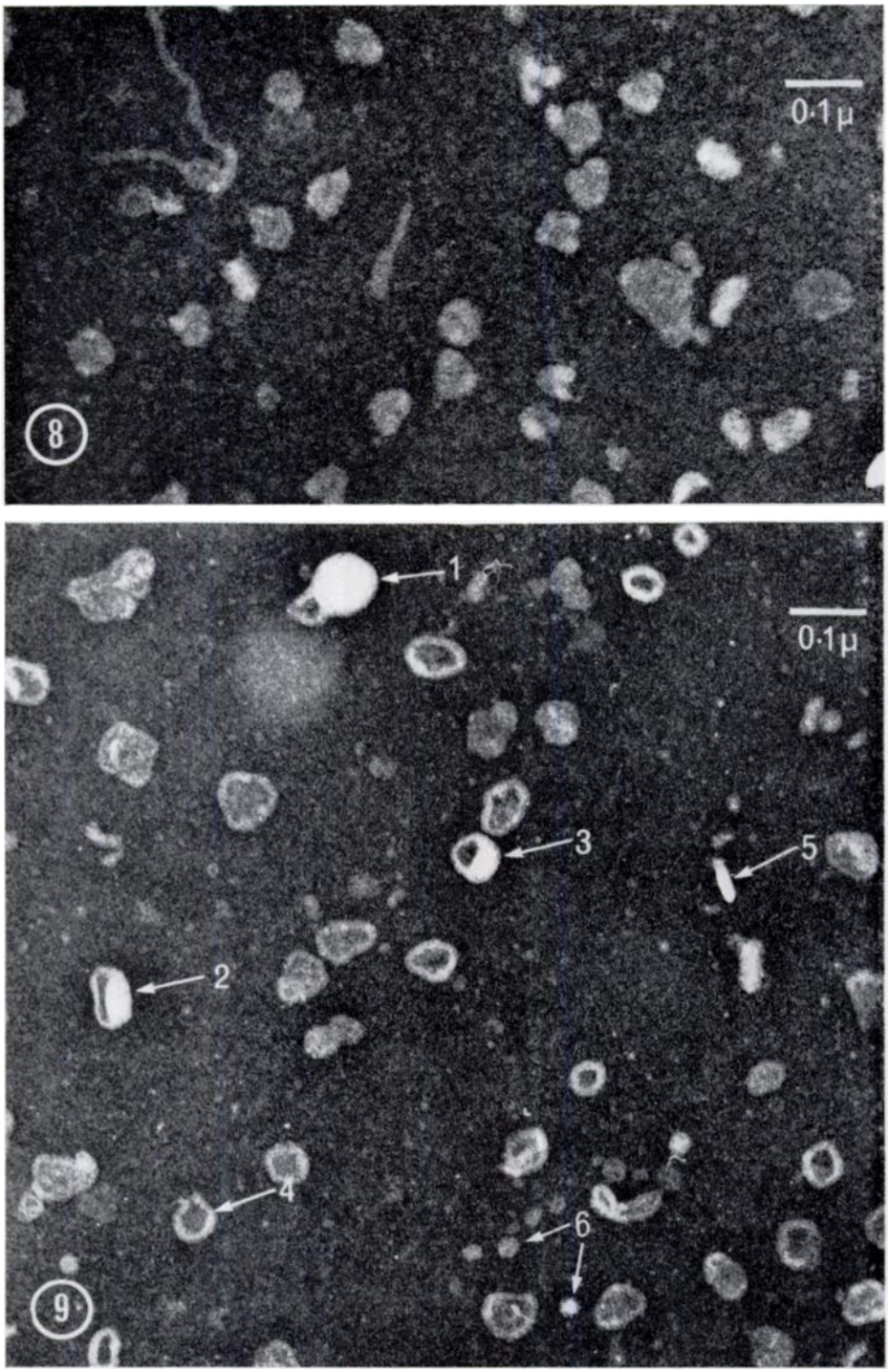

FIG. 6. Plate 8: Freshly prepared synaptic vesicles negatively stained with sodium phosphotungstate (38). Note absence of clear external membrane. Plate 9: Same preparation negatively stained after suspension in dilute phosphate buffer (38). Note clear membrane, vesicles with white cores (1–3), core substance (?) without external membrane (5) and disintegrating (6), and vesicle with break (4) apparently after extrusion of core.

only as a minor constituent. The proportion of granular vesicles is not increased when the preparations are induced to take up NE *in vitro* (23). 4) Vesicles derived from regions of the central nervous system containing little or no catecholamine may be shown to possess dense cores when suitably prepared (38). This point will now be briefly elaborated.

Synaptic vesicles isolated from the cerebral cortex (which contains extremely little NE, 5-HT, or dopamine but relatively high concentrations of acetylcholine) appear, in positive staining and thin section, to be exclusively of the agranular type (fig. 5, plates 4, 6) and identical in morphology with the vesicles seen in undisrupted nerve endings (fig. 5, plate 5). In negative staining, on the other hand, the external membranes of freshly prepared vesicles are seen indistinctly, or not at all (fig. 6, plate 7), even in formaldehyde-fixed preparations. Osmium fixation, ageing, or suspension in phosphate buffer (fig. 6, plate 8) permits the ingress of negative stain and the external membrane now becomes clearly visible. However, a proportion (about 10 %) of the vesicles are now seen to have areas of intense contrast within them, and sometimes the material responsible for this contrast appears without a surrounding membrane, or in a fragmented state. The material can also be seen in untreated vesicles but less clearly.

These images can be interpreted in various ways (38). One suggestion (15) is that the white material is some kind of a core substance, the presence of which in intact vesicles could account for the failure of negative stain to penetrate. The number of white-centred vesicles is greater (about 30 %) when acid rather than neutral phosphate is used. Vesicles treated with gold chloride show the core material particularly clearly, surrounded by the external membrane (fig. 5, plate 7).

The conditions under which the core substance is most clearly seen are also those which favour the precipitation of acetylcholine as an insoluble salt from isotonic solution. It is thus not inconceivable that what is being visualized is localized deposits of acetylcholine associated with vesicles from cholinergic endings. Further work will be necessary to substantiate this; meanwhile, it seems possible that all vesicles may possess some kind of core material which can be visualized in the electron microscope with appropriate reagents.

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