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THE SUBCELLULAR LOCALIZATION OF TRANSMITTER SUBSTANCES IN THE CENTRAL NERVOUS SYSTEM

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Abstract—The chemical nerve transmitter, acetylcholine, is thought to be contained in vesicles found in nerve endings. In support of this hypothesis nervous tissue was fractionated; acetylcholine and choline acetylase were found to have a similar distribution in sub-cellular fractions. Acetylcholine binding particles were easily separated from mitochondria by use of equilibrium centrifugation in a density gradient. About half of the bound acetylcholine was released from the particles by mechanical agitation, hypo-osmotic media or treatment with cobra venom, while the remaining bound acetylcholine fraction was freed by treatment with organic solvents, acids, supersonic vibrations or a temperature of 35 °C. The two types of bound acetylcholine are explained on the basis of a differential type of storage mechanism, one in solution and the other bound to the matrix of the particles by ionic bonds. The significance of the acetylcholine storage particles is discussed.

TRANSMISSION at nerve endings is being very much discussed at the present time in terms of what may be called the "synaptic vesicle theory" of synaptic transmission.[1] Briefly, it is assumed that chemical transmitters are not freely diffused throughout the axoplasm, but are localized in the inactive nerve terminal, inside small vesicles about 0·05 μ in diameter, which electron microscopic studies have shown to be present in large numbers at almost every type of nerve ending. These synaptic vesicles can be seen in Fig. 1 which is an electron micrograph of a nerve ending in lizard brain. The synaptic vesicles seen in mammalian nerve endings are similar in size and appearance. They are assumed to discharge their quantum of transmitter substance as the result of the arrival of a nerve impulse. A low rate of discharge may occur spontaneously all the time, but if so, the amount of transmitter substance released is sufficient to produce only a local "miniature" depolarization of the post-junctional membrane. The arrival of an impulse in the pre-junctional terminals enormously increases the rate of discharge and at the same time synchronizes it, so that the post-junctional potential reaches the proportions associated with a propagated discharge.

The evidence for the quantized release of transmitter comes mainly from the electrophysiological studies of Katz and co-workers[2] on the motor end plate. Katz observed that even in a resting junction, miniature end plate potentials of more or less uniform size made their appearance in random fashion. The size of these potentials indicated that they were being formed as the result of the release of "quanta" of acetylcholine in amounts of a few thousand molecules at a time. The normal end plate potential was shown to be the summed effect of several hundred quanta (about 300

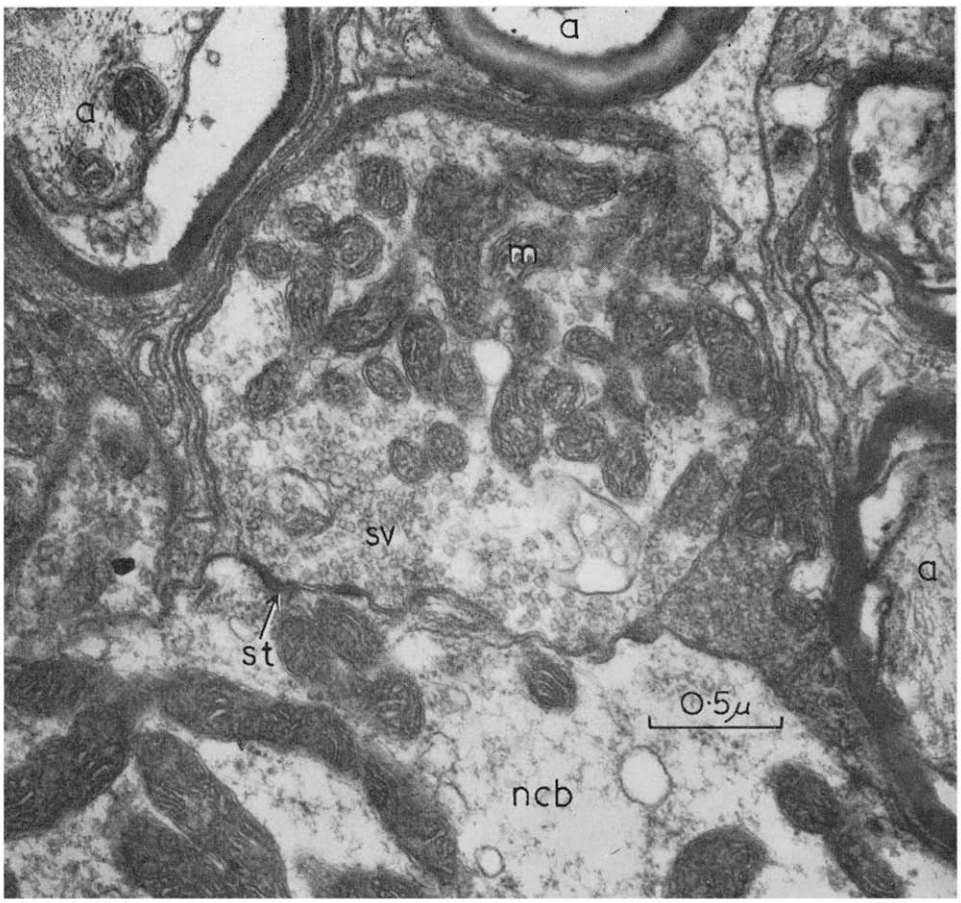

FIG. 1. Nerve endings in lizard brain. (*a*) Myelinated axon; (*m*) mitochondria; (*sv*) synaptic vesicles; (*st*) thickening of synaptic membranes; (*ncb*) neurone cell body. Electron micrograph prepared by B. B. Boycott, E. G. Gray and R. W. Guillery.

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according to the best estimates[3]) released suddenly over an interval of the order of 1 msec.

The electrophysiological and morphological evidence pointing to the existence of synaptic vesicles charged with transmitter substance is also supported by the biochemical evidence. It has long been known that substances such as acetylcholine exist in nervous tissue in a bound, pharmacologically-inactive form in association with the insoluble particulate fraction of the cell. While in this form they are immune from the destructive action of enzymes such as cholinesterase.

If synaptic vesicles really exist within the nerve cell as a distinct type of subcellular particle, it should be possible to isolate them by means of the same sort of techniques as have been successfully used by Schneider, de Duve, Blaschko and others for isolating nuclei, mitochondria, microsomes, lysosomes and chromaffin granules. Their successful isolation and a demonstration that they contain transmitter substance would not only be important supporting evidence for the synaptic vesicle theory; it would also, by enabling the biochemical make-up and properties of the particles to be studied *in vitro*, throw light on the nature of the transmission process itself, and the mode of action of drugs and toxins suspected of interacting with this process.

Work on these lines is being carried out at the Institute of Animal Physiology, Babraham, by Dr. C. O. Hebb and myself[4, 5]. We began by showing that bound acetylcholine and choline acetylase, the enzyme which synthesizes it, have a similar distribution in subcellular fractions prepared from homogenates of brain in iso-osmotic sucrose. The fractions were prepared by differential centrifugation and the greater part was recovered in the so-called mitochondrial fraction. We then showed that the particles containing bound acetylcholine could be separated from mitochondria, using equilibrium centrifugation in a density gradient. The simplified procedure now used[5] is as follows. The original homogenate of brain in 0·32 M sucrose is separated by centrifugation in integrated fields of 10^4 and 8×10^5 *g* min into a fraction comprised mainly of nuclei and cell debris, a crude mitochondrial fraction, and a cytoplasmic fraction consisting of microsomes suspended in the soluble constituents of cytoplasm diluted with sucrose. The crude mitochondrial fraction is then layered on top of a density gradient consisting of equal volumes of 1·2 M and 0·8 M sucrose, and the whole centrifuged at 39,000 rev/min for 1 hr in the SW39 head of the Spinco model L preparative ultracentrifuge. At the end of the run, the tubes have the appearance shown in Fig. 2. The crude mitochondrial fraction has separated into three well defined fractions. The first consists of particles less dense than 0·8 M sucrose (*A*-layer) which are visible under the phase contrast microscope as small particles of diameter 2–5 μ (Fig. 3(1)). The second (*B*-layer), intermediate in density between 0·8 and 1·2 M sucrose, consists of very small particles below the limit of resolution of the light microscope. The third (*C*-layer), denser than 1·2 M sucrose, consists of typical small rod-shaped mitochondria together with smaller particles.

Fig. 4 shows the distribution of bound acetylcholine and succinic dehydrogenase, a mitochondrial marker, in the various fractions. In the primary fractions, bound acetylcholine and succinic dehydrogenase have a similar distribution, about 70 per cent of each being recovered in the crude mitochondrial fraction. This parallel distribution shows that the bound acetylcholine content of the nuclear and microsomal fractions can be accounted for by the contamination of these fractions by particles broadly similar in sedimentation properties to mitochondria. By contrast, the distribution

of bound acetylcholine in the sub-fractions of the crude mitochondrial fraction is quite different from that of succinic dehydrogenase. The bound acetylcholine is seen to be associated mainly with the *B*-layer and the succinic dehydrogenase with the *C*-layer.

Under the electron microscope (Fig. 3(3)), the *C*-layer, as would be expected from its succinic dehydrogenase content, consists mainly of particles (*m*) having the typical internal structure of mitochondria. Compared with mitochondria fixed *in situ*, these appear vesicated, some have lost their outer membranes, or their cristae have shrunk and have been drawn to one pole. Many, however, are recognizable as reasonably intact mitochondria.

The *B*-layer (Fig. 3(2)) presents quite a different appearance. Here no mitochondria are visible. Instead we have large numbers of very small particles (*a*) appearing in section as simple vesicles (*v*) and varying in size from about 0·02 μ to 0·28 μ, with a

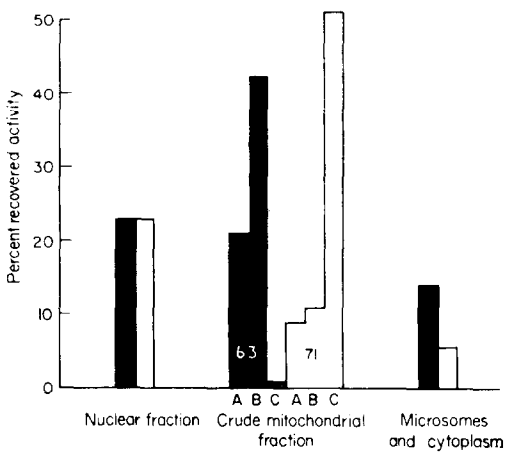

FIG. 4. Distribution of bound acetylcholine (black blocks) and succinic dehydrogenase (white blocks) in subcellular fractions of guinea pig brain.

median of 0·065 μ. Over 60 per cent of these particle fall within the size range 0·02 to 0·08 μ given by Palay[6] for synaptic vesicles. The differences in size range of the two populations of particles is shown in Fig. 5. Here the percentages of particles possessing a given size or less are plotted on a linear scale of probits as ordinates and the size on a logarithmic scale as abscissae. The particles of the *B*-layer (open circles) are seen to have a log-normal size distribution; those of the *C*-layer (solid circles) are also seen to be lognormally distributed except at the lower end of the range. However, if the *C*-layer in this experiment is assumed to be 10 per cent contaminated with particles of the *B*-layer, the corrected distribution (crosses) follows a lognormal distribution throughout its range. As shown in Fig. 3(3), the *C*-fraction contains some particles morphologically identical with those of the *B*-layer.

The characteristics of the *B*-layer, the size range of its particles, their simple vesicular structure and their high content of a transmitter substance, are all consistent with the idea that the *B*-layer represents a crude preparation of isolated synaptic vesicles.

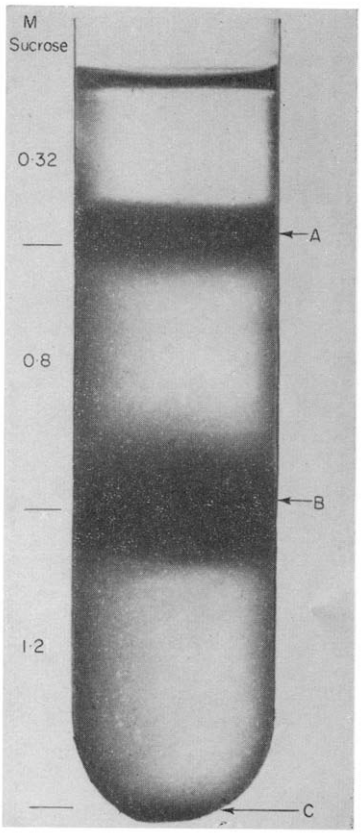

FIG. 2. Photograph of a centrifuge tube after separation of a crude mitochondrial fraction of guinea pig brain into three sub-fractions by equilibrium centrifugation in a density gradient. *A*, particles less dense than 0·8 M sucrose; *B*, particles intermediate in density between 0·8 and 1·2 M sucrose; *C*, particles denser than 1·2 M sucrose.

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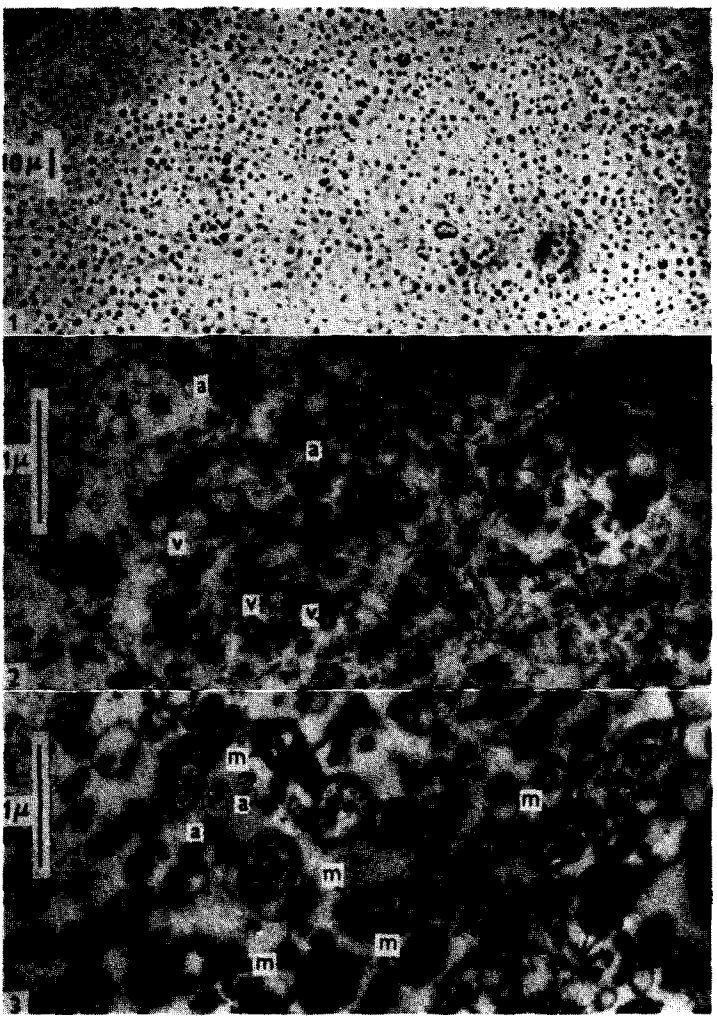

FIG. 3. (1) Phase contrast micrograph of fraction *A* of guinea pig brain. Wet preparation stained with osmium tetroxide. (2) Electron micrograph of thin sections of particles of the *B*-layer fixed and stained with osmium tetroxide and embedded in methylmethacrylate resin. (3) Particles from the *C*-layer similarly treated. The electron micrographs were prepared by Dr. K. M. Smith and Mr. G. J. Hills.

The acetylcholine-containing particles of the *B*-layer are quite stable for a few hours if kept in isotonic media at 0 °C. Amounts of purified cholinesterase capable of destroying an equivalent amount of free acetylcholine in a few seconds have no effect on particle-bound acetylcholine. On the other hand, if warmed to 37 °C or brought to below pH 4·5, all the acetylcholine is quickly released from the particles.

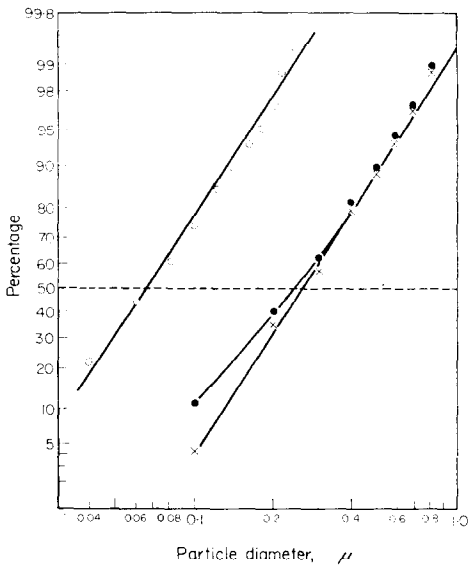

FIG. 5. Distribution of particle size in *B*- and *C*-fractions from guinea pig brain. Ordinates: percentage of total population having indicated diameter or below, plotted on linear scale of probits. Abscissae, particle diameters on logarithmic scale.

A closer study of the conditions bringing about release of acetylcholine support Brodkin and Elliott's[7] finding that about half the acetylcholine is bound in a particularly labile way. Mild disruptive treatments such as freezing and thawing, suspension in hypo-osmotic media, mechanical agitation or treatment with cobra venom release about 50–60 per cent of the total acetylcholine; the remainder requires more drastic treatment such as treatment with organic solvents, acids, supersonic vibrations or incubation at 37 °C for release. These findings are consistent with the idea that part of the acetylcholine is held inside the particle in simple solution and requires only a small permeability change in the particle membrane for release, while the other part is bound more firmly to the matrix of the particle, perhaps by ionic bonds. Experiments with ion exchange resins have suggested that ionic links between the acetylcholine cation and weak acid groups in the particle could, under certain circumstances, be strong enough to account for this latter form and would also account for the effect of pH on the binding.

If the *B*-layer is indeed a crude preparation of synaptic vesicles, we should expect other central nervous transmitters besides acetylcholine to be concentrated there. It was therefore of great interest to us to find that bound 5-hydroxytryptamine is also specifically concentrated in this layer as shown in Fig. 6. Hydroxytryptamine particles of the type described by Baker[8] were not present in brain. It is possible that the *B*-layer

is a rich storehouse of other pharmacologically active substances and that the presence of a substance there would be *prima facie* evidence for a transmitter action.

Bound hydroxytryptamine is released under much the same conditions as acetylcholine. There seem, however, to be important differences in the mechanism of storage.

The close parallelism between the distribution of acetylcholine and choline acetylase,[4] the "occluded" state of the latter in particles whose structural integrity has not

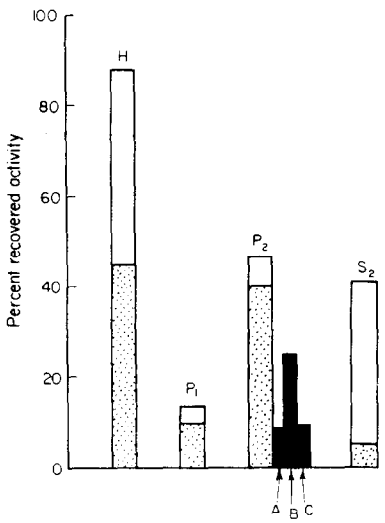

FIG. 6. Bound (dotted blocks) and free (white blocks) hydroxytryptamine in a sucrose homogenate of guinea pig brain (H) and in nuclear (P_1), crude mitochondrial (P_2) and supernatant (S_2) fractions derived from it. It will be seen that most of the free amine in H is recovered in the supernatant fraction while the bound is recovered in the crude mitochondrial fraction. The black blocks show the distribution of total hydroxytryptamine in the sub-fractions of P_2, the highest concentration being found in the B-fraction.

been destroyed[9] and the absence of "acetate activating enzyme" in the particles[10] suggest that acetylcholine is synthesized *in situ* from choline and acetylcoenzyme A. By contrast, a non-particulate location has been assigned to the enzyme 5-hydroxytrytophan decarboxylase responsible for the formation of hydroxytryptamine;[11] this implies that the hydroxytryptamine particles do not synthesize hydroxytryptamine *in situ* but that they take it up from the cytoplasm.

There is considerable evidence that adenosine triphosphate (ATP) is involved in some way in the binding of pressor amines to their storage granules. No studies have been made of the distribution of ATP in the brain fractions, though the absence of a typical component of the electron transport chain from the particles of the B-layer suggests that they are unable to synthesize it. Some preliminary experiments (Fig. 7) indicate, however, that they can take up hydroxytryptamine from the supernatant and that this process is ATP dependent. In contrast to the findings of Walaczek and Abood[12], reserpine does not appear to affect this process, nor does it deplete the particles of hydroxytryptamine *in vitro*, though it is possible to prepare depleted particles from animals previously treated with reserpine *in vivo*. These findings suggest

that reserpine does not act directly on the storage particles, but indirectly, perhaps by influencing the general level of availability of ATP. Walaczek and Abood's report[13] that reserpine is a decoupling agent in mitochondrial oxidative phosphorylation deserves further investigation in this connexion.

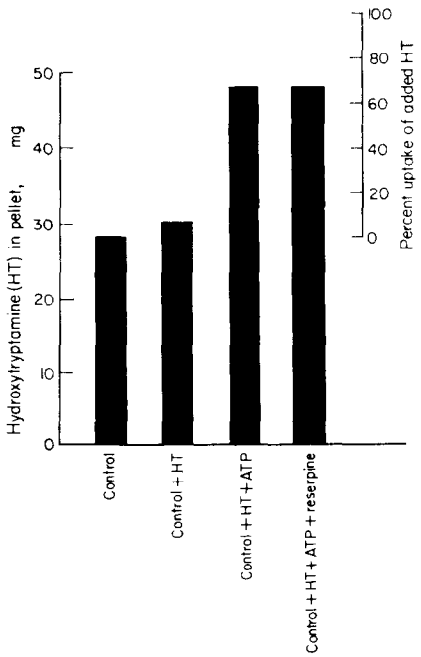

FIG. 7. Uptake of 5-hydroxytryptamine by brain particles in the presence of ATP. The crude mitochondrial (P_2) fraction from guinea pig brain (1 ml, equivalent to 450 mg original brain tissue) was kept at 0 °C for 1 hr alone (control) or with reserpine (25 μg), ATP (10 μmoles) and/or 5-hydroxytryptamine (30 μg free base) in a total volume of 1·48 ml 0·32 M sucrose as indicated. The samples were then centrifuged in an integrated field of 10^6 g min, the pellet taken up in 1 ml 0·32 M sucrose, heated for 10 min at pH 4·3 to release bound hydroxytryptamine and the latter assayed on the rat fundus strip[13].

Much more remains to be done in the study of these storage particles from brain, but I hope to have demonstrated how this may be expected to lead to further understanding of the mode of action of central nervous system drugs and the release of transmitter substances.

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Note added in proof: Recent work by E. G. Gray and V. P. Whittaker (*J. Physiol. Proc.*, 9th July 1960) using an improved electron microscopic technique has revealed that the smallest particles in the *B* layer are enclosed within thin membranes which become extremely fragile after osmication. The small particles within these larger membranes now closely resemble synaptic vesicles as seen in whole tissue sections. A comparison of the morphology of the the larger particles with that of nerve endings in mammalian cortex indicates that they are in fact pinched-off nerve endings. Occasionally particles are seen containing mitochondria in addition to synaptic vesicles and with a portion of post-synaptic membrane still adhering to their periphery.

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