CONTRIBUTION OF ELECTRONMICROSCOPY TO SOME NEUROPHARMACOLOGICAL PROBLEMS*

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THE use of the electron microscope on cells and tissues has permitted the discovery of a complex ultra-structural organization that extends down to the final resolving power of this instrument. The knowledge of this submicroscopic structure is of fundamental importance since many biologic phenomena are associated with physicochemical changes occurring at the level of macromolecules or molecular complexes. It is on this background that morphology can give a solid foundation to biochemistry, physiology and pharmacology. For example, the discovery of a highly organized macromolecular machinery in muscle with the actin and myosin myofilaments, has been of great importance in explaining the mechanism of cellular contraction at a molecular level.

The title of this paper may be misleading because it presupposes that a great deal of information is already available on electron microscopy applied to neuropharmacological problems and this is not the case. This field of research is just beginning but one can forsee the great possibilities that lie ahead.

We would like to show here a few examples of structures in neurons and related cells that are involved in neurosecretory functions, the study of which may have physiological and pharmacological implications.

1. SUBMICROSCOPIC ANALYSIS OF SECRETORY PROCESS IN ADRENOMEDULLARY CELLS

The various histochemical reactions that are characteristic of the adrenal medulla are due to the strong reducing properties of adrenaline, noradrenaline (NA) and other catecholamines present in the cell (Bennett, 1941; Lison, 1953; Bachmann, 1954). Among these reactions the rapid reduction of osmium tetroxide noted by Plenick in 1902, has been repeatedly used as an indicator of catecholamines (Cramer, 1928). Since

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osmium tetroxide became the standard fixation procedure for study of tissues with the electron microscope, its use in the adrenal medulla provides a means for the localization of catecholamines. This is a special situation in which cytochemical results can be obtained with electron microscopy.

Sjöstrand and Wetzstein (1946) first demonstrated the presence of catechol-containing granules, with the electron microscope, in adrenal cells of the cat, mouse and guinea pig. These granules were found to be different from mitochondria thus disproving the suggestion made by Hillarp *et al.* (1954), that the granules were comparable to mitochondria. Sjöstrand and Wetzstein (1956) and later Wetzstein (1957) reached the conclusion, previously maintained by Hillarp *et al.* 1954), that the specific catechol-containing structures were not secretory granules, but permanet cell organoids. De Robertis and Vaz Ferreira (1957) studied adrenal glands of the rabbit in the normal condition, after section of the splanchnic nerve and under stimulation of the nerve with supramaximal pulses of different frequency. Later the structure of the adrenal medulla of the hamster was also studied (De Robertis and Sabatini, 1960).

In the rabbit, 24 hr after section of the splanchnic nerve, there is a heavy accumulation of catechol-containing droplets (granules) which is greater than that of normal glands. These droplets can be easily differentiated from mitochondria. They are circular with a mean diameter of 1600 Å and a range between 870 and 2300 Å. A few smaller droplets of about 400 Å can be observed in the deeper regions of the cell and there are all gradations in size with the larger ones. All of them show a similar structure with a single limiting membrane and a dense osmium deposit. Between both there is a zone of lower electron density of about 100–200 Å (Fig. 1).

The presence of a membrane explains some of the physicochemical properties of the droplets isolated by cell fractionation methods, such as the permeability changes by different physical, chemical or enzymatic agents (Hillarp *et al.*, 1954; 1955). The large clear spaces (Höfe) around the central deposit described by Sjöstrand and Wetzstein (1956) were considered to be artifacts by De Robertis and Vaz Ferreira (1957).

De Robertis and Sabatini (1960) described the early stages of formation of the catechol-containing droplets (*ccd*) in the adrenal cells of the hamster. The smallest *ccd* appear in the deep region of the cytoplasm near the folded nuclear membrane and in direct relationship with the Golgi vesicles. It is within some of these vesicles of about 400 Å that the electronically dense material (catechol) first appears.

In this region of the Golgi complex all stages of the development of the *ccd* can be followed until they reach the maturation size indicated above. At the same time the secretion material is moved toward the peripheral region of the cytoplasm and frequently becomes accumulated

in the vicinity of the surface membrane, where they are excreted (Fig. 2A).

De Robertis and Vaz Ferreira (1957) studied the excretion of the catechol droplets by electrical stimulation of the splanchnic nerve in the rabbit. With frequencies of 40–100 supramaximal pulses per second for 10 min changes take place that in some cells lead to a complete excretion of the *ccd*.

These results cannot be interpreted by the hypothesis postulated by Hillarp *et al.* (1954) and Sjöstrand and Wetzstein (1956) that the *ccd* are permanent organoids of the cell that can store and discharge their content in catecholamines. In the case of the rabbit it can be shown that in certain stimulated cells the *ccd* disappear entirely or leave empty membranes, while after section of the splanchnic nerve they increase in number. This finding is supplemented by the observations of new small *ccd*, which are found in the Golgi region (Fig. 2 B). It seems to us that there may be species differences in the mechanism of excretion of the *ccd*. In the case of the rabbit there is clear proof that the secretion droplets undergo a cycle by which they are formed in the deeper region of the cell and evacuated at the periphery. These facts are better interpreted considering the *ccd* as secretory inclusions and not as permanent organoids. The number of *ccd* present at a definite moment is the result of a balance between the process of elaboration and storage and the excretion of the secretory product.

2. MICROVESICLES IN ADRENERGIC NERVES

In a recent electron microscope study of adrenergic nerve fibres of the sympathetic system innervating the pineal gland and in branches of the splenic nerve abutting spenic arteries, De Robertis and Pellegrino de Iraldi (1961, *a*) have described a characteristic plurivesicular component. These adrenergic vesicles are similar in size and shape to the typical synaptic ones, found in synaptic endings (De Robertis and Bennett, 1954), but some of them differ in their content. While some vesicles have a homogeneous interior of relative low density the others have a heterogeneous content characterized by the presence of a dense osmium deposit. In the splenic nerve of the rat, which is purely adrenergic, some plurivesicular material can be found along the axon, but this is more abundant in the small and finer branches endings within the smooth muscle tissue (Fig. 3). The adventitia of the splenic arteries are unusually rich in nerve endings containing plurivesicular material.

The presence of these heterogeneous vesicles with a granule strongly reducing osmium tetroxide may be indicative that this component, as in the case of the adrenal medulla, is the specific site of storage of NA and other reducing amines of the adrenergic nerves and endings. The size distribution of these vesicles suggest that they may contain the

adrenergic transmitter in definite multimolecular or quantal units. The finding of a specific component in adrenergic nerves opens the possibility for studying histophysiologically the mechanisms of storage and release of this chemical transmitter at the level of resolution of the electron microscope.

3. SECRETORY PROCESS OF THE PINEAL GLAND

Giarman and Day (1958) made the interesting observation that the pineal gland has a high concentration of biogenic amines. Histamine, catecholamines and serotonin (5-hydroxytryptamine) are all exceptionally concentrated in this tissue. Giarman *et al.* (1960) have found that the serotonin levels in human and simian glands are highest ever reported for any neural structure of any species so far examined.

In addition to these amines there is the skin-lightening hormone melatonin described by Lerner *et al.* (1958). This substance, which antagonizes the skin-darkening effect of the melanocyte-stimulating-hormone, is chemically a 5-methoxy-N-acetyltryptamine and probably derives from 5-hydroxytryptamine by O-methylation and N-acetylation. Axelrod (1961) has found that the two enzymes involved in these chemical transformations are found concentrated in the pineal gland.

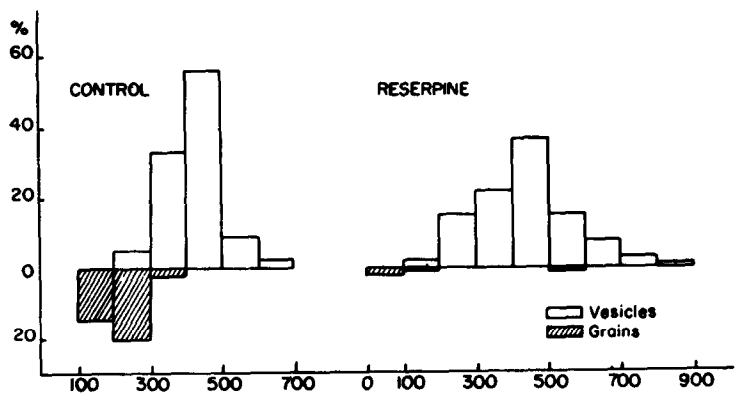

FIG. 5. Histograms of sizes of vesicles (in Ångstroms) in the secretory processes of the pinealocytes in a control rat and in a rat 2 hr after injection of reserpine. In the upper part percentage distribution of sizes of vesicles, below distribution of sizes of dense grains/100 vesicles. See the almost complete disapperance of grains with reserpine (only 22 grains in 600 vesicles). (Pellegrino de Iraldi and de Robertis, 1961).

Recently de Robertis and Pellegrino de Iraldi (1961, *b*) have observed that the main morphological characteristic of the pinealocyte is the presence of club-shaped perivascular expansions connected to the cell edge by thin pedicles. These expansions contain mitochondria and, as in the case of the adrenergic nerves, they have a plurivesicular secretory material.

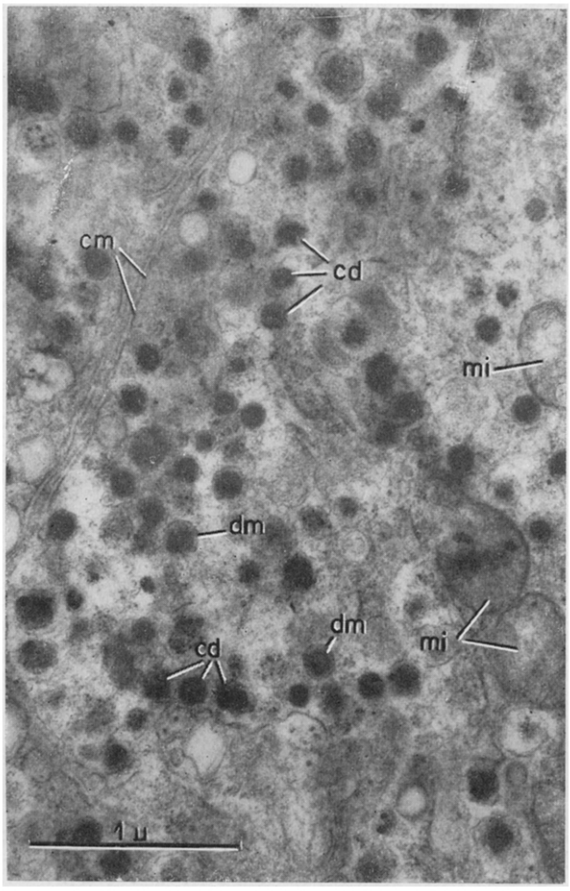

FIG. 1. Part of an adrenomedullary cell of the rat. The dense content of the droplets (*cd*) is rather heterogeneous in this animal. The droplet membrane (*dm*) is observed in nearly all granules. *cm* cell membrane. Ribosomes and cisternae of the endoplasmic reticulum and mitochondria (*mi*) may be seen. Osmium tetroxyde fixation and Araldite embedding. 51,500.

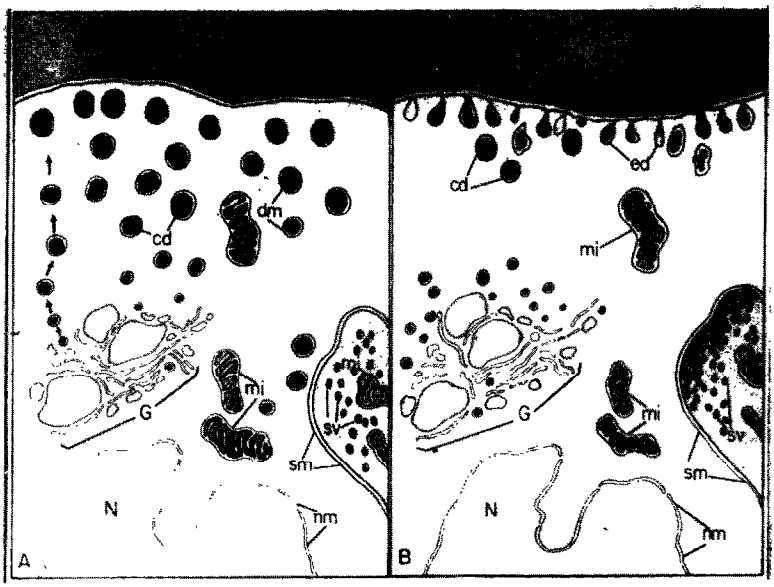

FIG. 2. Diagrammatic interpretation of the mechanism of secretion in the chromaffin cell. A. Cell in the resting condition showing the storage of mature catechol droplets in the outer cytoplasm. Near the nucleus within the Golgi complex new secretion is being formed at a slow rate. At the right a portion of a nerve terminal showing the synaptic membrane (*sm*), synaptic vesicles (*sv*) and mitochondria (*mi*) B. Cell after strong electrical stimulation by way of the splanchnic nerve. The catechol droplets (*cd*) have largely disappeared and the few left are seen in different stages of their excretion into the intercellular cleft. The Golgi region is now forming new droplets at higher rate. The nerve endings show an increase of synaptic vesicles with accumulation at "active points" on the synaptic membrane. (de Robertis and Sabatini, 1960).

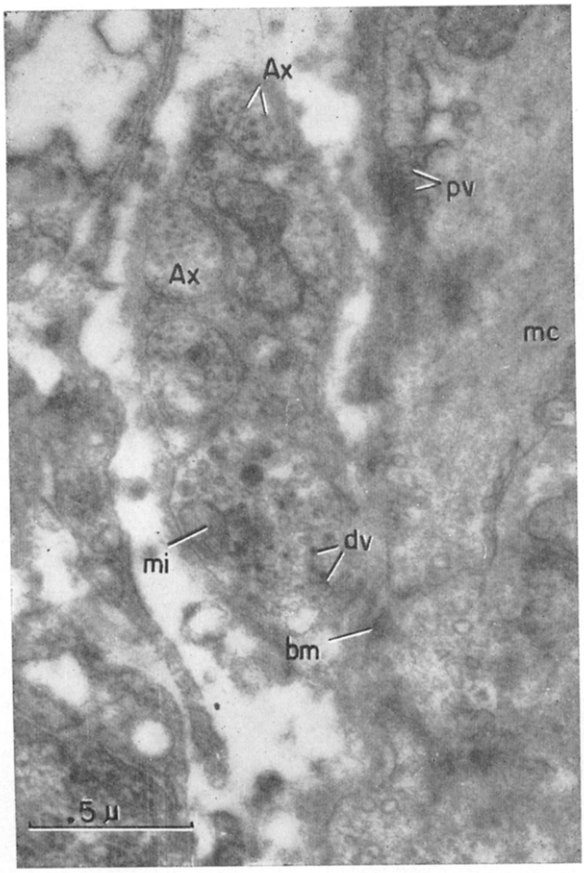

FIG. 3. Adrenergic nerve axons (*Ax*) of the splenic nerve of the rat. The axon at the bottom abuts a smooth muscle cell (*mc*) of an artery. See the increase in number of vesicles, some of which show a dense content (*dv*). *mi* mitochondria, *bm* basement membrane, pv pinocytotic vacuoles in the muscle cell. × 70,000. (de Robertis and Pellegrino de Iraldi, 1961, *a*).

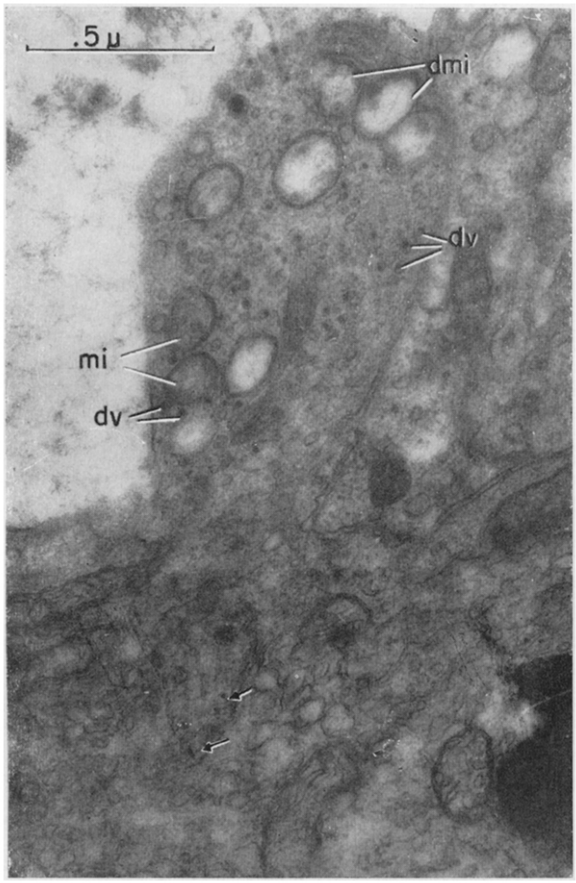

FIG. 4. Electronmicrograph of an unusually large plurivesicular secretory process of the pineal gland of a rat united to the cell by a thick pedicle. Numerous vesicles with light homogeneous content and others with a dark osmium granule (*dv*) make the main content of the process. Some of these vesicles are in the cytoplasm (arrows). There are also mitochondria (*mi*), some of them showing degeneration (*dmi*). × 80,000. (de Robertis and Pellegrino de Iraldi, 1961, b).

This is made of round or oval vesicles, with a mean diameter of 425 Å and having a definite limiting membrane. These elements resemble the synaptic vesicles and have about the same size distribution (Fig. 4). However a striking difference is that there are two types of vesicles according to their content with probable intermediary forms. Most of the vesicles have a homogeneous content, but interspersed there are other having a very dense and round osmium deposit with a mean diameter of 210 Å (Fig. 5). These characteristics explain the name of *plurivesicular secretory processes* coined for these cellular expansions (de Robertis and Pellegrino de Iraldi, 1961, *b*).

The ratio of heterogeneous/homogeneous vesicles varies, but it is usually between 40 and 50 per cent in the normal gland. This ratio can be changed considerably in conditions that are known to increase or to diminish catecholamines and serotonin in the nervous tissue. Unpublished observations by Pellegrino de Iraldi and de Robertis have shown that this ratio may increase in animals treated with the amino-oxidase inhibitor iproniazid and with the injection of catecholamine or serotonin precursors. Pyrogallol, an inhibitor of methyltransferase (Axelrod, 1961), also induces an increase in the ratio of heterogeneous/homogeneous vesicles.

Of even greater interest is the depletion of heterogeneous vesicles observed after injection of reserpine (Pellegrino de Iraldi and de Robertis, 1961). Between 2 and 48 hr after a single injection of 5 mg/kg of reserpine there is an almost complete disappearance of heterogeneous vesicles containing the dense osmium deposit. At the same time, there is a decrease in size of the homogeneous vesicles, some of which resemble sections of the tubular elements of the pedicle. The effect of the drug can be observed as early as 10 min after the injection, reaching the maximal effect after 2 hr.

The restoration of the heterogeneous vesicles is evident after 3 days and the numbers increase to normal levels by the sixth to eighth day. The curve of grain recovery is strikingly similar to that obtained by Shore and Brody (1957) for the restoration of serotonin and noradrenalin in the brain of rabbits after injection of reserpine (Fig. 6). Chronic administration of reserpine leads to an almost complete disappearance of the dense granules and to other profound changes in the vesicular components of the secretory processes.

These observations bring definite evidence that pinealocytes are secretory cells. The secretion represented by the small plurivesicular material is produced and accumulated preferentially within the perivascular expansions of the cell although sometimes they can be observed in the cytoplasm proper.

The experiments reported above suggest that this plurivesicular material contains some of the biogenic amines of the pineal gland. The

facts that reserpine produces the release of serotonin (Shore *et al.*, 1957), noradrenaline (Carlsson *et al.*, 1957) and dopamine (Bertler and Rosengren, 1959) and that all these amines reduce osmium tetroxide intensely, makes difficult at the present time a finer morphological discrimination of the site of storage of these different amines. Further pharma-

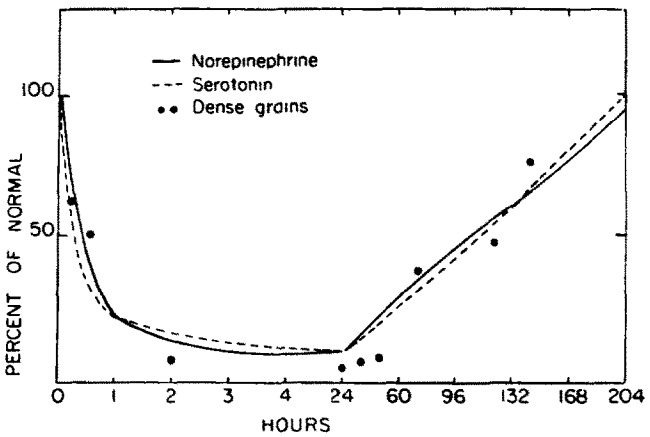

FIG. 6. Curve of Shore and Brodie (1957) showing the concentration of serotonin and noradrenaline (in per cent of the normal) in the nervous tissue after a single injection of reserpine. On this curve the points indicate the per cent change of the normal in dense granules observed in the pineal gland after reserpine. (100% represent the number of dense grains/100 vesicles found in the normal controls) (From Pellegrino de Iraldi and de Robertis, 1961).

cological studies with drugs that may influence the metabolism of each one of these biogenic amines, should be carried out in order to establish with better bases the storage and the mechanism of release of these amines in the pineal gland.

The synthesis of complex biogenic amines such as melatonin (Axelrod, 1961) involves the interplay of several enzymes whose exact intracellular localization is barely known (Lerner *et al.*, 1958). It is conceivable that the finding of a specific submicroscopic structure for the pinealocyte may lead to a better understanding of the mechanism of synthesis, release and inactivation of biogenic amines in the pineal gland.

The type of research illustrates the potentialities of electron microscopy in the study of pharmacological problems.

IV ISOLATION OF CHOLINERGIC AND NON-CHOLINERGIC NERVE ENDINGS OF THE C.N.S

The electron microscopic observations, of the central nervous tissue shows an extraordinary complexity of organization. More than half of

the volume of gray matter is occupied by tightly packed synaptic endings, axons, dendrites, glial processes and so forth, most of which are of submicroscopic dimensions and thus can not be analyzed with the light microscope.

For a study of subcellular fractions of the brain, more than in other tissues, the electron microscopic observations are indispensable. In the last two years our laboratory has been engaged in the effort of perfecting methods for isolation of fractions that may be recognized to be as homogeneous as possible under the electron microscope. It has been thought that this study may provide firm bases for a morpho-biochemical correlation at a subcellular level of structure and for studying the localization and metabolism of biochemical components which are important in nerve function.

Thus the isolation of nerve endings and eventually of synaptic vesicles and other components of the synaptic region may be of paramount importance for the exact localization and isolation of transmitters receptor proteins, enzymes and other materials, that are involved in synaptic function.

The literature on chemical transmission in the C.N.S. will not be considered in detail here (see the reviews by Perry, 1953; Feldberg, 1957; Crossland, 1957; and Koelle, 1959). As stated by Feldberg (1957) "acetylcholine is a central transmitter, but it is not the universal one. In other words, the central nervous system is built up of cholinergic and non-cholinergic neurons". Several non-cholinergic agents such as adrenaline, noradrenaline and 5-hydroxytryptamine have been found in the C.N.S with a preferential localization in certain regions (Vogt, 1954) and have been considered as neurohumoral transmitters (see Shore and Brodie, 1957). A series of inhibitory substances are apparently involved in the so-called factor I extracted from brain and other nerve tissues (see Florey, 1960, *a*, *b*). Finally there are other compounds such as substance P, ATP and some unidentified factors whose possible participation as transmitters is less evident.

Acetylcholine and some of the other transmitter substances of the C.N.S. are mainly in a "bound" form suggesting an association with some subcellular particle. Feldberg (1945) gave some of the first evidence that acetylcholine is bound with a protein particulated component, which was generally identified with mitochondria. Hebb and Whittaker (1958), upon further sedimentation of the mitochondral fraction, could demonstrate that acetylcholine and choline-acetylase is bound to a subcellular fraction that is distinct from mitochondria, nuclei and microsomes.

Whittaker (1959) continued this line of work on the assumption that the acetylcholine-rich fraction could be associated with the synaptic vesicles of nerve endings, first described by de Robertis and Bennett

(1954) (see de Robertis, 1959). He could separate the mitochondrial fraction into three subfractions A, B, C, which were later identified, respectively, as myelin, synaptic endings and mitochondria (Gray and Whittaker, 1960). Fraction B contained most of the particle-bound ACh and 5-hydroxytryptamine.

In a more recent work of de Robertis *et al.* (1961, c) fractions of the rat brain were studied under the electron microscope and their acetylcholinesterase (AChE) and succinicdehydrogenase (SDH) activity and content of bound acetylcholine (ACh) was determined.

After mild homogenation in isosmolar sucrose three primary fractions (nuclear, N, mitochondrial, M and supernatant, S) were isolated by centrifugation. The M fraction was centrifuged in a density gradient of sucrose varying between 0·8 and 1·4 M and five subfractions: A,B,C,D and E, were isolated.

Under the electron microscope subfraction A consists of myelin fragments, B is a complex formed by small fragments of nerve endings containing synaptic vesicles, curved membranes of synaptic type, some myelin fragments and vesicles of microsomal size; C is formed essentially by pinched off nerve endings containing numerous synaptic vesicles; D is the most pure and abundant fraction of nerve endings, (Fig. 7), some of which contain mitochondria, and E is practically a pure fraction of free mitochondria.

The study of AChE in the subfractions of M shows that A contains no AChE, B and C are rich in AChE, D is low and E very low. SDH is practically nil in A, B and C, increases slightly in fraction D and specially in E.

The content of bound ACh is almost nil in A, rises in B, reaches a maximum in C and decreases considerably in D and E. The results of the AChE and ACh indicate that subfraction C contains cholinergic nerve endings and D non-cholinergic nerve endings (Fig. 8). This last subfraction is larger than the former.

These results bear upon the possible localization and functions of AChE at the synaptic region. The finding of a considerable activity in subfraction B, coinciding with the presence of curved membranes and subsynaptic structures, may be indicative of a postsynaptic localization. More significant are the results of subfraction C, in which AChE reaches the maximum coinciding with the highest concentration of ACh. Although in this case one may conclude that AChE is associated with cholinergic endings the fact that these carry the attached subsynaptic membrane precludes a final localization at the pre- or postsynaptic sides or in both.

Because of the lack of true extracellular spaces the conditions for diffusion of transmitter substances in the C.N.S. are certainly very different then in the peripheral nerve tissue and it is not possible to

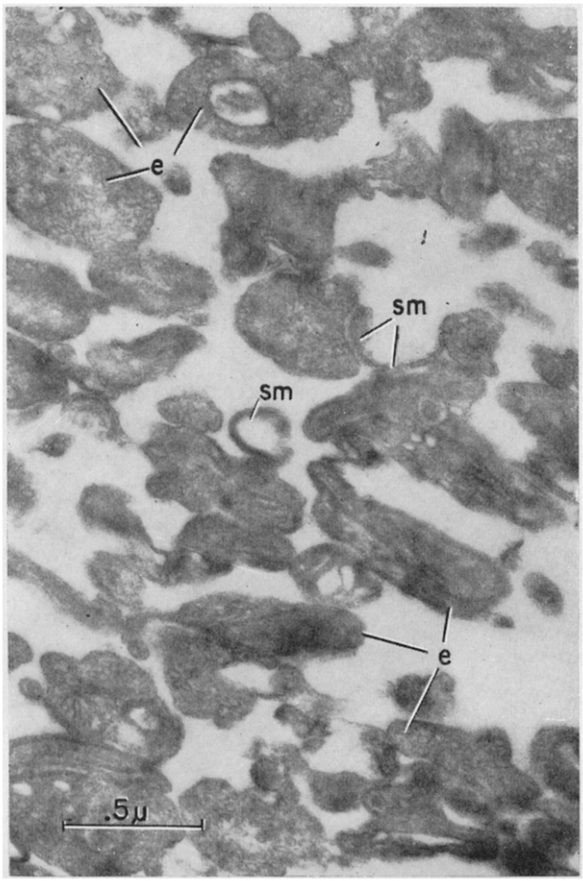

FIG. 7. Electronmicrograph of subfraction D containing exclusively nerve endings (*e*), a few of which have the attached subsynaptic membranes (*sm*). Notice the presence of mitochondria in some of the endings and the numerous synaptic vesicles. × 60,000. (From de Robertis *et al.*, 1961).

extrapolate from what is known in certain peripheral synapses, in which AChE seems to act only presynaptically (see Koelle, 1959). It might well be that in the C.N.S. AChE acts both at the presynaptic side to prevent excess accumulation of ACh at the ending and postsynaptically on the ACh released by the nerve impulse.

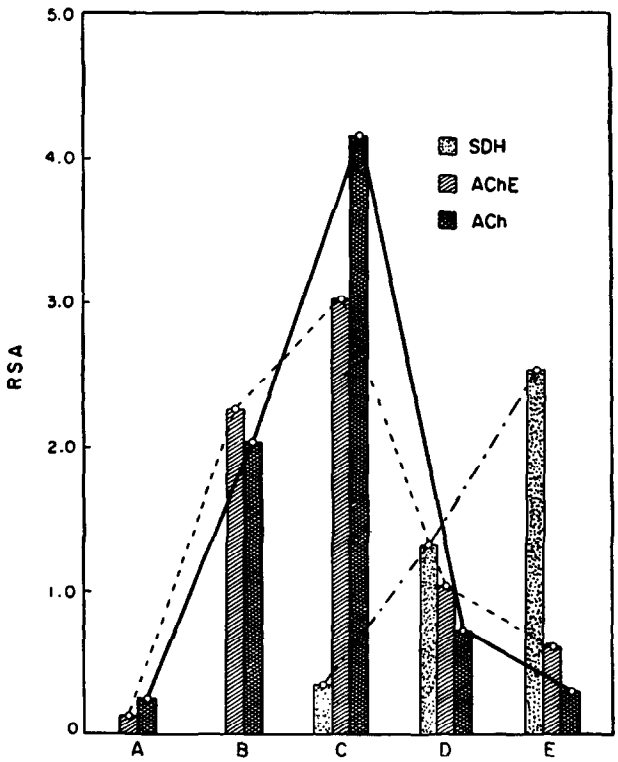

FIG. 8. Graphic representation of the specific activity (RSA) of the five subfractions (A,B,C,D,E) for succinicdehydrogenase SDH), acetylcholinesterase (AChE) and acetylcholine (ACh). The values are shown by the bars of the histogram. The lines are only to emphasize the general trend of distribution of the three components. The *relative specific activity* is defined as the quotient of the percentage of recovered ACh, AChE and SDH to the percentage of the recovered protein of each fraction. (From de Robertis *et al.*, 1961).

The results presented above suggest that two populations of nerve endings can be isolated from the brain tissue. These endings although rather similar in structure under the electron microscope can be differentiated by the content of bound ACh and the AChE activity.

The nerve endings of subfraction C contain 13 per cent of protein, 36 per cent of AChE and 50 per cent of bound ACh of the total "mitochondrial" or M-fraction. The relative specific activity (RSA), that meas-

ures the enzyme activity or the concentration of ACh in comparison with the values for the total homogenate, is about 3·0 for AChE and 4·1 for ACh.

Apart from C, only in subfraction B has there been a concentration of AChE (RSA 2·25) and bound ACh (RSA 2·0) indicating the presence of cholinergic components. These may be small fragments of endings containing a few synaptic vesicles which together with some synaptic membranes are observed by electron microscopy.

The population of non-cholinergic endings is concentrated in subfraction D, which is morphologically the most pure fraction of nerve terminals. By protein content this subfraction is about twice the size of C. This agrees with the general concept that there are more non-cholinergic than cholinergic endings in the brain.

While for the moment we can only define this subfraction by a negative character, experiments are underway to investigate the nature of these nerve endings. The study of the localization of catecholamines, 5-hydroxytryptamine or the inhibitory substances in the different subfractions might possibly show a pattern of distribution, which may elucidate the now obscure significance of this large population of non-cholinergic endings.

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DISCUSSION

Dr. EDITH BULBRING: I would like to ask Dr. de Robertis whether, in his beautiful electronmicrograph of the sympathetic nerve ending of smooth muscle of the spleen, he has seen synaptic vesicles of the type which are supposed to contain acetylcholine side by side with the dark granules presumably containing noradrenaline, I mean both types in the same nerve-ending?

Dr. de ROBERTIS: Indeed the electron microscope observations of adrenergic nerve endings show two types of vesicles: one homogeneous with a clear content and the other heterogeneous with a dark content. The possibility that two types of transmitters may be present in adrenergic nerves is thus not excluded.

Dr. O. H. LOWRY: It is my impression that mitochondria, as seen in sections of intact brain, are very variable in size and shape; that mitochondria, in axons, for example, are long and slender; and yet the mitochondria which you have isolated appear quite homogeneous.

Dr. de ROBERTIS: Free brain mitochondria show a tendency to swell while those contained within the nerve endings do not.