PHARMACOLOGICAL STUDIES WITH ISOLATED CELL COMPONENTS

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UNTIL recently, biochemists have been mainly concerned with those aspects of the chemical machinery of cells which are common to all cells and tissues: with, for example, the details of anaerobic glycolysis and fermentation, oxidative phosphorylation and the electron transport chain, with lipid metabolism and protein synthesis. Although much remains to be done in these fields, there are signs that this historic phase is now coming to an end, and that greater attention is being paid to those structural and functional components of cells which distinguish one cell or tissue from another. It is these aspects which are of particular interest to the pharmacologist and which must be understood if an adequate explanation of drug action in biochemical terms is to be achieved.

One way of identifying the components of a cell which are essential for its special function is to isolate the functionally differentiated portions of the cell and then to determine what components are present in these portions which are not present in the rest of the cell. For this purpose, the technique of homogenization and differential centrifugation has proved extremely useful. In this paper I will describe some work in which this technique has been applied to the problem of chemical transmission at nerve endings in the central nervous system. This work was begun in collaboration with Dr. C. O. Hebb[1] and has been continued with Drs. E. G. Gray[2,3], M. Nyman and I. A. Michaelson and Miss J. Cleugh. To begin with, it was largely concerned with acetylcholine, now generally accepted as a C.N.S. as well as a peripheral transimitter ,but more recently we have been concerned with 5-hydroxytryptamine, substance P and other substances as well.

Just before we started this work, early in 1957, it had been suggested, on the basis of the electrophysiological findings of Katz and co-workers[4] and the morphological studies of synapses by Sjöstrand[5], de Robertis and Bennett[6], Palay[7] and others, that acetylcholine and other transmitters are stored within the presynaptic nerve terminals in small vesicles, the synaptic granules or vesicles, which are visible in large

numbers in electron micrographs of nerve endings. Our approach was different. We decided to study the distribution of acetylcholine in subcellular fractions of brain tissue with no preconceived ideas as to which particulate fraction, if any, it might be found in, and then to attempt to identify any structures with it appeared to be specifically associated.

We found that most of the acetylcholine of the tissue is recovered in a particulate fraction of intermediate particle size, usually referred to by biochemists as the "mitochondrial" or "large granule" fraction. On submitting this fraction to density gradient separation, the acetylcholine-containing particles were readily separable from mitochondria[1,8] as shown in Fig. 1.

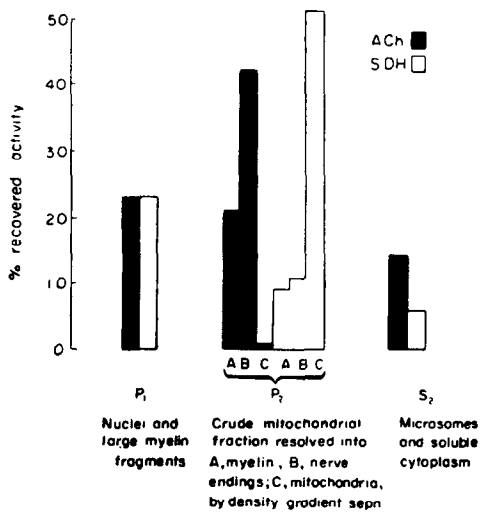


FIG. 1. The distribution of acetylcholine (black blocks) and succinic dehydrogenase (white blocks) in subcellular fractions of guinea-pig brains. On refractionating the crude mitochondrial fraction into three sub-fractions A, B and C, most of the succinic dehydrogenase (included as a mitochondrial marker) migrated to the densest region (fraction C), whereas acetylcholine stayed with a fraction of intermediate density (fraction B).

Dr. Gray found, early in March 1960, that the fraction containing the acetylcholine consisted largely of pinched-off nerve endings, packed with synaptic vesicles and not infrequently seen with a portion of post-synaptic membrane still adhering[2]. This finding led us to make a thorough morphological investigation of the various fractions in the electron microscope[3]. Some of our results were communicated to the fourth International Neurochemical Symposium in June 1960[9] and to the Physiological Society in July 1960[2].

As a result of our work we distinguish, as shown diagrammatically in Fig. 2, five main types of particle in brain homogenates: nuclei, mye-

lin fragments, mitochondria, microsomes and nerve ending particles. The term "microsome" here covers a variety of membrane fragments and small vesicles, derived from cell membranes, endoplasmic reticulum and possibly broken nerve endings. In addition, detached RNP particles (ribosomes), bundles of neurofilaments and spine apparatuses (described

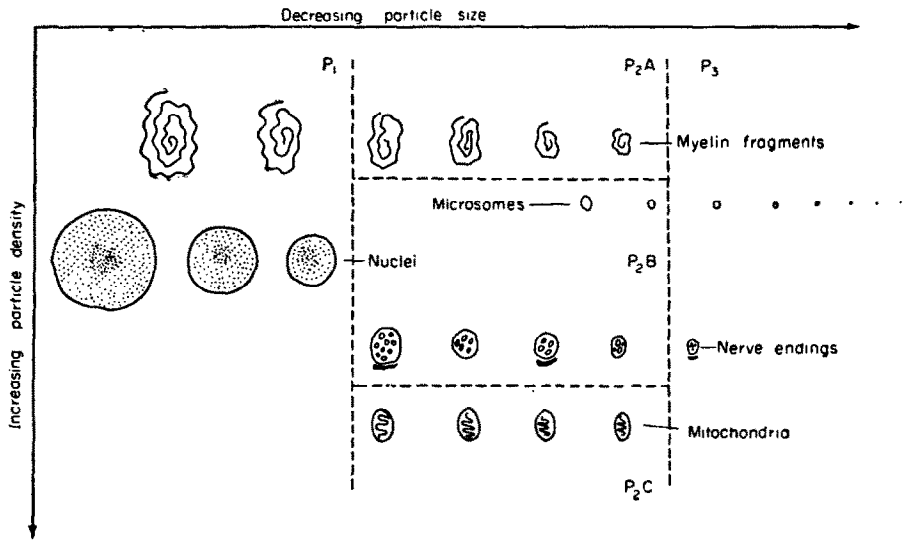


FIG. 2. The main particulate constituents of brain homogenates. Also observed: ribosomes, neurofilaments, spine apparatuses.

below) can also be identified. Many of these particles overlap in size and the separation of the initial homogenate into relatively homogeneous fractions necessitates a combination of differential centrifugation and density gradient separation. Thus, myelin fragments float on 0·8 M sucrose and can be separated from nuclei, nerve-ending particles or mitochondria of comparable size by density gradient separation. Nerve-ending particles float on 1·2 M sucrose and so can be separated from mitochondria. The so-called mitochondrial fraction from brain is thus not a homogeneous fraction and it will be necessary to re-evaluate the many metabolic studies carried out with this fraction.

THE NERVE-ENDING FRACTION

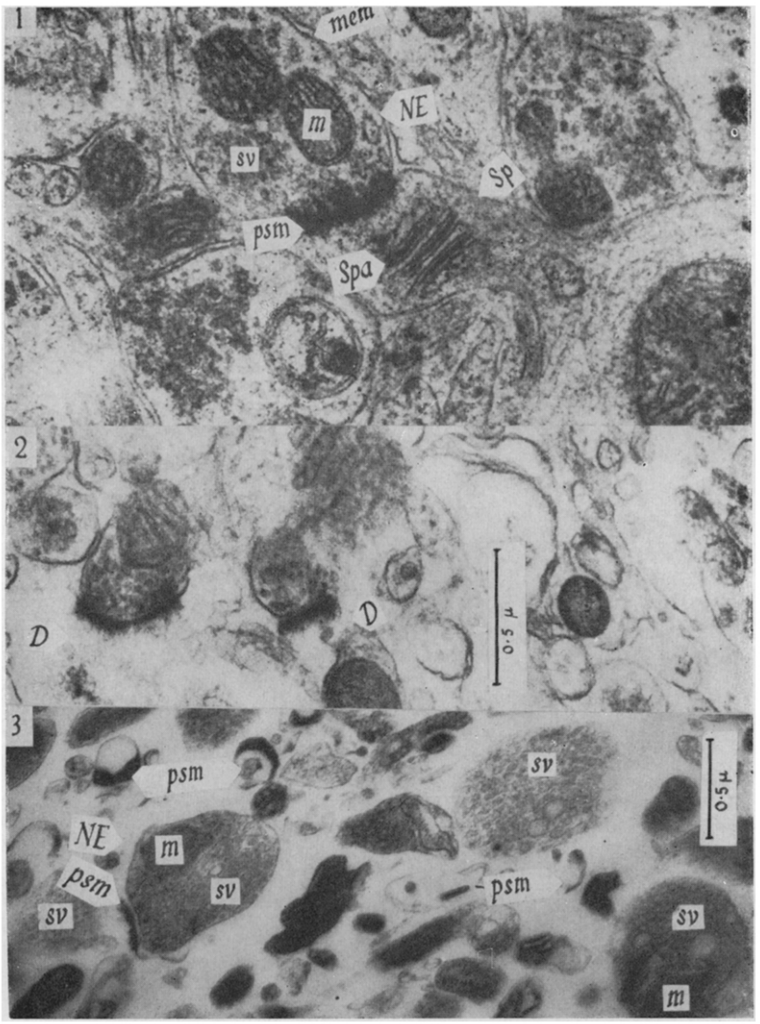
Most of our work has naturally centred on the nerve-ending fraction. In whole tissue sections, nerve endings (*NE*) are seen (Pl. 1, Fig. 1) as club-like structures surrounded by a thin membrane (*mem*) and containing synaptic vesicles (*sv*) and small mitochondria (*m*). The post-synaptic membrane (*psm*) is usually thickened in the region of synaptic contact, and this contact may be made directly with a cell body or

dendrite but is often with a specialized projection from the dendrite. called a dendrite spine (*Sp*). Inside the spine is seen a peculiar layered arrangement of flattened hollow vesicles and thick membranes of unknown function known as the spine apparatus (*Spa*)[10].

Examination of the torn edges of whole tissue sections (Pl. 1, Fig. 2) shows that the club-like nerve ending has considerable mechanical strength and readily tears away from the disintegrating dendrite (*D*) carrying its *psm* with it. This indicates that the presynaptic element is firmly "glued" to its *psm;* the glue does not readily stain with osmic acid but can sometimes be seen as a faintly staining band of material (Pl. 2, Fig. 6). We believe[2,3] that during homogenization a similar process occurs and that the nerve endings are "pinched-off" to give nerve-ending particles which retain the various structural features of the nerve ending (Pl. 1, Fig. 3). Occasionally a spine is torn away with the remains of the spine apparatus still inside (Pl. 2, Fig. 4); some of the mitochondrion-like bodies seen in the nerve-ending fraction may be detached spine apparatuses (Pl. 2, Fig. 5). A great variety of endings are seen, with and without attached *psm's*; in some, compound vesicles (*cv*) are visible, as in intact nerve endings (Pl. 2, Fig. 5). With the smooth walled Aldridge[11] homogenizer used in this work few broken nerve endings are seen though some membrane fragments, including detached *psm's,* are present (Pl. 1, Fig. 3).

When exposed to hypertonic sucrose, the endings shrink and may fuse into black masses (Pl. 2, Fig. 7) but the process is reversible on return to isotonic conditions if it has not gone too far. In hypotonic conditions, by contrast, the endings swell and become empty shells (Pl. 2, Fig. 8); simultaneously acetylcholine is released and destroyed, unless eserine is present. The particles are very sensitive to autolysis; after a brief exposure to temperatures above 0° they appear as empty shells with masses of broken vesicles between (Pl. 3, Fig. 9); acetylcholine is again liberated and in the absence of an anticholinesterase, destroyed. On osmication, the particles become extremely fragile and are readily broken up (Pl. 3, Fig. 10); the outer membrane is lost and the cytoplasm breaks up into particles of about the same size range as synaptic vesicles[8]. Osmication also causes release of acetylcholine.

Many studies have been made with different types of density gradient. These indicate that the efficiency of separation obtained with hypertonic sucrose is partly the result of a differential loss of water from the particles. With more complex gradients than we normally use[8], the smaller membranes present in the nerve ending fraction can be partially removed and the large nerve endings partially separated from the smaller. The distribution of acetylcholine suggests that it may be associated with the smaller endings; however, a sharp separation



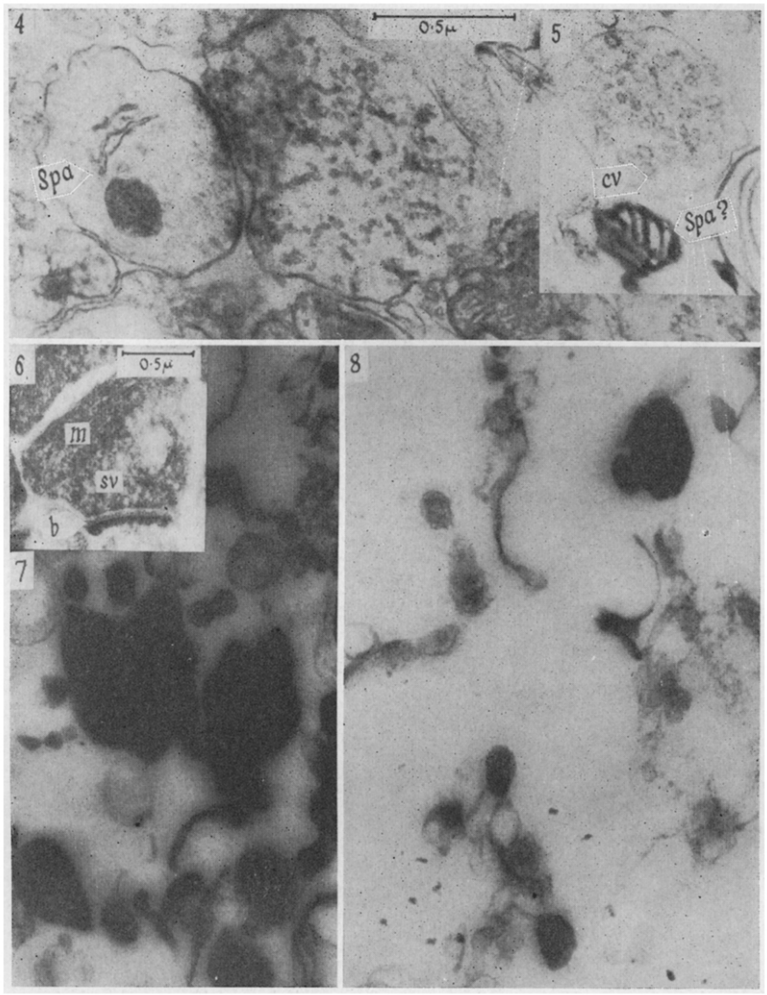
All plates are electron micrographs of thin sections of whole tissue or subcellular particles fixed and stained in osmium tetroxide and phosphotungstic acid and embedded in Araldite.

The electron micrographs of Pl. 1, Fig. 1 & 2 from ref. 10, Pl. 1, Fig. 3 and Pl. 2, , Fig 6 and 7 from ref. 3 and Pl. 3, Fig. 10 from ref. 8. Permission to publish is gratefully acknowledged.

Pl. 1, Fig. 1. Whole tissue section of rat cerebral cortex showing nerve ending (NE) containing mitochondria (*m*) and synaptic vesicles (*sv*) enclosed within a thin membrane (*mem*). The post-synaptic membrane (*psm*) is part of a dendrite spine (*Sp*) containing the spine apparatus (*Spa*). Scale as in Fig. 2.

Pl. 1, Fig. 2. Torn edge of whole tissue section similar to Fig. 1. Disintegrated dendrite at *D*.

Pl. 1, Fig. 3. Particles of acetylcholine-containing fraction isolated between 0·8 and 1·2 M sucrose (fraction B). Note mitochondria (*m*) and small vesicles (*sv*) enclosed by thin membrane (*mem*), to which a thick membrane (*psm*) is sometimes seen adhering, as in particle labelled *NE*. The whole complex closely resembles the nerve endings in Fig. 1.



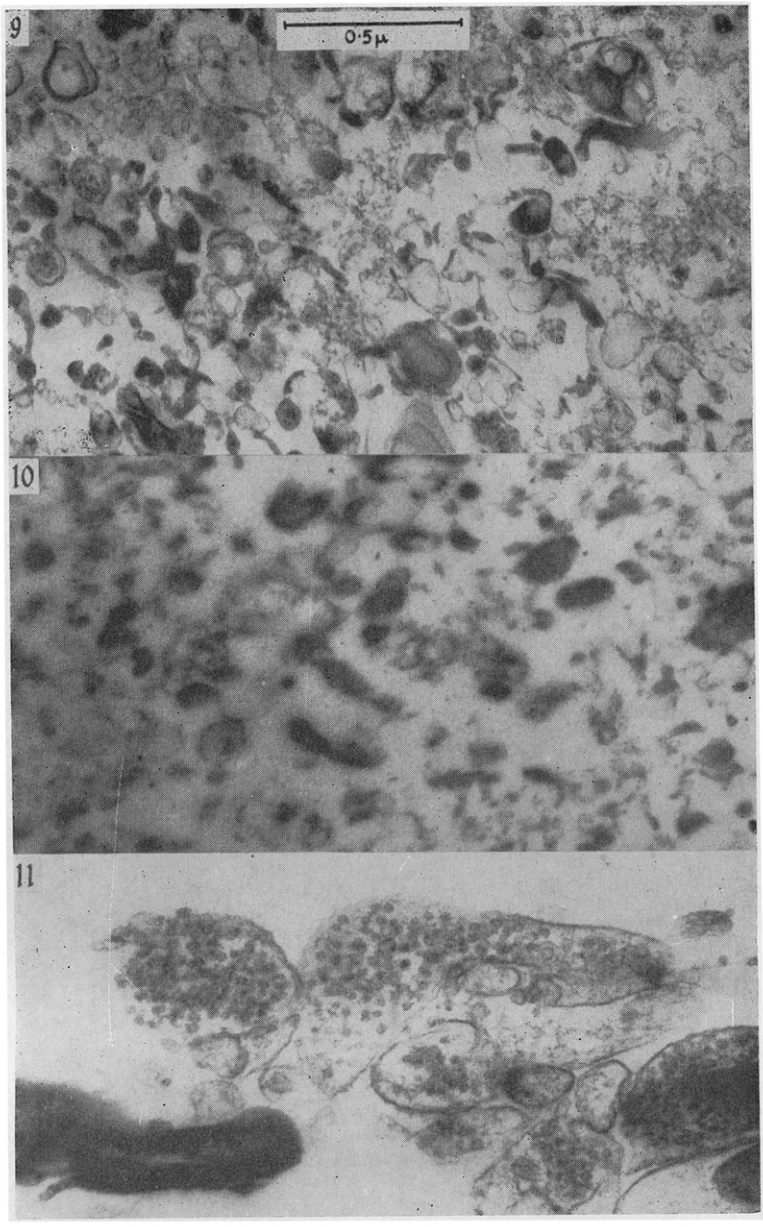
PL. 2, FIG. 4. Nerve ending particle (*NE*) with attached dendrite spine and remains of spine apparatus (*Spa*).

PL. 2, FIG. 5. Nerve ending particles showing compound vesicles (*cv*) and (?) detached spine apparatus (*Spa?*). Scale as in Fig. 4

PL. 2, FIG. 6. Nerve ending particle with faintly staining band (*b*) of material in synaptic cleft. Mitochondrion (*m*) and synaptic vesicles (*sv*) visible within nerve ending.

PL. 2, FIG. 7. Shrunken nerve-ending particles from hypertonic sucrose. Scale as in Fig. 4.

PL. 2, FIG. 8. Swollen and empty membranes of nerve-endings exposed to hypotonic sucrose (0·032 M). Scale as in Fig. 4.



PL. 3, FIG. 9. Effect of incubation at 37° on nerve-ending particles. Note empty shells and clumps of broken vesicles.

PL. 3, FIG. 10. Small particles obtained by mechanical disintegration of osmicated nerve-ending particles. Scale as in Fig. 9.

PL. 3, FIG. 11. Synaptic vesicles embedded in cytoplasm of torn nerve ending in whole tissue section of cerebral cortex of dog. Scale as in Fig. 9.

of nerve endings into morphologically and chemically distinct types has not been achieved[27].

Attempts to prepare intact monodispersed synaptic vesicles free from the enclosing membrane of the nerve ending particle have so far been unsuccessful. Until this has been done it cannot be established whether transmitters are actually localized within the synaptic vesicles or not. We believe that the vesicles are embedded at 0° in a stiff jelly-like cytoplasm. This may be inferred from Pl. 3, Fig. 11 which shows a broken nerve ending in a whole tissue section with the vesicles adhering in a mass.

THE SUBCELLULAR DISTRIBUTION OF PHARMACOLOGICALLY ACTIVE SUBSTANCES

Table I lists some of the substances we have found to be present mainly in the nerve ending fraction. Acetylcholine has already been mentioned. Hydroxytryptamine is similarly distributed. Dr. I. A. Michaelson[27] has recently checked my earlier results[8] using the spectrofluoro-

TABLE I

Substances found mainly in the nerve-ending fraction

Substance	Author
Acetylcholine (bound)	Hebb and Whittaker[1] Whittaker[8]
Hydroxytryptamine (bound)	Whittaker[8] Michaelson[27] Baker[12]
Noradrenaline	Chruściel[13]
Substance P	Cleugh
ATP	Nyman

photometric method and obtains excellent agreement. He has also confirmed that hydroxytryptamine more readily leaks out from the particles during preparation than acetylcholine. We are not sure yet whether this means that the mode of binding of hydroxytryptamine is different from that of acetylcholine or whether the particles containing it are more easily broken. Recently Miss J. Cleugh has been working on substance P. This exists almost entirely in the bound form in sucrose homogenates and is recovered mainly in the nerve-ending fraction.

The presence of hydroxytryptamine[8,12] and noradrenaline[13] in the nerve-ending fraction made us wonder whether adenosine triphosphate (ATP) was also present in this fraction. ATP has been implicated in the

binding of pressor amines to the much heavier storage granules found in platelets[14], adrenal medulla[15], duodenal mucosa[16] and sympathetic nerves[17], but not in brain. Dr. M. Nyman has carried out a very careful study of this point in my laboratory during the past few months using the fire-fly luminescence method. She finds, in confirmation of other workers, that there is a rapid fall in brain ATP 2–3 min after death to about 10% of the initial value. This relatively stable fraction of ATP also survives homogenization in sucrose. The results I am about to present apply to this fraction only, since there is no way of knowing which cellular compartment the labile 90% of ATP belongs to.

Most of the ATP was found to be particle-bound, however, a small but significant amount was present in the completely particle-free supernatant which had to be released with trichloroacetic acid in the same way as particle-bound ATP before it could be detected by the assay method. Probably this soluble bound ATP is protein-bound. All forms of ATP were found to be very unstable and the recoveries of 60-96% obtained in the fractionation experiments required good refrigeration and minimal delay in centrifugation.

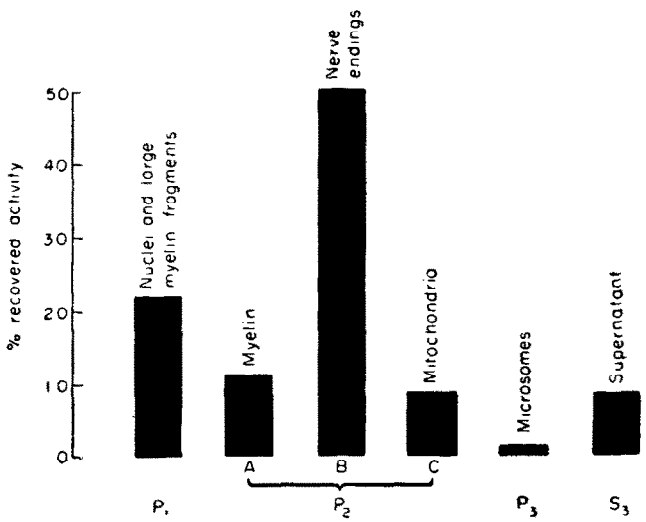


FIG. 3. Subcellular distribution of ATP in guinea-pig brain (average of 6 experiments). Content of homogenate, 188-240 (mean, 214) μg/g tissue; recovery 60%.

As shown in Fig. 3, most of the particulate ATP is recovered in the nerve ending fraction. However, the amount present in this fraction (about 210 n mole/g tissue) is greatly in excess of hydroxytryptamine (1 nmole/g tissue); thus the ratio of ATP to hydroxytryptamine is much greater than the 4 : 1 ratio of ATP to pressor amine found in adrenal medullary granules and elsewhere. Further (Fig. 4) there is no correla-

tion between the distribution of ATP and pressor amines in different areas of the guinea pig and dog brain, and reserpine, while greatly depressing the amine content of the homogenate and the nerve-ending fraction, does not alter its ATP content, in contrast to results with adrenal medulla[18,19].

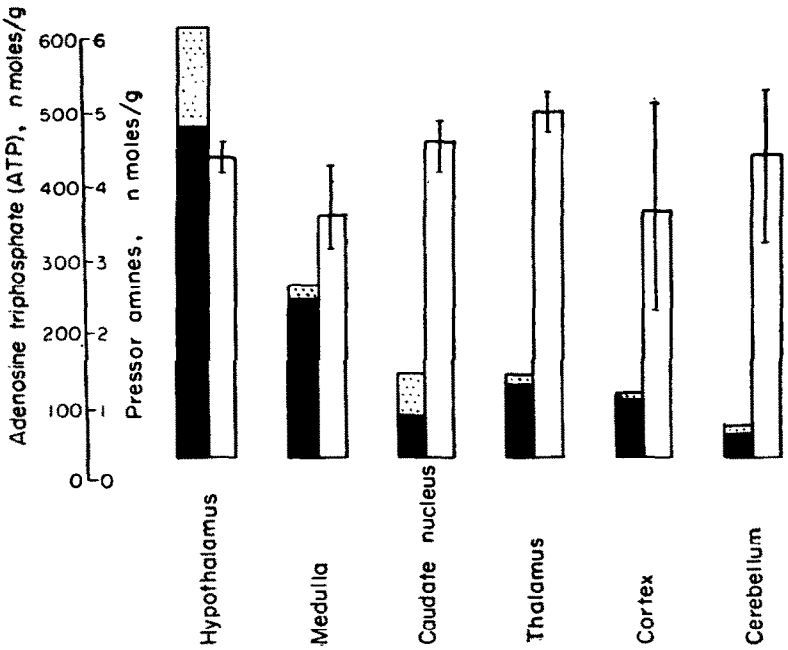


FIG. 4. Topographical distribution of ATP (white blocks) in guinea-pig brain. The bars on the white blocks give the range of values observed. Similar results were obtained with dog brain. For comparison, the distribution of noradrenaline[23] (black blocks) and hydroxytryptamine[21] (dotted blocks) are also shown (additively).

ATP is not present exclusively in the nerve ending fraction. Its level in white matter is as high as in grey and it is found in appreciable amounts in the nuclear, myelin and mitochondrial fractions.

The results, therefore, are inconclusive regarding a specific pressor amine binding function for ATP in brain. If ATP does bind amines stoichiometrically in this tissue, the levels actually found are high enough to obscure it.

Another possible role for ATP is as a transmitter substance[20]. Dr. Nyman has found that ATPase is present in the nerve-ending and other fractions in high concentration: ATP rapidly disappears if the fraction is kept above 0° or disrupted in other ways. Thus ATP conforms to the biochemical criteria which have been laid down by Paton[21], Koelle[22] and others for transmitter substances. However, microelectrode exper-

iments in the mammalian cortex and spinal card have not as yet disclosed any transmitter-like action of ATP.

To sum up, it is now possible to isolate nerve endings in good yield from the central nervous system. The fraction containing these endings is rich in pharmacologically active substances and ATP. It provides a preparation which may well prove useful for the discovery of new transmitters and other specific components, for investigating the mechanism of transmitter release and for studying the mode of action of drugs and toxins on nerve endings.

Addendum. Electron micrographs of brain mitochondrial fractions have been published by Petrushka and Giuditta[24] and by de Robertis, de Iraldi, Rodriguez and Gomez[25], who have drawn attention to the presence of nerve ending particles in this fraction. The "*x particles*" of Petrushka and Giuditta may be altered nerve-ending particles. The microsomal fraction of brain contains vesicles of such a wide size-range that the identification of any of them with synaptic vesicles[26] on the basis of appearance alone is conjectural.

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