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THE SUBCELLULAR LOCALIZATION OF DOPAMINE AND ACETYLCHOLINE IN THE DOG CAUDATE NUCLEUS

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The distribution of dopamine (3,4-dihydroxyphenylethylamine) between the subcellular fractions from homogenates of dog caudate nucleus was compared with the distributions of acetylcholine, 5-hydroxytryptamine and lactate dehydrogenase activity. The distributions of noradrenaline and 5-hydroxytryptamine between the subcellular fractions from homogenates of dog hypothalamus were also determined. Most of the dopamine, in contrast to acetylcholine, occurred in the soluble supernatant fraction; the remainder was associated with the fractions rich in pinched-off nerve ending particles, but localization in any one fraction was not as sharp as that of acetylcholine. Evidence is presented that suggests that the dopamine occurs in a free or easily released form throughout cell cytoplasm.

In a study of the subcellular distribution of dopamine (3,4-dihydroxyphenylethylamine) in the brain stem of the rabbit, Weil-Malherbe & Bone (1957b, 1959) found that after high speed centrifugation the amine was equally distributed between the particulate material and the supernatant fractions and that approximately 40% of the total dopamine in the brain stem was associated with that fraction of the particulate material which contained the mitochondria. More recently, Bertler, Hillarp & Rosengren (1960), using caudate nuclei from cats and rabbits, and Weil-Malherbe, Posner & Bowles (1961), using rabbit brain, have found that after subcellular fractionation only 25% of the dopamine was recovered in the particulate material so that the majority of the dopamine seemed to occur in the "cytoplasmic sap." A cytoplasmic localization for dopamine was also found in bovine splenic nerves by Schümann (1958).

Originally, it was thought that the amines acetylcholine and 5-hydroxytryptamine found in the central nervous system were associated with subcellular structures similar to or identical with mitochondria (Bodian, 1942; Brodkin & Elliott, 1953; Walaszek & Abood, 1957). More recent work using techniques of differential and density gradient centrifugation have demonstrated that acetylcholine (Hebb & Whittaker, 1958; Whittaker, 1959), 5-hydroxytryptamine (Whittaker, 1959; Michaelson & Whittaker, 1963) and noradrenaline (Chruściel, 1960; Michaelson, unpublished) are associated with a distinct subfraction of brain homogenates which

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has been shown by electron microscopy to consist mainly of pinched-off nerve ending particles (Gray & Whittaker, 1962); for these particles the name "synaptosome" has recently been adopted (Whittaker, Michaelson & Kirkland, 1963b).

The present investigation was undertaken to examine the subcellular localization of dopamine in the dog caudate nucleus and to obtain information as to the nature of the subcellular structure with which dopamine is associated. This region was selected because of its known high content of dopamine. A comparison was made with the subcellular distributions of acetylcholine and 5-hydroxytryptamine, also present in this tissue in relatively high concentrations. Lactate dehydrogenase was used as a cytoplasmic marker and comparisons were made with the distribution of noradrenaline in the hypothalamus of the dog.

METHODS

Preparation of fractions

Mongrel dogs anaesthetized with ether, chloroform or chloralose were bled from a carotid artery. The brain was removed and the caudate nuclei (and hypothalamus) were rapidly excised and weighed; the tissues were then homogenized in 0.32 M-sucrose solution using a glass and Perspex homogenizer (Aldridge, Emery & Street, 1960) to give a homogenate containing 10% (w/v) of tissue. A sample of homogenate was kept for analysis.

In initial experiments, this homogenate was separated into total particulate material (*P*) and high-speed supernatant fluid (*S*), by centrifugation at 100,000 *g* for 1 hr in the AH 40 head of a Spinco Model L preparative ultracentrifuge. In other experiments the homogenate was separated initially into three primary particulate fractions essentially as described by Whittaker (1959). The first particulate fraction (P_1) was obtained by centrifuging for 11 min at 1,000 *g* in a Servall bench centrifuge. This P_1 fraction was washed twice with 5 ml. of 0.32 M-sucrose solution and the washings were added to the supernatant fluid from the P_1 preparation. The next particulate fraction (P_2) was separated from the supernatant fluid of the P_1 preparation by centrifuging at 17,300 *g* for 1 hr in a Servall RC-2 refrigerated automatic centrifuge. A further small particulate fraction (P_3) was separated from the supernatant fluid of the P_2 preparation by centrifugation at 100,000 *g* for 1 hr in a Spinco Model L preparative ultracentrifuge, leaving a final high-speed supernatant fraction (S_3) containing the soluble constituents of the cell cytoplasm diluted with sucrose solution.

Subfractionation of the P_2 fraction by equilibrium density gradient centrifugation was carried out as described by Whittaker (1959). The P_2 pellet was resuspended in 0.32 M-sucrose solution so that 1 ml. of suspension corresponded to 500 mg of original tissue. The suspension (1 ml./tube) was layered on top of a discontinuous density gradient, prepared 1 hr before use, consisting of 2 ml. of 0.8 M-sucrose solution layered over an equal volume of 1.2 M-sucrose solution, and centrifuged at 100,000 *g* for 1 hr in the SW 39 swing-out bucket head of the Spinco Model L preparative ultracentrifuge. Three distinct subfractions were obtained; the first (*A*) consisted of particles less dense than 0.8 M-sucrose solution, the second (*B*) of particles of density intermediate between 0.8 M- and 1.2 M-sucrose solution, and the third (C) of particles denser than 1.2 M-sucrose solution. All operations were carried out at 0° to 4° C.

Analysis of fractions

Each primary particulate fraction was resuspended in 1 ml. of 0.32 M-sucrose solution. After removal of small samples for electron microscopy, acetylcholine assay and estimation of lactate dehydrogenase activity, each fraction was diluted to 2 ml. with 0.01 N-hydrochloric acid and the protein precipitated with 2.0 ml. of 0.8 N-perchloric acid. The S_3 fraction was adjusted to 0.4 N with respect to perchloric acid with 12 N-perchloric acid. The samples were then centrifuged and the acid supernatant fractions separated from their packed solids. The

perchloric acid was removed by neutralizing each of the acid supernatant fractions with 3 N-potassium carbonate solution to *p*H 4.0 and centrifuging in the cold.

Separation of the catechol amines by ion exchange column chromatography was carried out as described by Bertler, Carlsson & Rosengren (1958): the neutralized perchloric acid extracts were passed through a column of Dowex 50 X-8 (200–400 mesh, 3×25 mm) equilibrated with 1.0 N-sodium acetate buffer (*p*H 6). For extracts of caudate nuclei the column was washed with 6 ml. of 0.4 N-hydrochloric acid to remove any dihydroxyphenylalanine or noradrenaline, and the dopamine eluted with 8 ml. of 2 N-hydrochloric acid. For hypothalamic extracts the column was washed with 4 ml. of water and the noradrenaline eluted with 8 ml. of 0.4 N-hydrochloric acid.

Estimation of the dopamine was carried out fluorimetrically after condensation with ethylene diamine, as described by Weil-Malherbe & Bone (1952, 1957a). The fluorescence derived from the dopamine in the final eluate was compared in each case with the fluorescence produced by a standard amount of dopamine added to a fraction of the same eluate to ensure the correct development of the fluorescence. In control experiments the recovery of dopamine added to tissue samples was usually greater than 70%.

Because of the small amounts of tissue available, acetylcholine was assayed on the leech muscle micro-preparation described by Szerb (1961) using physostigmine (1 mg/100 ml.) as the anticholinesterase in the bathing fluid. The samples to be assayed were heated to 100° C at *p*H 4 to release bound acetylcholine and diluted with leech Locke solution before assay. In experiments in which the homogenization was carried out in the presence of physostigmine it was found that physostigmine was carried through the extraction procedure for dopamine and formed a highly fluorescent compound with ethylene diamine. Thus it was not possible to estimate the dopamine in these experiments.

The 5-OR indolyl compounds were measured as described by Ashcroft & Sharman (1962). Whenever possible, the compounds were separated into basic (presumably 5-hydroxytryptamine) and acidic (presumably 5-hydroxyindolylacetic acid) fractions. In the case of the primary particulate fractions (P_1,P_2,P_3), which were also assayed for dopamine, only a small quantity of the protein-free extract was available; this was analysed for total 5-OR indolyl compounds.

Estimation of noradrenaline was carried out fluorimetrically as described by Sharman, Vanov & Vogt (1963). Lactate dehydrogenase activity was measured spectrophotometrically as described by Johnson (1960) and is expressed as the change in extinction of the reaction mixture at 340 mμ (ΔE_{340}) with time.

The concentration of amines (or enzyme) in the tissue fractions was expressed as mμmoles (or ΔE_{340}/min)/volume of fraction derived from 1 g of fresh tissue. The distribution of constituents between fractions was expressed as the percentage of the total recovered constituent found in each fraction. The results of similar experiments are expressed as the mean value and, with more than four observations, the standard deviation from the mean was calculated.

Electron microscopy

This was carried out by the negative staining method as described by Horne & Whittaker (1962). Preparations were fixed at 0° C by the addition of equal volumes of 10% (w/v) formaldehyde in 0.32 M-sucrose solution previously neutralized to *p*H 7.2 with 0.33 N-sodium hydroxide solution. The mixture was diluted with six times its volume of ice-cold 1% (w/v) aqueous phosphotungstic acid which had been brought to *p*H 7.2 with 2 N-sodium hydroxide and was then transferred to grids with a micropipette. Most of the droplet was removed from the grid with filter paper. A thin film of suspension remained which dried rapidly leaving particles embedded in solid sodium phosphotungstate. In this method particles are seen as a whole and not in section.

RESULTS

Distribution between particulate-bound and soluble material

A number of experiments were carried out in which an homogenate of dog caudate nuclei was separated into total particulate material (*P*) and a high-speed supernatant fluid (*S*) which were then analysed. The results for dopamine, 5-OR indolyl compounds, acetylcholine and lactate dehydrogenase activity are summarized in Table 1. It will be seen that dopamine resembles lactate dehydrogenase and

TABLE 1

DISTRIBUTION OF CONSTITUENTS OF DOG CAUDATE NUCLEUS HOMOGENATES BETWEEN THE TOTAL PARTICULATE MATERIAL (*P*) AND THE HIGH SPEED SUPERNATANT (*S*) FRACTION

The homogenate content is expressed in mμmoles/g of original tissue ; lactate dehydrogenase activity is expressed as ΔE_{340}/min/g of original tissue. *These fractionation experiments were carried out using sucrose solution containing physostigmine sulphate (32·2 mg/l.) to preserve any free acetylcholine

	No. of experiments	Mean homogenate content	Distribution (% of recovered activity)	
			P	*S*
Dopamine	3	40	37	63
Lactate dehydrogenase activity	3	85	34	66
Acetylcholine*	2	17	81	19
Basic 5-OR indolyl compounds (5-hydroxytryptamine)	3	1·6	70	30
Acidic 5-OR indolyl compounds (5-hydroxyindolylacetic acid)	4	1·7	26	74

5-hydroxyindolylacetic acid in being recovered predominantly in fraction *S* representing the soluble constituents of the cell sap diluted with suspension medium. This is in contrast to the localization of acetylcholine and 5-hydroxytryptamine, which are recovered mainly in the total particulate fraction. It was also found that homovanillic acid, the major acid metabolite of dopamine in the dog caudate nucleus (Sharman, 1963), occurred predominantly in the high-speed supernatant fraction.

Distribution between particulate fractions

The results of analyses of the primary particulate fractions separated from dog caudate nucleus and dog hypothalamus are shown in Table 2. Since most of the acidic 5-OR compounds present is found in the high speed supernatant fluid (Table 1), the analyses of the total 5-OR compounds given in this section will represent predominantly the basic 5-OR compounds, that is 5-hydroxytryptamine.

It will be seen from Table 2A that the distribution of acetylcholine among the primary particulate fractions from dog caudate nucleus differed appreciably from that of dopamine, the acetylcholine being much more sharply localized in the P_2 fraction.

In the experiments analysing the subcellular distribution of noradrenaline and 5-OR indolyl compounds in dog hypothalamus it was found that both amines were principally associated with the P_2 fraction (Table 2B). It is of interest to note that the ratio of the total particulate-bound material to soluble material for dopamine

TABLE 2

PERCENTAGE DISTRIBUTION OF DOPAMINE, ACETYLCHOLINE, 5-OR INDOLYL COMPOUNDS, NORADRENALINE AND LACTATE DEHYDROGENASE ACTIVITY BETWEEN THE PRIMARY FRACTIONS FROM DOG CAUDATE NUCLEUS AND HYPOTHALAMUS HOMOGENATES

The homogenate content is expressed in m.moles/g of original tissue ; lactate dehydrogenase activity is expressed in ΔE_{340}/min/g of original tissue. Values are means and standard deviations.

	No. of experiments	Homogenate content	Ratio of total particulate bound material to material in supernatant ($P_1+P_2+P_3 : S_3$)	Percentage of total particulate bound material recovered in			Percentage recovery of homogenate content in combined fractions
				P_1	P_2	P_3	
A. *Dog caudate nucleus*							
Dopamine	8	44± 7	41 : 59	33±11	54±11	13± 6	80±17
Total 5-OR indolyl compounds	7	—	—	27± 5	53±11	20±10	—
Acetylcholine	5	19± 3	95 : 5	9± 2	86± 6	5± 3	88± 9
Lactate dehydrogenase activity	2	100	51 : 49	27	67	6	89
Acidic 5-OR indolyl compounds	1	—	—	23	70	7	—
B. *Dog hypothalamus*							
Noradrenaline	3	7	68 : 32	10	64	26	90
Total 5-OR indolyl compounds	3	—	—	16	67	17	—

in caudate nuclei is the inverse of that for noradrenaline in the hypothalamus. In the particulate fractions from both tissues the 5-hydroxytryptamine distribution parallels the distribution of the corresponding catechol amine.

Subfractionation of the P_2 fraction by density gradient centrifugation led to a further distinction between dopamine and acetylcholine (Table 3). It was found that the acetylcholine was again more sharply localized, in this case in the *B* sub-fraction.

Electron microscopy

Electron microscopic examination of the various fractions from the caudate nucleus showed that these were similar in composition to, but more heterogeneous than, the whole guinea-pig brain preparations previously examined by Gray & Whittaker (1962) and Horne & Whittaker (1962). Besides nuclei, myelin and fragments of incompletely homogenized tissue, the P_1 fraction contained free mitochondria, synaptosomes and many small membrane fragments. Some of the synaptosomes were very large and so would have been expected to sediment in this fraction. This has been observed with the large synaptosomes formed from mossy fibre endings in homogenates of cerebellar cortex (Whittaker, 1962).

The P_2 fraction contained, as with whole guinea-pig brain preparations, myelin fragments, mitochondria, synaptosomes and many small membrane fragments mostly oval or circular in outline, down to microsomal dimensions. The synaptosomes varied greatly in size. Subfraction *A* of P_2 consisted mainly of myelin and small membrane fragments; however, synaptosomes were also present in considerable numbers. Subfraction *B* was a relatively homogeneous fraction

TABLE 3

PERCENTAGE DISTRIBUTION OF DOPAMINE, ACETYLCHOLINE AND LACTATE DEHYDROGENASE ACTIVITY AMONG SUBFRACTIONS OF THE P_2 PRIMARY FRACTION

Values are means with standard deviations.

	Dopamine	Acetylcholine	Lactate dehydrogenase	Particles identified in the electron microscope
No. of experiments	5	4	2	
% in fraction *A*	45± 7	19	33	Myelin, small membrane fragments, many small synaptosomes
% in fraction *B*	47±14	79	56	Mainly synaptosomes, some shrunken
% in fraction *C*	8± 7	2	11	Mainly mitochondria, a few shrunken synaptosomes
Content of P_2/g of original tissue	7·8±1·9 mμmoles	15 mμmoles	ΔE^{30}_{340}/min	
% recovery of P_2 content in combined fractions	70±19	81	95	

consisting mainly of synaptosomes, many large (2 μ or more in diameter), often with considerable lengths of axons attached, others shrunken and bizarre in shape and densely packed with vesicles. This shrinking was caused by exposure to hypertonic sucrose solution. Noteworthy was the almost complete absence of free mitochondria in *A* and *B*, though small mitochondria were frequently present inside synaptosomes. By contrast, the *C* subfraction consisted almost entirely of mitochondria, often morphologically abnormal as previously observed in positively stained thin sections of the corresponding whole guinea-pig brain fraction. Occasional particles in this fraction poorly penetrated by phosphotungstate and often possessing long axon-like " tails " were tentatively identified as synaptosomes whose cytoplasm had fused to a dense mass as a result of dehydration in hypertonic sucrose solutions (the " black bodies " in the positively stained preparations of Gray & Whittaker, 1962).

Fraction P_3 consisted mainly of vesicular membrane fragments (microsomes) of varying size from about 0.02 to 0.07 μ ; there were also a few small synaptosomes and mitochondria. Two fractions obtained by subfractionating P_3 on a density gradient did not differ except that the smaller particles were more plentiful in the light fraction and the synaptosomes and the mitochondria were recovered with the larger membrane fragments in the denser fraction.

DISCUSSION

Dopamine

The topographical distribution of dopamine among the various parts of the brain (Bertler & Rosengren, 1959) does not correspond to that of noradrenaline (Vogt, 1954). It is possible that dopamine ocurs in the brain other than as a precursor of noradrenaline. Information about the subcellular distribution of dopamine in brain tissue might help in elucidating its function.

In the present study the subcellular distribution of dopamine in the caudate nucleus of the dog between total particulate and high-speed supernatant fractions was somewhat similar to that observed by Weil-Malherbe & Bone (1959) using rabbit brain stem, but it differed from the distributions of acetylcholine and

5-hydroxytryptamine in the caudate nuclei fractions, and also from that of noradrenaline in dog hypothalamus homogenates. The distribution of these latter amines in dog tissue corresponds well, however, with the distributions of acetylcholine (Whittaker, 1959), 5-hydroxytryptamine (Michaelson & Whittaker, 1963) and noradrenaline (Michaelson, unpublished observations) in guinea-pig whole brain tissue. The dopamine distribution between total particulate and supernatant fractions was closely paralleled by the distribution of lactate dehydrogenase activity, and was also matched by the distribution of 5-hydroxyindolylacetic acid. This difference in distribution between dopamine and the other amines must reflect a difference in the subcellular localization or metabolism of dopamine when compared with the other amines.

The percentage distribution of dopamine in the three primary particulate fractions from caudate nuclei is similar to that found for 5-hydroxytryptamine in this tissue, both being less sharply localized than acetylcholine. Dopamine from caudate nucleus homogenates, unlike 5-hydroxytryptamine in whole guinea-pig brain (Michaelson & Whittaker, 1963), is not readily lost from the particulate material during the prolonged manipulation in sucrose media involved in the fractionation procedure, as shown by the similarity in the ratios P/S (Table 1) and $(P_1+P_2+P_3)/S_3$ (Table 2).

Upon subfractionation of the "crude mitochondrial" (P_2) fraction by density gradient centrifugation dopamine was not as closely associated with any one subfraction as was observed for acetylcholine. In the present study dopamine was equally distributed between subfractions *A* and *B* whereas acetylcholine was preferentially localized in *B*. It would appear then that the structures containing dopamine in the dog caudate nucleus have a larger range of densities than those associated with acetylcholine. Electron microscopic examination of the subfractions from the dog caudate nucleus revealed that the density gradient separation failed to resolve the P_2 fraction into such relatively homogeneous subfractions as was observed for guinea-pig brain (Gray & Whittaker, 1962). Both the *A* and *B* subfractions of P_2 contained many pinched-off nerve endings. It would appear that the synaptosomes in the dog caudate nucleus have a larger range of sizes than the synaptosomes derived from guinea-pig brain tissue.

Lactate dehydrogenase activity and dopamine had similar relative distributions. Lactate dehydrogenase is thought to be associated with the cell cytoplasm (Johnson, 1960; Johnson & Whittaker, 1963). This association suggests that dopamine is also distributed through the cell cytoplasm and that the dopamine associated with the particulate material is trapped in the cytoplasm of the pinched-off nerve ending particles. Additional evidence for a cytoplasmic localization of dopamine came from two preliminary experiments in which the particulate fractions containing dopamine were subjected to water-treatment and density gradient separation (Whittaker *et al.*, 1963a, b). After water-treatment 80% of the particulate-bound dopamine was recovered in the supernatant fraction. It appears that the dopamine is contained in a loosely bound or free form in a subcellular particle that can be disrupted by water-treatment. In contrast, acetylcholine was still associated with particulate material after water-treatment.

Acetylcholine

The homogenates of caudate nuclei contained a relatively high concentration of acetylcholine. Even in the presence of physostigmine, which preserves any free acetylcholine initially present, there was very little acetylcholine in the high-speed supernatant fluid. Thus nearly all of the acetylcholine was associated with particulate material. On fractionation, this particle-bound acetylcholine was localized in the P_2 fraction and the *B* fraction derived from it, in spite of the presence of considerable numbers of synaptosomes in the P_1 and *A* fractions. It is not known for certain whether the free acetylcholine in brain tissue homogenates represents acetylcholine released from cholinergic nerve endings which have been disrupted during homogenization or acetylcholine originally present in other parts of the cholinergic neurone. The latter appears probable, since Hebb & Whittaker (unpublished) found that the acetylcholine in homogenates of ventral spinal columns, a region in which cholinergic neurones are represented mainly by the cell bodies of motor neurones and in which the only known cholinergic endings are those of the recurrent collateral fibres to Renshaw cells, was mainly in the free form. If this interpretation is correct, the results with caudate nuclei strongly imply that cholinergic neurones are here represented almost exclusively by endings which break off on homogenization to form a rather uniform population of synaptosomes containing acetylcholine. This assumption is supported by the recent work of Shute & Lewis (1963) who demonstrated histochemically in the rat that there is a dense cholinergic neuropil in the caudate nucleus and putamen contributed by the striatal radiation from the ventral midbrain tegmentum. Comparisons between the morphology of the synaptosomes of fraction *B* and the cholinergic neuropil are clearly needed.

It may be concluded from these experiments that the subcellular particle with which part of the dopamine is associated is similar to but distinguishable from that with which acetylcholine is associated. It was not possible to separate particles containing dopamine and 5-hydroxytryptamine. However, the distribution ratios of the latter two amines between the particulate and high-speed supernatant fractions are quite different. A possible explanation is that both these amines are in the same cell but that 5-hydroxytryptamine is confined to the synaptosomes and that dopamine is distributed throughout the cell. It cannot be excluded, however, that dopamine and 5-hydroxytryptamine are in different cells.

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REFERENCES

ALDRIDGE, W. N., EMERY, R. C. & STREET, B. W. (1960). A tissue homogenizer. *Biochem. J.*, **77**, 326–327.

ASHCROFT, G. W. & SHARMAN, D. F. (1962). Drug-induced changes in the concentration of 5-OR indolyl compounds in cerebrospinal fluid and caudate nucleus. *Brit. J. Pharmacol.*, **19**, 153–160.

BERTLER, Å., CARLSSON, A. & ROSENGREN, E. (1958). A method for the fluorimetric determination of adrenaline and noradrenaline. *Acta physiol. scand.*, **44**, 273–292.

BERTLER, Å., HILLARP, N.-A. & ROSENGREN, E. (1960). "Bound and "free" catecholamines in the brain. *Acta physiol. scand.*, **50**, 113–118.

BERTLER, Å. & ROSENGREN, E. (1959). Occurrence and distribution of catecholamines in brain. *Acta physiol. scand.*, **47**, 350–361.

BODIAN, D. (1942). Cytological aspects of synaptic function. *Physiol. Rev.*, **22**, 146–169.

BRODKIN, E. & ELLIOTT, K. A. C. (1953). Binding of acetylcholine. *Amer. J. Physiol.*, **173**, 437–442.

CHRUSCIEL, T. J. (1960). Observations on the localization of noradrenaline in homogenates of dog's hypothalamus. In *Ciba Foundation Symposium on Adrenergic Mechanisms*, ed. VANE, J. R., WOLSTENHOLME, G. E. W. & O'CONNOR, M. p. 539. London: Churchill.

GRAY, E. G. & WHITTAKER, V. P. (1962). The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat. (Lond.)*, **96**, 79–88.

HEBB, C. O. & WHITTAKER, V. P. (1958). Intracellular distribution of acetylcholine and choline acetylase. *J. Physiol. (Lond.)*, **142**, 187–196.

HORNE, R. W. & WHITTAKER, V. P. (1962). The use of the negative staining method for the electron-microscopic study of subcellular particles from animal tissue. *Z. Zellforsch*, **58**, 1–16.

JOHNSON, M. K. (1960). The intracellular distribution of glycolytic and other enzymes in rat brain homogenates and mitochondrial preparations. *Biochem. J.*, **77**, 610–618.

JOHNSON, M. K. & WHITTAKER, V. P. (1963). Lactate dehydrogenase as cytoplasmic marker in brain. *Biochem. J.*, **88**, 404–409

MICHAELSON, I. A. & WHITTAKER, V. P. (1963). The subcellular localization of 5-hydroxytryptamine in guinea-pig brain. *Biochem. Pharmacol.*, **12**, 203–211.

SCHÜMANN, H. J. (1958). Über die Verteilung von Noradrenalin und Hydroxytyramine in sympathischen Nerven (Milznerven). *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmakol.*, **234**, 17–25.

SHARMAN, D. F. (1963). A fluorimetric method for the estimation of 4-hydroxy-3-methoxyphenyl acetic acid (homovanillic acid) and its identification in brain tissue. *Brit. J. Pharmacol.*, **20**, 204–213.

SHARMAN, D. F., VANOV, S. & VOGT, M. (1962). Noradrenaline content in the heart and spleen of the mouse under normal conditions and after administration of some drugs. *Brit. J. Pharmacol.* **19**, 527–533.

SHUTE, C. C. D. & LEWIS, P. R. (1963). The cholinergic corticopetal radiations of the forebrain. *J. Anat.(Lond.)*., **97**, 476–477.

SZERB, J. C. (1961). The estimation of acetylcholine, using leech muscle in a micro bath. *J. Physiol. (Lond.)*, **158**, 8*P*.

VOGT, M. (1954). The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs. *J. Physiol. (Lond.)*, **123**, 451–481.

WALASZEK, E. J. & ABOOD, L. J. (1957). Fixation of 5-hydroxytryptamine by brain mitochondria. *Fed. Proc.*, **16**, 113.

WEIL-MALHERBE, H. & BONE, A. D. (1952). The chemical estimation of adrenaline-like substances in blood. *Biochem. J.*, **51**, 311–318.

WEIL-MALHERBE, H. & BONE, A. D. (1957a). Intracellular distribution of catecholamines in the brain. *Nature (Lond.)*, **180**, 1050–1051.

WEIL-MALHERBE, H. & BONE, A. D. (1957b). The estimation of catecholamines in urine by a chemical method. *J. clin. Path.*, **10**, 138–147.

WEIL-MALHERBE, H. & BONE, A. D. (1959). The effect of reserpine on the intracellular distribution of catecholamines in the brain stem of the rabbit. *J. Neurochem.*, **4**, 251–263.

WEIL-MALHERBE, H., POSNER, H. S. & BOWLES, G. R. (1961). Changes in the concentration and intracellular distribution of brain catecholamines. *J. Pharmacol. exp. Ther.*, **132**, 278–286.

WHITTAKER, V. P. (1959). The isolation and characterization of acetylcholine containing particles from brain. *Biochem. J.*, **72**, 694–706.

WHITTAKER, V. P. (1962). The separation of subcellular structures from brain tissue. In *Biochem. Soc. Symp. No.* **23**., *Methods of Separation of Subcellular Components*, ed. GRANT, J. K., p. 109. Cambridge University Press.

WHITTAKER, V. P., MICHAELSON, I. A. & KIRKLAND, R. J. (1963a). The separation of synaptic vesicles from disrupted nerve ending particles. *Biochem. Pharmacol.*, **12**, 300–302.

WHITTAKER, V. P., MICHAELSON, I. A. & KIRKLAND, R. J. (1963b). The separation of synaptic vesicles from nerve ending particles (synaptosomes). *Biochem. J.*, in the press.