The Separation of Synaptic Vesicles from Nerve-Ending Particles ('Synaptosomes')

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When brain tissue is homogenized in media iso-osmotic to plasma, the club-like presynaptic nerve endings resist disruption and are snapped or torn off from their attachments to form discrete particles (nerve-ending particles) in which all the main structural features of the nerve ending are preserved. For these particles we propose the name 'synaptosomes' in order to emphasize their relative homogeneity and their resemblance in physical properties to other subcellular organelles. They can be separated as a distinct fraction by differential and density-gradient centrifuging (Gray & Whittaker, 1960, 1962; Whittaker, 1960). This fraction contains most of the particle-bound acetylcholine (Hebb & Whittaker, 1958; Whittaker, 1959), choline acetyltransferase (choline acetylase) (Hebb & Whittaker, 1958), hydroxytryptamine (Whittaker, 1959; Michaelson & Whittaker, 1962, 1963) and noradrenaline (Chruściel, 1960) of the tissue. Since acetylcholine is now well established, by all the classical criteria, as a central as well as a peripheral transmitter (for a review see Gaddum, 1961), it seems reasonable to conclude that the particle-bound acetylcholine and choline acetyltransferase of the fraction represent acetylcholine and enzyme localized within synaptosomes derived from cholinergic neurones. Similarly, the 5-hydroxytryptamine and noradrenaline in this fraction are probably due to the presence of synaptosomes derived from neurones containing these amines. This view has been strengthened by the findings of Carlsson, Falck & Hillarp (1962), who have obtained histochemical evidence for the neuronal localization of these amines in the brain.

The nerve endings and the synaptosomes derived from them have a complex fine structure when examined under the electron microscope with positive staining and thin sectioning (see review by Whittaker & Gray, 1962) or negative staining (Horne & Whittaker, 1962). They are seen to consist (Plate 1*d*) of thin-walled bags containing cytoplasm packed with synaptic granules or vesicles (Sjöstrand, 1953; Palay & Palade, 1954;

Robertson, 1956; De Robertis & Bennett, 1955; Morán, 1957); frequently (though not in this example) one or more mitochondria are also present. The region of the post-synaptic membrane immediately adjacent to the ending is thickened; on homogenization it may remain adherent and accompany the synaptosome through the various steps of the fractionation procedure. Synaptic vesicles appear to be of at least three main kinds: 'hollow', 'dense-cored' and 'compound' (Plate 1*d*). They have been proposed as the actual binding sites of transmitters within the nerve endings (De Robertis & Bennett, 1955; del Castillo & Katz, 1955, 1956; De Robertis, 1958) and as the morphological counterpart of the 'quantized' release of acetylcholine detected electrophysiologically (Fatt & Katz, 1952). The dense-cored vesicles are numerous in peripheral adrenergic nerve endings, and have there been proposed as the binding sites of noradrenaline.

We have for some time been studying the disruption of synaptosomes with the object of obtaining, as separate fractions, synaptic vesicles, intraneuronal mitochondria, external and post-synaptic membranes and the soluble constituents of the nerve-ending cytoplasm for biochemical and pharmacological analysis (Whittaker, 1961). For the morphological control of the various disruptive procedures in the electron microscope, negative staining was found to avoid many of the limitations inherent in conventional methods of positive staining, embedding and thin sectioning when applied to this kind of material (Horne & Whittaker, 1962; Whittaker, 1963*a*).

Suspension of synaptosomes in media hypo-osmotic to plasma was found to result in the bursting of about 80 % of the synaptosomes with the survival of intact synaptic vesicles and about 50 % of the bound acetylcholine. By contrast, frozen-and-thawed preparations contained fewer ruptured synaptosomes and almost no intact synaptic vesicles unconfined by an external membrane (Johnson & Whittaker, 1962, 1963). In this work, lactate dehydrogenase was used as a marker for the easily-diffusible water-soluble components of the nerve-ending cytoplasm.

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It was therefore decided, in the current series of experiments, to use suspension in water as a means of releasing the vesicles. To separate the vesicles from membrane fragments, mitochondria and incompletely disrupted synaptosomes, a density-gradient procedure was devised. The tendency of vesicles to remain clumped together after release as though embedded in a sticky cytoplasm (Whittaker, 1961, 1963*a*) proved troublesome and decreased the yield of free vesicles. Preliminary accounts of this work have already been given (Whittaker, 1963*b*; Whittaker, Michaelson & Kirkland, 1963).

While this work was in progress, De Robertis, Arnaiz & de Iraldi (1962), De Robertis, Salganicoff, Zieher & Arnaiz (1963*b*) and De Robertis, Arnaiz, Salganicoff, de Iraldi & Zieher (1963*a*) claimed to have isolated synaptic vesicles by a simpler procedure than ours. Reasons are given, in the Results section of the present paper, for believing that the preparations of De Robertis and co-workers are heterogeneous and are heavily contaminated with small membrane fragments and intact and partially disrupted synaptosomes. We consider that their electron-microscopic methods greatly underestimate the amount of this contamination and that their conclusions on the biochemical make-up of the isolated vesicles are therefore open to question.

METHODS

Preparation of tissue fractions

Primary fractions. All operations were conducted at 0–4°, with analytical-grade reagents and freshly prepared glass-distilled water. Guinea-pig forebrains were homogenized in 0.32M-sucrose and separated into fractions P_1 (nuclei, large myelin fragments, tissue debris), P_2 (mitochondria, synaptosomes, small myelin fragments, some microsomes) and S_2 (microsomes, some small mitochondria and synaptosomes) as described by Gray & Whittaker (1962), except that, to speed preparation and to diminish microsomal contamination, P_1 was not washed and P_2 was separated at 10000*g* for 20 min. instead of at 17000*g* for 60 min. This resulted in a lower yield of synaptosomes (and therefore of acetylcholine and choline acetyltransferase) in fraction P_2 but this was immaterial for our purposes. In some experiments the P_2 pellet was washed by resuspension in 0.32M-sucrose and recentrifuging at 10000*g* for 30 min. In other experiments the resuspended P_2 pellet was further fractionated into subfractions *A* (myelin, some synaptosomes and microsomes), *B* (synaptosomes and some microsomes) and *C* (mitochondria) by means of a discontinuous density gradient consisting of equal volumes of 0.8M- and 1.2M-sucrose as described by Gray & Whittaker (1962). The *A* and *B* fractions were diluted with equal volumes of water and centrifuged at 40000 rev./min. (100000*g*) for 60 min. to give well-packed pellets.

For comparison, fractions were prepared under identical conditions from guinea-pig liver.

Disruption of fractions. The P_2 or *B* pellets were dis-

rupted (1) by freezing-and-thawing ten times, or (2) by suspending in water (2 ml./g. of original tissue). When the latter method was used, the water suspension, *W*, was usually centrifuged at 10000*g* for 20 min. to remove the larger mitochondria and myelin fragments as a loosely packed pellet, W_p , leaving a cloudy supernatant, W_s , for further fractionation. In some experiments, the water contained eserine sulphate (0.2%). This had the effect of preserving the 50% of the acetylcholine originally present in the P_2 fraction which was released into the free state by the water treatment (Whittaker, 1959) and which was otherwise destroyed by the cholinesterase present in the preparation.

In a few experiments, CaCl_2 (0.001–0.1%) was added to the sucrose or the water, or both, used in the fractionations. Concentrations of CaCl_2 less than about 0.01% had no effect, but concentrations greater than this tended to cause particulate material to form coacervates, thereby decreasing yields.

Density-gradient separation of disrupted fractions. Suspensions of disrupted material (5–6 ml., corresponding to 1.5–2.5 g. of tissue/tube) were transferred to discontinuous density gradients usually consisting of five successive layers (5 ml./tube) of sucrose differing in concentration by 0.2M and varying from 0.4M at the top to 1.2M at the bottom of the gradient. In an abbreviated procedure, when only the lightest fractions were required, the gradient consisted of 0.4M-sucrose (5 ml./tube) layered over 20 ml. of 0.6M-sucrose. The gradients were set up about 1 hr. before use in Lustroid tubes of the SW25 head of the Spinco model L preparative ultracentrifuge. After centrifuging at 25000 rev./min. (53500*g*) for 2 hr. the tubes and contents were sliced in a tube cutter to give a number of separate fractions. Volume recoveries were 85–92%.

Analysis of fractions

Total nitrogen. This was determined by the micro-Kjeldahl method and expressed in milligrams.

Potassium. This was kindly determined by Dr M. W. Smith, using an EEL model A flame photometer. Results are expressed in micrograms.

Lactate dehydrogenase. This was determined spectrophotometrically as described by Johnson (1960) by following the change in extinction at 340 m μ , accompanying the transfer of hydrogen from NADH_2 to sodium pyruvate, with a Unicam SP. 700 recording spectrophotometer. Lactate dehydrogenase has been shown to be a soluble cytoplasmic enzyme in brain; the portion of the enzyme associated with particles is a marker for the soluble cytoplasm of synaptosomes (Johnson & Whittaker, 1962, 1963). Enzyme activities are expressed as the change in extinction at 340 m μ (ΔE_{340})/min.

Succinate dehydrogenase. This was used as a mitochondrial marker and was determined manometrically with potassium ferricyanide as the electron acceptor, as described by Whittaker (1959). The enzyme activity is expressed as μ moles of succinate oxidized/hr.

Choline acetyltransferase. This was determined after activation with ether (Hebb & Smallman, 1956) by the procedure of Berry & Whittaker (1959). The enzyme activity is expressed as μ moles of acetylcholine formed/hr.

Cholinesterase. This was determined manometrically by the method of Ammon (1933), with acetylcholine as substrate in a final concentration of 10 mM. A decrease in

activity was observed with higher concentrations of acetylcholine, indicating that the enzyme was mainly acetylcholinesterase. The enzyme activity is expressed as μ moles of acetylcholine hydrolysed/hr.

Acetylcholine. The acetylcholine content of fractions was assayed on a small (8 mm. $\times$ 0.25 mm.) slip of the dorsal muscle of the leech mounted in a horizontal organ bath of 0.05 ml. capacity as described by Szerb (1962), except that eserine (1 mg./100 ml.) added to the medium was used to sensitize the preparation instead of a short exposure to sarin. Contractions were isotonic. The thickness of the muscle slip was found to be fairly critical; slips thicker than 0.5 mm. did not relax well. The threshold was usually 0.5–2 μ moles of acetylcholine (i.e. about 0.1–0.4 μ mg. of acetylcholine chloride)/ml. Sucrose fractions were heated at pH 4 and 100° for 10 min. to release any bound acetylcholine, diluted 1:10 or more with medium and neutralized, if necessary, with 0.33 N-NaOH before assay. That muscle responses were due to acetylcholine (or a related ester) was shown by tests in which the activity was abolished by the addition of erythrocyte cholinesterase. G. Dowe & V. P. Whittaker (unpublished work) have shown that at least 95% of the acetylcholine-like activity of guinea-pig brain is due to acetylcholine itself.

Expression of results. Activities of fractions are expressed (1) in units/vol. of fraction equivalent to 1 g. of original tissue (units/g.), (2) as a percentage of the total recovered activity, and (3) as relative specific activity, i.e. as (2) divided by the nitrogen content of the fraction expressed as a percentage of the total recovered nitrogen.

Nitrogen rather than protein content was used to calculate relative specific activities because the protein content of fractions consisting mainly of phospholipid membranes may be extremely low. Relative specific activities determined on a protein basis may be subject to considerable error. Relative specific activities of fractions low in nitrogen into which soluble active material may have diffused are also misleading.

Percentage recoveries were corrected for losses of material during tube slicing.

Electron microscopy

Samples were prepared for examination in the Siemens Elmiskop I μ m 11 electron microscope by using the technique of negative staining as adapted for mammalian sub-cellular fractions by Horne & Whittaker (1962). Dilute suspensions of particles were usually fixed by the addition of an equal volume of ice-cold 10% (w/v) formaldehyde in 0.32 M-sucrose previously neutralized to pH 7.4 with 0.33 N-NaOH. A drop of the fixed suspension was placed on a collodion-carbon-coated specimen grid grasped in forceps and most of the liquid withdrawn with a piece of filter paper lightly applied to the side of the grid. A drop of 1–2% (w/v) phosphotungstic acid previously neutralized to pH 7.4 with 2 N-NaOH was then placed on the grid and similarly almost completely removed. Some grids were found to be markedly hydrophobic and withdrawal of most of the droplet did not leave the thin film of liquid evenly deposited over the surface of the grid which was essential for successful negative staining. Such grids were rejected. Prior coating of the grids with a thin film of aqueous 1% (w/v) bovine serum albumin promoted spreading but reduced resolution. Interference by sucrose was occasionally troublesome. If not diluted out sufficiently by phos-

photungstate it disrupted the negative-staining pattern, forming hard-edged patches of light contrast. When present in low concentrations it sometimes gave rise, in regions of thick phosphotungstate deposition, to a pattern of small regular white patches superficially similar to small membrane fragments. With practice, however, such patches could be readily distinguished from biological structures by their locus and hard contrast.

Negatively stained preparations of free synaptic vesicles were found to be unstable and were best examined as soon as possible after preparation. Larger structures (e.g. mitochondria) were stable for several days.

RESULTS

Fractionation of water-treated synaptosomes on a density gradient

When the supernatant of the water-treated P_2 fraction, W_s , was submitted to density-gradient separation, the appearance of the tubes after centrifuging was as shown in Fig. 1.

The tube and contents were separated into seven fractions in a manner dictated by the distribution of material in the gradient. Fraction *O* was the water-clear topmost zone, corresponding to the position of fraction W_s at the beginning of the run. Fraction *D* corresponded approximately to the

Fig. 1. Appearance of Lustroid tube of Spinco SW 25 head after centrifuging the 10000g supernatant of water-treated P_2 fraction (fraction W_s) at 25000 rev./min. (53500g) for 2 hr. The gradient was separated into seven fractions as shown.

Table 1. *Morphology of fractions prepared from water-treated P₂ fractions of guinea-pig brain by density-gradient separation*

For nomenclature of the fractions see the text and Fig. 1. The density of the particles of the fractions is expressed as the concentrations of sucrose in molar units whose density is equal to that of the particles after centrifuging.

Fraction	Particle density	Morphological identification
<i>O</i>	<0.4	No organized structures
<i>D</i>	0.4	Synaptic vesicles, occasional microsomes
<i>E</i>	0.4-0.6	Microsomes, some synaptic vesicles, often in clumps, occasional myelin fragments
<i>F</i>	0.6-0.8	Synaptosome ghosts, myelin fragments, non-vesicular membrane fragments
<i>G</i>	0.8-1.0	Synaptosome ghosts, membrane fragments
<i>H</i>	1.0-1.2	Damaged synaptosomes
<i>I</i>	>1.2	Small mitochondria, some shrunken synaptosomes

layer of 0.4 M-sucrose immediately below the initial position of fraction *W*, and had a faintly-hazy bluish appearance such as would be caused by light-scattering by extremely small particles. Fractions *E*, *F*, *G* and *H* corresponded to well-marked cream-coloured bands of particulate material floating between 0.4 M- and 0.6 M-sucrose, 0.6 M- and 0.8 M-sucrose, 0.8 M- and 1.0 M-sucrose, and 1.0 M- and 1.2 M-sucrose respectively. Fraction *H* included all the 1.2 M-sucrose layer. Fraction *I* was the tan-coloured pellet of particles denser than 1.2 M-sucrose which had sedimented to the bottom of the tubes, and was resuspended in 0.32 M-sucrose for analysis.

Morphology of fractions. Each fraction was examined by negative staining in the electron microscope. The results are summarized in Table 1; representative electron-micrographs are reproduced in Plates 1 (a)–1 (c), 2 (a)–2 (d) and 3 (a).

Fraction *O* contained no organized structures. The pattern of phosphotungstate deposition was not entirely uniform and was broken up in patches. This could have been caused by the presence of soluble protein or lipid deposited in amorphous masses on drying.

Fraction *D* (Plates 1a–1c) consisted almost entirely of small vesicular structures about 500 Å in diameter together with a few larger oval membrane fragments of a kind that were abundant in the next fraction *E*. The small vesicles were identical in size and shape with the synaptic vesicles seen within negatively-stained nerve-ending particles when these had been sufficiently disrupted to permit the ingress of phosphotungstate (Plate 1d), and all the various types were represented.

The mean diameter ($\pm$ s.d.) of 351 small vesicles of the preparation shown in Plates 1 (a) and 1 (b) was 469 ± 110 Å, close to the value of 451 ± 108 Å (406) calculated from Fig. 84A of Andersson-Cedergren (1959) for whole-tissue sections and to the value of 439 ± 137 Å (70) for the small sample represented by the synaptosome in Plate 1 (d).

Fraction *E* (Plate 2a) consisted mainly of circular or oval membrane fragments about 0.1–0.3 μ in diameter similar to those seen in the microsomal fraction of brain homogenates by Gray & Whittaker (1962). Synaptic vesicles were, however, also present, sometimes in small clumps, and there were occasional myelin fragments.

Fractions *F* and *G* (Plates 2b and 2c) were similar. They contained membrane fragments of various shapes and sizes, from microsomal dimensions to those of intact synaptosomes. Occasionally synaptic vesicles could be seen within the larger fragments which are probably the outer membranes of synaptosomes with some cytoplasm and synaptic vesicles remaining within ('synaptosome ghosts'). These synaptosome ghosts

EXPLANATION OF PLATES 1 AND 2

PLATE 1. Unless otherwise stated, all electron-micrographs shown in Plates 1–4 are of material negatively stained as described in the text. (a) Low-magnification electron-micrograph of particles of fraction *D* showing large numbers of synaptic vesicles about 500 Å in diameter together with smaller numbers of larger microsomes 0.1–0.2 μ in diameter. (b) High-magnification electron-micrograph of same preparation as (a). (c) Portion of pellet *D*, obtained by centrifuging fraction *D* and showing hollow (sv₁), dense-cored (sv₂) and compound (csw) synaptic vesicles. (d) Synaptosome negatively stained under slightly hypo-osmotic conditions showing synaptic vesicles of the three main types [see legend to (c)] *in situ*. A portion of axon (a) remains attached, but this synaptosome does not contain a mitochondrion.

PLATE 2. (a) Fraction *E* showing large numbers of microsomes, a few scattered synaptic vesicles and a portion of a myelin fragment (my). (b) Fraction *F* showing membrane fragments including non-vesicular fragments (psm?) that could be detached post-synaptic thickenings. (c) Fraction *G* showing larger oval membrane fragments (g) believed to represent synaptosome ghosts. (d) Fraction *H* showing partially disrupted synaptosomes (ds) and small mitochondria (m) often surrounded by small vesicles and membrane fragments.

were more abundant in fraction *G* than in fraction *F*. Thick non-vesicular membranes were also common, resembling post-synaptic membranes. These were more plentiful in fraction *F* than in fraction *G*.

Fraction *H* (Plate 2*d*) contained a confused mass of membranes of greatly different sizes, resembling the parent fraction *W_s*, and is believed to represent incompletely disrupted synaptosomes. It is difficult to convey an adequate impression of this fraction in a single electron-micrograph. Frequently, small mitochondria of a size similar to those seen within synaptosomes could be seen surrounded by vesicular masses resembling synaptic vesicles and disrupted external membranes. It is known from previous work (summarized by Whittaker, 1963*a*) that the synaptic vesicles of ruptured endings behave as though they are embedded in a sticky cytoplasm. The equilibrium densities of the *G* and *H* fractions correspond to that of intact synaptosomes (Gray & Whittaker, 1960, 1962; Michaelson & Whittaker, 1962; Johnson & Whittaker, 1962, 1963).

Fraction *I* (Plate 3*a*) consisted almost entirely of small mitochondria with the characteristic fine structure (Whittaker, 1963*a*) of swollen mitochondria. It also contained unpenetrated structures tentatively identified with the highly osmophilic shrunken synaptosomes seen in intact preparations exposed to hyperosmotic sucrose and referred to as 'black bodies' by Gray & Whittaker (1962).

All the structures visible in fractions *O*–*I* could be seen in fraction *W_s*. Occasionally, drifts of isolated synaptic vesicles could be seen, but incompletely disrupted synaptosomes (Plate 3*b*), microsomes, synaptosome ghosts, mitochondria

and non-vesicular membrane fragments were all visible in adjacent areas.

Fraction *W_p* contained mainly large mitochondria and myelin fragments; however, considerable numbers of partially damaged synaptosomes were also present. Its morphology was consistent with the relatively high proportion of the mitochondrial marker, succinate dehydrogenase, and relatively low proportions of other components sedimenting in this fraction (Table 2).

Activity of fractions. The lactate-dehydrogenase, succinate-dehydrogenase, choline-acetyltransferase, cholinesterase and acetylcholine activities of the various fractions are presented in Tables 2 and 3. The rather large standard deviations in Table 2 were due to the difficulty experienced in duplicating conditions exactly each time. One source of variation was the difference in the weights of tissue processed, but the main variation was probably due to the difficulty of cutting the tubes in exactly the same way each time. Nevertheless the general pattern of distribution in each experiment was the same.

The most noteworthy finding was that acetylcholine (Table 2, 1. 5) was bimodally distributed in the density gradient, the fractions with the highest activities being the synaptic-vesicle fraction, *D*, and the fraction containing incompletely disrupted synaptosomes, *H*. The acetylcholine in these fractions was in the bound form and could be sedimented with the particles contained in them by high-speed centrifuging. Further details of the properties of the acetylcholine of the *D* fraction are given below.

Lactate dehydrogenase (Table 2, 1. 1) was recovered mainly in fraction *O*, with some activity in fraction *D* and little or none in the other fractions. This distribution was similar to that found for the potassium of the fraction (Table 2, 1. 7) and for the soluble protein bovine serum albumin after a dummy run in which 28.5 mg. of albumin in 5 ml. of water was placed on top of a density gradient, centrifuged and sampled in the same way as in a normal experiment (Table 2, 1. 8). This showed that soluble components do not diffuse throughout the whole gradient in 2 hr. Fraction *O* thus contains most of the soluble cytoplasmic constituents of the synaptosome, and lactate dehydrogenase and potassium behave as one would expect of soluble cytoplasmic markers.

Succinate dehydrogenase (Table 2, 1. 2) was recovered mainly in fractions *I* and *H*, as would be expected of a mitochondrial marker. Almost half the original activity of fraction *W* had already been removed in fraction *W_p* by the initial low-speed centrifuging.

Cholinesterase, shown to be a microsomal marker by Toschi & Hanzon (1959) and Aldridge &

EXPLANATION OF PLATES 3 AND 4

PLATE 3. (a) Fraction *I* consisting mainly of swollen mitochondria (m). (b) Fraction *W_s* showing partly damaged synaptosome (ds) containing a mitochondrion (m) and synaptic vesicles, synaptosome ghosts (g), isolated synaptic vesicles (sv), microsomes (mi) and non-vesicular membrane fragments (psm?). (c) Appearance of fraction *D* after brief exposure to acid (pH 4 for 1–2 min. at 0°). The synaptic vesicles have clumped and partially fused. (d) Same preparations as in (c) at higher magnification.

PLATE 4. (a) Electron-micrograph of *M_s* preparation of De Robertis *et al.* (1963*a*) showing isolated synaptic vesicles (sv) mixed with damaged synaptosomes (ds) and membrane fragments of varied shape and size. (b) The same preparation as in (a), negatively stained by suspending an osmic acid-treated pellet in ammonium acetate and diluting with sodium phosphotungstate as described by De Robertis *et al.* (1963*a*). The large membrane fragments and damaged synaptosomes of (a) are absent.

Table 2. *Distribution of enzymes, acetylcholine, nitrogen and potassium in fractions prepared from water-treated P₂ fractions of guinea-pig brain by density-gradient separation*

For nomenclature of the fractions see the text and Fig. 1. Values are mean values $\pm$ s.d.; numbers in parentheses indicate the numbers of experiments averaged when this differs from that given in column 3.

Component	Unit	No. of experiments	W (units/g.)	Percentage distribution										Recovery (% of W_0)
				Percentage distribution		Percentage distribution								
				In W_p	In W_s	Recovery (% of W)	In O	In D	In E	In F	In G	In H	In I	
Lactate dehydrogenase	$\Delta E_{330}/\text{min.}$	3	13.4 $\pm$ 4.1	9 $\pm$ 4	91 $\pm$ 4	123 $\pm$ 42	74 $\pm$ 18	16 $\pm$ 14	3 $\pm$ 3	1 $\pm$ 1	0	2 $\pm$ 2	3 $\pm$ 3	71 $\pm$ 17
Succinate dehydrogenase	$\mu\text{moles/hr.}$	3	193.0 $\pm$ 70 (2)	45 $\pm$ 10	55 $\pm$ 10	80 $\pm$ 2 (2)	5 $\pm$ 5	5 $\pm$ 5	4 $\pm$ 4	5 $\pm$ 4	6 $\pm$ 2	22 $\pm$ 5	52 $\pm$ 20	66 $\pm$ 16
Cholinesterase	$\mu\text{moles/hr.}$	4	63.0 $\pm$ 29	17 $\pm$ 8	83 $\pm$ 9	91 $\pm$ 11	5 $\pm$ 2	9 $\pm$ 3	20 $\pm$ 7	27 $\pm$ 5	21 $\pm$ 6	10 $\pm$ 4	8 $\pm$ 7	88 $\pm$ 22
Choline acetyltransferase	$\mu\text{moles/hr.}$	3	0.46 $\pm$ 0.07	9 $\pm$ 4	91 $\pm$ 4	116 $\pm$ 16	73 $\pm$ 6	8 $\pm$ 1	2 $\pm$ 1	5 $\pm$ 3	5 $\pm$ 1	4 $\pm$ 2	3 $\pm$ 1	83 $\pm$ 37
Acetylcholine	$\mu\text{m-moles}$	4	2.70 $\pm$ 0.39	24 $\pm$ 10	76 $\pm$ 10	100 $\pm$ 68	2 $\pm$ 2	30 $\pm$ 8	8 $\pm$ 5	5 $\pm$ 5	11 $\pm$ 5	35 $\pm$ 11	9 $\pm$ 6	81 $\pm$ 19
Nitrogen	mg.	11	3.24 $\pm$ 1.8	28 $\pm$ 6	72 $\pm$ 6	99 $\pm$ 15	28 $\pm$ 6	12 $\pm$ 2	8 $\pm$ 1	12 $\pm$ 3	12 $\pm$ 2	15 $\pm$ 4	13 $\pm$ 5	84 $\pm$ 7
Potassium	$\mu\text{g.}$	1	305	13	87	98	75	14	4	5	2	0	0	100
Bovine serum albumin*	—	1	—	—	—	—	76	24	0	0	0	0	0	111

(5.7 mg./ml.)

* Dummy run.

Johnson (1959), was rather diffusely distributed throughout the density gradient but was mainly present in fractions *E*, *F* and *G*, all rich in small membrane fragments. The highest relative specific activity was associated with the predominantly microsomal fraction *E* (Table 3, 1. 3).

In the primary fractions of brain homogenates, the distribution of choline acetyltransferase closely parallels that of acetylcholine (Hebb & Whittaker, 1958). After water-treatment, choline acetyltransferase behaves like lactate dehydrogenase, potassium and bovine serum albumin, being recovered mainly in fraction *O*. As with these other components, the concentration in fraction *D* is consistent with diffusion from fraction *O*. High-speed centrifuging (100 000*g* for 60 min.) or fraction *D* gave a pellet, *D_p*, and a slightly opalescent supernatant, *D_s*; 87% of the recovered choline acetyltransferase remained in fraction *D_s* whereas 80% of the recovered acetylcholine was sedimented in fraction *D_p*. Thus choline acetyltransferase, unlike acetylcholine, is not a constituent of the synaptic vesicles but is localized in the soluble cytoplasm of the synaptosome.

As seen in Table 3, the separation of fraction *W_p* from fraction *W_s* in the initial low-speed run produced an enrichment with respect to fraction *W* in fraction *W_s* of all the activities except that of succinate dehydrogenase. The enrichment was greatest, as would be expected, with the soluble cytoplasmic constituents, lactate dehydrogenase, potassium and choline acetyltransferase, which had been largely (87–95%) released by the water-treatment, less with the microsomal cholinesterase and least with acetylcholine, nearly a quarter of which remained sequestered within the damaged synaptosomes sedimenting in fraction *W_p*. Nearly half the succinate dehydrogenase was carried down with fraction *W_p*, which is consistent with the high mitochondrial content of this fraction.

The other results in Table 3 serve to show that, in general, the fractions with the highest activities are also the fractions with the highest relative specific activities.

Effect of inhibiting cholinesterase. When eserine sulphate (0.2%) was present in the water in which the *P₂* pellet was suspended, the free acetylcholine released was not destroyed and was recovered, as one would expect, mainly in the *O* and *D* fractions with smaller amounts in other fractions.

Preparation of vesicles from fraction B. When fraction *B*, consisting almost entirely of synaptosomes, was suspended in water and fractionated on the density gradient, fractions similar to those described above were obtained. The exposure of the synaptosomes to hyperosmotic sucrose during their isolation from the *P₂* fraction did not prevent vesicles from being released.

Table 3. *Relative specific activities of density-gradient fractions prepared from water-treated P₂ fractions from guinea-pig brain*

For nomenclature of the fractions see the text and Fig. 1. The relative specific activity is (percentage of total recovered activity)/(percentage of total recovered nitrogen). Fractions *W_s* and *W_p* are referred to fraction *W* taken as unity, and fractions *O-I* to fraction *W_s* taken as unity. Values are obtained from the mean values in Table 2.

Activity	Relative specific activity								
	In <i>W_p</i>	In <i>W_s</i>	In <i>O</i>	In <i>D</i>	In <i>E</i>	In <i>F</i>	In <i>G</i>	In <i>H</i>	In <i>I</i>
Lactate dehydrogenase	0.32	1.26	2.64	1.33	0.37	0.08	0	0.13	0.23
Succinate dehydrogenase	1.61	0.76	0.18	0.42	0.50	0.42	0.50	1.47	3.77
Cholinesterase	0.61	1.15	0.18	0.75	2.50	2.25	1.75	0.67	0.61
Choline acetyltransferase	0.32	1.26	2.61	0.67	0.25	0.42	0.42	0.27	0.23
Acetylcholine	0.86	1.06	0.07	2.50	1.00	0.42	0.92	2.33	0.69
Potassium	0.46	1.21	2.68	1.17	0.50	0.42	0.17	0	0

Fractionation of frozen-and-thawed and untreated preparations

For comparison with the experiments described in the preceding section, washed *P₂* fractions were divided and one half frozen-and-thawed ten times. The frozen-and-thawed and untreated preparations were then placed on to the usual density gradients and separated as described above.

The appearance of the tubes after the centrifuging was essentially the same for both preparations but different from those in which water-treated material had been fractionated. The *D* band was no longer visible, the *E* band was faint and the *H* band was intensified. Most of the lactate dehydrogenase, choline acetyltransferase, acetylcholine and nitrogen were recovered in the *H* band; however, with the frozen-and-thawed preparations significant amounts of lactate dehydrogenase (23%) and choline acetyltransferase (20%) were recovered in the region 0–0.4 M-sucrose corresponding to the *O* and *D* bands of the water-treated preparations. These results are consistent with previous conclusions (Johnson & Whittaker, 1962, 1963) that freezing-and-thawing, though disrupting a certain proportion of synaptosomes, does not release intact synaptic vesicles.

Properties of the acetylcholine of the synaptic-vesicle fraction

Evidence for the association of acetylcholine and vesicles. The acetylcholine of the *D* fraction was in a particulate bound state as evinced by the following observations: (1) It was immune from the action of cholinesterase, until released by any of the methods mentioned below. If the method of release did not simultaneously destroy the cholinesterase present in the preparation, the prior addition of a cholinesterase inhibitor was essential to stabilize the released acetylcholine. (2) On diluting fraction *D* with an equal volume of water and centrifuging at 100 000g for 1 hr. most of the acetylcholine was sedimented along with most of

the synaptic vesicles. Examination of the supernatant in the electron microscope showed that a few synaptic vesicles had not been sedimented. (3) The acetylcholine in the *D* fraction was inactive on the leech preparation unless first released by an appropriate disruptive technique.

It was decided to compare the stability of the bound acetylcholine in the isolated synaptic vesicles of the *D* fraction with that of synaptosomes (Whittaker, 1959). This study is not yet complete but the following preliminary observations have been made.

Spontaneous release. The vesicles appeared to be stable for a period of at least 2–3 hr. after preparation at 0°. On incubation at 20° about 60% of the bound acetylcholine was released after 20 min. and 100% after 60 min. At 35°, release was still more rapid, 60% having been released after 5 min. and 100% after 30 min.

Effect of osmotic strength of suspension medium. Synaptic vesicles, in contrast with synaptosomes, do not appear to be osmotically sensitive. This is indicated by their method of preparation and by an experiment in which there was no increase in the rate of spontaneous release from the *D* fraction (sucrose concn. approx. 0.4 M) when this was diluted with an equal volume of water.

Effect of acid. Brief (1–2 min.) exposure at 0° to acid conditions (pH 4.0) released all the bound acetylcholine of the fraction. This release was accompanied by a remarkable change in the morphology of the vesicles (Plates 3c and 3d). They clumped together and apparently began to fuse. These experiments showed that it is not necessary to heat the *D* fraction at 100° for 10 min. to release all the bound acetylcholine, as was in fact done as a routine.

Are the particles of fraction D an artifact?

A problem that must be seriously considered is the possibility that the synaptic vesicles of fraction *D* are simply lipoprotein micelles that have arisen as the result of the degradation of lipoprotein

membranes by water-treatment and mechanical disruption during preparation. The presence of bound acetylcholine might then be accounted for by the uptake by the lipoprotein micelles of small amounts of acetylcholine released from storage sites during manipulation.

It may be difficult to disprove such a proposition until analyses on the lipid composition of the isolated vesicles are complete. Nevertheless, two types of experiments have been carried out in an attempt to test it.

Attempted preparation of 'synaptic vesicles' from liver. A liver-mitochondrial fraction was suspended in water and submitted to density-gradient fractionation by exactly the same procedure as was used for preparing fraction *D* from brain. The density gradient, illustrated diagrammatically in Fig. 2, had a very different appearance from that shown in Fig. 1. At the top was a thin layer of fat. The regions corresponding to most of fraction *O* and the whole of fractions *D* and *E* were clear and lemon-yellow in colour; in the electron microscope they were found to be free from organized membranous structures. Particulate material corresponding to fractions *F*, *G*, *H* and *I* was present, but only the pellet (corresponding to fraction *I*) contained much material. This consisted of badly damaged mitochondria often with disintegrating matrices. Swollen mitochondria also accounted for most of the layer corresponding to fraction *H*. The mitochondria were more damaged than in brain which is in accordance with the known greater fragility of liver mitochondria. The regions equivalent

to fractions *F* and *G* appeared to consist mainly of mitochondria fragments, but there were also occasional larger granular bodies which might have been lysosomes. Other work has shown that liver mitochondria are much more readily disrupted in water than brain mitochondria. It seems clear that particles like the isolated synaptic vesicles of the *D* fraction from brain do not arise spontaneously from lipoprotein membranes generally.

Effect of emulsified lecithin. Acetylcholine ($5\mu\text{m-moles/ml.}$) and lecithin ($2\mu\text{moles/ml.}$) were shaken together in aqueous solution and the ensuing emulsion tested on the frog rectus-abdominis-muscle preparation. No evidence of binding was obtained. The micelles formed by lecithin under these conditions vary greatly in size and are readily distinguishable from the isolated synaptic vesicles in negative staining (Dr A. D. Bangham, personal communication).

Do synaptic vesicles contain choline acetyltransferase?

Our findings, reported above, show that the isolated vesicles of fraction *D* contain acetylcholine but not choline acetyltransferase. The choline acetyltransferase of fraction *D* is considered to originate by diffusion from fraction *O*.

These results are at variance with those published by De Robertis *et al.* (1963*a*). In their procedure, a crude mitochondrial fraction from rat brain (corresponding to our P_2 fraction) is suspended in water, coarse particulate material is removed at $11500g$ for 20 min. (fraction M_1 corresponding to our fraction W_1) and the supernatant (corresponding to our W_2 preparation) is centrifuged at $100000g$ for 30 min. to give a pellet, M_2 , and a supernatant, M_3 . Fraction M_2 contains cholinesterase, choline acetyltransferase and acetylcholine, and is stated to consist of free synaptic vesicles mixed with some 'curved membranes'. Their published electron micrographs support this statement.

Thinking that relatively small differences of procedure might account for the divergence in the two sets of results, we have attempted to repeat experiments of De Robertis and co-workers as closely as their published account allows. Since, in their latest paper (De Robertis *et al.* 1963*a*) though not in their previous ones (De Robertis *et al.* 1962, 1963*b*), they stress the importance of the presence of a low concentration of Ca^{2+} ions, sucrose solutions were deionized before use by passage through a mono-bed resin (Amberlite MB-1) to remove traces of Ca^{2+} and other ions, and calcium chloride was added to give a final concentration of $10\mu\text{M}$. The water used for resuspending the P_2 fraction also contained this concentration of calcium chloride.

Fig. 2. Diagram showing the appearance of the density gradient when an experiment similar to that shown in Fig. 1 was performed with guinea-pig liver.

Table 4. Comparison of distribution of activity in fractions of water-treated P_2 fractions of guinea-pig brain by using different proceduresFor nomenclature of the fractions see the text. (a) Results of De Robertis *et al.* (1963a); (b) this paper.

	In M_1		In M_2		In M_3		Percentage recovery		In W_p	In W_s	Percentage recovery
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	W_p (b)	W_s (b)	
Choline acetyltransferase	33	0	52	2	15	98	63	103	9	91	116
Acetylcholine	35	21	37	27	28	52	100	61	24	74	100

Our results (Table 4) were similar to those obtained with our W_p and W_s fractions prepared in the absence of Ca^{2+} ions, on the assumption that fraction W_s is equivalent to fraction M_2 plus fraction M_3 . Electron microscopy, by our usual technique (Plate 4a), confirmed this. Fraction M_2 was extremely heterogeneous, containing disrupted synaptosomes, membrane fragments, clumps of synaptic vesicles and detached vesicles. Fraction M_3 was similar to fraction M_2 except that it was much more dilute, microsomes and synaptic vesicles predominated and there were fewer larger membrane fragments present.

Electron microscopy was also carried out on fraction M_2 by using the negative-staining technique of De Robertis and co-workers. The M_2 fraction was resuspended and treated with 2% (w/v) osmium tetroxide in 0.32M-sucrose and the osmic acid-treated particles were centrifuged to form a pellet. This pellet was resuspended in ammonium acetate and diluted 1:5 with 2% (w/v) phosphotungstic acid neutralized to pH 6.7. Droplets were applied to grids in the usual way.

The electron-micrographs showed an astonishing difference (Plate 4b) from those prepared by our usual method. The partially disrupted synaptosomes had disappeared, leaving clumps of synaptic vesicles and membrane fragments, selected areas of which resembled the electron-micrographs published by De Robertis and co-workers. It is evident that the resuspension of the osmic acid-treated particulate material of fraction M_2 had led to extensive disruption of the more organized structures, as indeed was predicted (see Discussion in Whittaker, 1963b) from the work of Gray & Whittaker (1960, 1962) and Horne & Whittaker (1962). These workers found that osmic acid-treated synaptosomes are extremely fragile and disintegrate on resuspension liberating vesicles stained with osmic acid. It is this property which accounts for the appearance of the previous electron-micrographs of the B fraction (Whittaker, 1959) in which resuspension techniques had been used and in which 60% of the particles were within the size range of synaptic vesicles.

We believe, therefore, that De Robertis and co-

workers may have seriously underestimated the degree of organization of their M_2 fraction and are mistaken in thinking that it is a reasonably homogeneous preparation of isolated synaptic vesicles. We do not attach great significance to the fact that in our hands this fraction contained a considerably lower proportion of the total choline acetyltransferase than they found, as this could be accounted for by a higher proportion of undamaged synaptosomes in their preparation. However, it is surprising that the percentage of acetylcholine which they recovered in fraction M_2 is not also proportionately higher. This might be explained by assuming that the less-stable synaptosomes had already been broken during their initial homogenization. Vesicles released at this stage, because of their tendency to stick together (Whittaker, 1963a), might well be recovered in fraction M and subsequently in fractions M_2 and M_3 , but the choline acetyltransferase released from these broken synaptosomes would be recovered in fraction S_2 and so would not be included in their results.

DISCUSSION

The results given in the present paper show that it is now possible to resolve fractions containing isolated nerve endings into a number of subfractions which represent its main structural features: synaptic vesicles, soluble cytoplasm, mitochondria and post-synaptic membrane. Of these, the last-named fraction is less well characterized than the others. In addition, fractions containing partially ruptured endings and microsomes are obtained. The microsomes probably represent contamination of the original preparation augmented by membrane fragments formed from larger structures during disruption. The markers lactate dehydrogenase and potassium (soluble cytoplasm) and succinate dehydrogenase (mitochondria) have assisted the characterization of the fractions.

The results clearly indicate the separate localization within the synaptic region of the three components (acetylcholine, choline acetyltransferase and cholinesterase) of the acetylcholine system. The transmitter itself appears to have a multiple

localization within the neurone. When brain tissue is homogenized in sucrose containing eserine, a portion (20–30 %) of the total acetylcholine of the tissue is recovered in the soluble supernatant fraction (S_3). The remainder, representing bound acetylcholine, is recovered, under our conditions of homogenization, in a fraction identified as largely consisting of pinched-off nerve endings. To emphasize the relatively homogeneous nature of this fraction, and the similarity in physical properties of the isolated nerve endings to subcellular organelles, we have proposed the term 'synaptosomes' for the nerve-ending particles.

Disruption of the synaptosomes by exposure to hypo-osmotic media and other relatively mild conditions has led to the recognition of two kinds of bound acetylcholine, the 'labile' and 'stable' fractions (Whittaker, 1959). There are thus in all three states in which acetylcholine is present in nervous tissue: (a) that which is released on initial homogenization in iso-osmotic media; (b) the labile bound fraction of synaptosomes; (c) the stable bound fraction of synaptosomes.

It is well known that choline acetyltransferase is distributed throughout the length of cholinergic neurones. It seems reasonable to assume that a low steady-state concentration of acetylcholine exists in the cytoplasm of these neurones which represents the balances of synthesis and destruction by intracellular cholinesterase or spontaneous hydrolysis or both. This acetylcholine may give rise to the free acetylcholine of the S_3 fraction. The 'labile' bound acetylcholine released by hypo-osmotic treatment may represent the fraction of this free cytoplasmic acetylcholine sequestered within the synaptosomes. The 'stable' bound acetylcholine may represent acetylcholine bound to synaptic vesicles or contained within them, providing a device for raising the effective steady-state acetylcholine concentration at the cholinergic terminal. It is likely, however, that, even if these different acetylcholine 'compartments' exist, shifts from one 'compartment' to another may occur during the formation and subsequent disruption of the synaptosomes.

Although acetylcholine is stored, at any rate in part, in the synaptic vesicles, its synthesis in the cholinergic nerve ending appears to take place in the cytoplasm. In our experiments choline acetyltransferase is localized in the soluble cytoplasm; L. A. Mounter & V. P. Whittaker (unpublished work) also found a cytoplasmic localization for acetate-coenzyme A ligase (adenosine monophosphate) (acetylthiokinase), which provides the acetyl-coenzyme A for the acetylation of choline.

The yield of isolated synaptic vesicles cannot at present be estimated with any certainty but is probably low. About 10 % of the acetylcholine

originally present in the P_2 fraction is eventually recovered in the D band. If it is assumed that all the acetylcholine was originally present in the vesicles and that no gains or losses occurred subsequent to water-treatment, the yield would also be about 10 %. If the vesicles were not the sole 'compartment' or acetylcholine were lost from them during isolation, or both, the yield might be considerably higher. Work is continuing in an attempt to raise the yield.

SUMMARY

1. A density-gradient procedure has been devised for fractionating pinched-off nerve endings ('synaptosomes') after disruption by suspension in water.

2. Seven fractions are obtained. These consist of soluble cytoplasmic components, synaptic vesicles, microsomes, larger membrane fragments, partially disrupted synaptosomes and mitochondria.

3. The various fractions have been characterized morphologically and biochemically. Lactate dehydrogenase (known to be a soluble-cytoplasmic marker), potassium and choline acetyltransferase were recovered mainly in the soluble-cytoplasmic fraction; succinate dehydrogenase in the mitochondrial fraction; and cholinesterase in the microsome and large-membrane fractions. Bound acetylcholine was distributed bimodally, part being recovered in the synaptic-vesicle fraction and part in the fraction containing incompletely disrupted synaptosomes.

4. The isolated synaptic vesicles had a mean diameter of $469 \pm 110 \text{ \AA}$, close to values reported in the literature for synaptic vesicles in whole-tissue preparations.

5. The acetylcholine in the vesicle fraction was in the bound form, but was readily released on incubation or acid treatment. The specific activity of this fraction was higher than that of preparations of intact synaptosomes.

6. The 'compartmentalization' of acetylcholine, choline acetyltransferase and cholinesterase in the cholinergic synapse is discussed in the light of these findings.

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Biochem. J. (1964) **90**, 303

Comparative Studies of 'Bile Salts'

18. THE CHEMISTRY OF CYPRINOL*

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The bile alcohol cyprinol has been isolated from four species of fish of the family Cyprinidae and was shown by feeding experiments on captive carp to be very probably made *in vivo* from cholesterol (Haslewood, 1955, 1959). The infrared-absorption spectrum of cyprinol was recognized as showing the presence in the molecule of the nucleus of allocholic acid and chromic oxidation gave a mixture

probably containing 3,7,12-trioxoallocholic acid (Briggs & Haslewood, 1962).

Cyprinol was also made from carp bile by Hoshita (1962), who isolated, after alkaline hydrolysis, the diacetate of anhydrocyprinol. Hoshita (1962) oxidized the acetylated anhydrocyprinol with chromic acid at 70°: the product after hydrolysis was found to contain allocholic acid and also a substance that had the mobility on paper chromatograms of 25-homocholic acid. Lithium

* Part 17: Bridgwater, Haslewood & Watt (1963).