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Exchangeability of Radioactive Acetylcholine with the Bound Acetylcholine of Synaptosomes and Synaptic Vesicles

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1. The exchangeability with added radioactive acetylcholine of the acetylcholine in isolated presynaptic nerve terminals (synaptosomes) and isolated synaptic vesicles was studied by a Sephadex-column method. 2. A substantial proportion of the synaptosomal acetylcholine is exchangeable with added radioactive acetylcholine. It is liberated by hypo-osmotic shock and ultrasonic treatment, and behaves as though it occupies the cytoplasmic compartment of synaptosomes. 3. Methods of isolating vesicles from hypo-osmotically ruptured synaptosomes in optimum yield are discussed. 4. The acetylcholine of synaptic vesicles isolated on a sucrose density gradient is released by hypo-osmotic conditions, suggesting that it is enclosed by a semi-permeable membrane; however, it is not easily released by ultrasonic treatment. 5. Added radioactive acetylcholine does not exchange with vesicular acetylcholine under a variety of different conditions. These include addition of ATP and Mg^{2+}, and pre-loading of the synaptosome with radioactive acetylcholine before hypo-osmotic rupture. This failure to exchange is discussed in terms of the possible storage mechanism of vesicular acetylcholine.

Acetylcholine (ACh†) is a transmitter at many peripheral and central synapses (for review see Hebb & Krnjević, 1962). Its storage in nervous tissue and the mechanism of its release are of fundamental importance for understanding of the processes of chemical transmission. It is therefore of interest to study the biochemistry of bound ACh.

When brain cortex is homogenized in 0·32M-sucrose presynaptic nerve terminals are pinched off and may be separated by density-gradient centrifugation as discrete particles, about 0·5μ in diameter. These particles have been termed 'synaptosomes' and they contain ACh protected from the cholinesterases in the preparation that would otherwise destroy it (for review see Whittaker, 1965).

Synaptosomes are sensitive to hypo-osmotic conditions. They rupture, liberating soluble cytoplasmic constituents as well as the structures that are usually seen inside them. Prominent among the latter are the synaptic vesicles approx. 500Å in diameter. When synaptosomes are ruptured not all of their ACh is converted into the free form accessible to hydrolysis by cholinesterase. Some 50% of it remains bound (Whittaker, 1959; Whittaker, Michaelson & Kirkland, 1964), and by centrifuging the disrupted preparation it is possible to recover the bound form in the fraction containing synaptic vesicles. The release of ACh from vesicles into the synaptic cleft is thought to be an important step in impulse transmission at a cholinergic chemical synapse.

Three distinct kinds of ACh in a brain homogenate are believed to exist. (i) True 'free' ACh, external to the synaptosome; this is usually destroyed by cholinesterase during the preparative procedure for synaptosomes unless a cholinesterase inhibitor is present. (ii) Synaptosomal ACh that is easily liberated by hypo-osmotic treatment (termed 'labile bound' ACh; Whittaker *et al.* 1964). (iii) Synaptosomal ACh that remains bound under the hypo-osmotic conditions used to rupture synaptosomes and that may be isolated in synaptic vesicles by further centrifugation (vesicular ACh).

Methods have already been described for measuring the synaptosomal content of small-molecular-weight substances by using Sephadex columns (Marchbanks, 1966). When synaptosomes were passed through a column equilibrated with a solution iso-osmotic to the medium in which they were suspended they retained their internal Na^+ and K^+, as shown by the emergence of these ions

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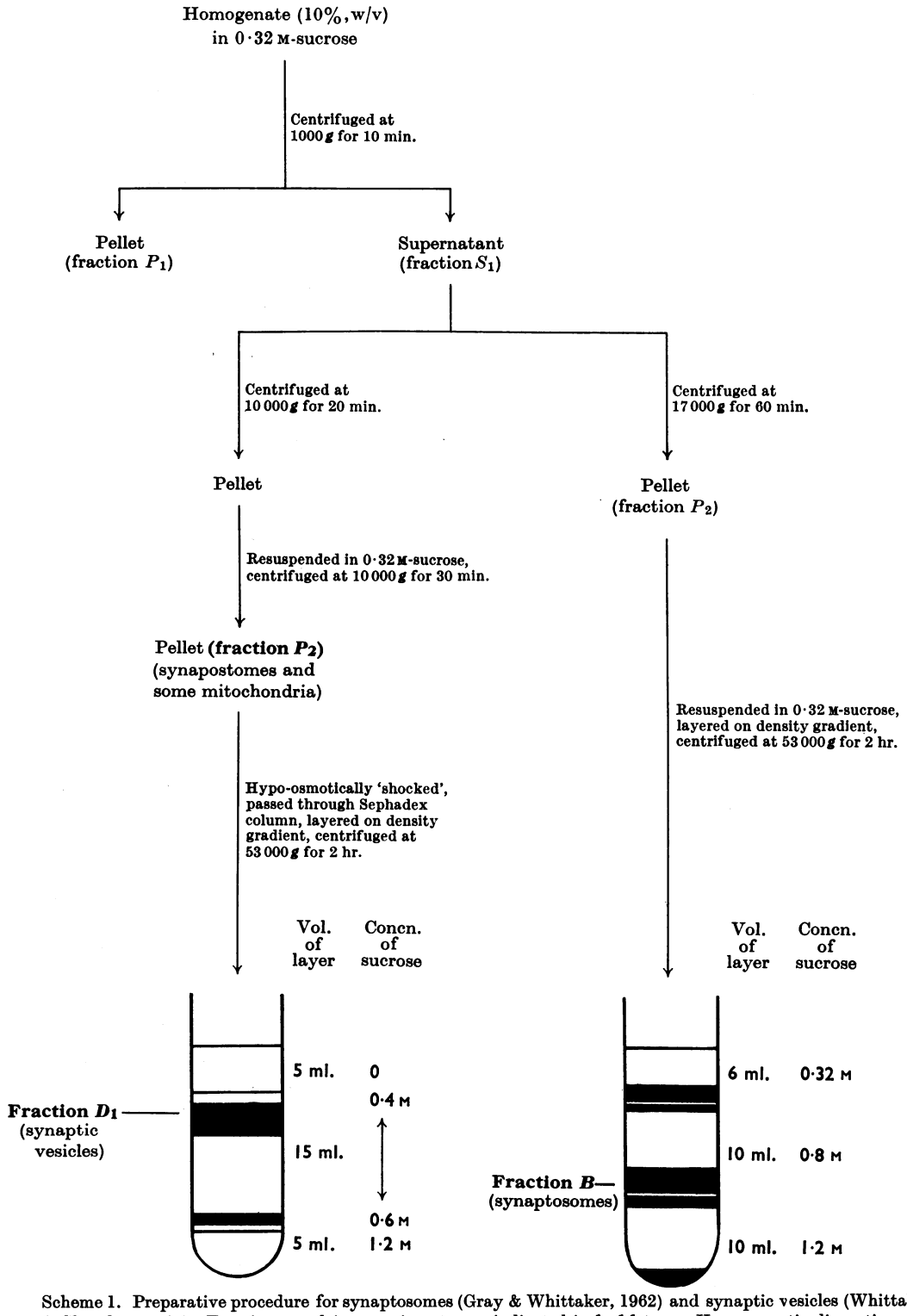
† Abbreviation: ACh, acetylcholine.



Scheme 1. Preparative procedure for synaptosomes (Gray & Whittaker, 1962) and synaptic vesicles (Whittaker & Sheridan, 1965). Fractions used in experiments are indicated in **bold** type. Hypo-osmotic disruption was carried out by resuspending the pellet derived from 1 g. wet wt. of cortex, with 2 ml. of water (additions as specified in the text), and then passing over a column containing 2 g. of Sephadex G-50 equilibrated with water: 5 ml. of the void volume effluent was layered on the continuous density gradient.

in the void volume. When the columns were eluted with hypo-osmotic solutions the synaptosomes burst and their content of Na^+ and K^+ was liberated and retained in the interior volume of the column. Evidence has been presented that the Na^+ and K^+ thus released occupy the compartment that is enclosed by the external membrane of the synaptosomes. Similar methods have been used to examine the properties of synaptosomal and vesicular ACh, and to study the exchangeability of the forms of bound ACh with added radioactive ACh. A preliminary account of these experiments has been given (Marchbanks, 1967*a*).

METHODS

Preparations

Isolation from tissue. Synaptosomes were prepared from scraped guinea-pig cerebral cortex by the method of Gray & Whittaker (1962) and synaptic vesicles were prepared from the same tissue by the method of Whittaker & Sheridan (1965). The procedures used and nomenclature of fractions are shown as a flow chart (Scheme 1). For the initial homogenization the pestle was rotated at 840 rev./min.; the diameter of the pestle was 29·5 mm. and the clearance between pestle and mortar was 0·25 mm. Experiments were done on the preparations immediately after isolation, and unless otherwise reported were carried out at 5°. Sucrose solutions were not buffered, but the pH was always checked and found to be in the range 6·0–6·5. Amounts of preparation derived from 0·2–0·5 g. wet wt. of cortex were usually used.

Ultrasonic treatment. Preparations were submitted to ultrasonic vibrations at 20000 cyc./sec. from a 60 w Mullard–MSE disintegrator (type 7685/2 with a titanium probe) for various times at 2°.

Electron microscopy. Synaptic vesicles that had been passed through a Sephadex column were examined by negative staining. Small drops of suspensions were treated with 2% (w/v) phosphotungstic acid previously neutralized with 2 N-NaOH, as described by Whittaker *et al.* (1964), and examined in the Siemens Elmiskop I ÜM11 electron microscope.

Analytical methods

Determination of potassium. K^+ was determined on an EEL model A flame photometer. Neither protein, sucrose nor Na^+ in the concentrations usually present were found to interfere with the estimation of K^+.

Determination of ACh. ACh was extracted by bringing preparations to pH 4·0 with HCl and heating at 100° for 10 min. The precipitated protein was then removed by centrifuging. The ACh content of the extract was assayed on a small (8 mm. × 0·25 mm.) slip of the dorsal muscle of the leech mounted in a vertical organ bath of 0·1 ml. capacity, essentially as described by Szerb (1962) and Whittaker *et al.* (1964). Eserine (1 mg./100 ml.) was added to the medium to sensitize the preparation.

If a positive response was found the sample was neutralized with $NaHCO_3$, 2 units of acetylcholinesterase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) in 0·1 ml. were added and the sample was incubated at 37° for 30 min. (1 unit of acetylcholinesterase hydrolyses 0·1 μmole of ACh/min. at 37° and pH 7·4). The sample was then re-assayed to ensure that no substances other than ACh were causing the muscle to contract. The presence of substances that inhibit the muscle response to ACh was also tested for by adding internal standards after the initial assay (Feldberg & Hebb, 1947). Responses to substances other than ACh, or inhibition of the response by substances present in the sample, were only rarely encountered and were corrected for.

If the concentration of ACh was sufficient (more than 1 mμmole/ml.) it was assayed on the frog rectus-muscle preparation essentially as described by MacIntosh & Perry (1950). ACh is expressed in μμmoles.

Measurement of radioactivity. The radioactivity of [^{14}C]ACh labelled either in the choline or the acetyl group was measured in a Packard automatic scintillation counter with the scintillation medium described by Bray (1960). Changes in relative counting efficiency due to quenching were always checked and corrected for by adding internal standards after the initial count. The specific radioactivity of ACh in a sample was determined by assaying for radioactivity and ACh and then determin ng the percentage of the total radioactivity in the sample behaving as ACh on an ion-exchange column. Columns (9 cm. × 0·7 cm.) of the resin XE-97 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) buffered to pH 4·3 with 0·1 M-NaH_2PO_4 (Whittaker, 1963) were used. The ACh peak was eluted in 15 ml. Radioactive ACh of specific activity 32 μc/μmole was purchased from The Radiochemical Centre, Amersham, Bucks.

Determination of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, Sussex) as standard.

Determination of osmotically sensitive K^+ *and ACh.* Small columns (11 cm. high × 0·8 cm. diam.) containing 0·5 g. of Sephadex G-50 (bead form) were used. The iso-osmotic columns were equilibrated with and eluted with a sucrose solution iso-osmotic to that in which the synaptosomes or synaptic vesicles were suspended. This was 0·8 M for the former and 0·4 M for the latter. The hypo-osmotic columns were eluted with glass-distilled water. A sample (0·5 ml.) of the preparation was added to the top of the column and allowed to drain down; 0·8 ml. of eluting solution of the appropriate molarity was added to the top of the column and allowed to drain down similarly; then a further 1·0 ml. was added and the effluent collected, the whole procedure taking 3–5 min. The recovery of protein in the effluent was 80%, and that of unbound small molecules such as K^+, Na^+ and ACh was less than 1·0%. The columns were then washed with 50 ml. of eluting solution and used again. All column operations were conducted at 5°. The difference between the amount of ACh or K^+ present in the effluent from the column eluted iso-osmotically and that eluted hypo-osmotically is termed the osmotically sensitive ACh or K^+ content of the preparation. The specific radioactivity of osmotically sensitive ACh was calculated from the expression:

$$\frac{\text{Radioactivity due to ACh in iso-osmotic effluent} - \text{radioactivity due to ACh in hypo-osmotic effluent}}{\text{ACh in iso-osmotic effluent} - \text{ACh in hypo-osmotic effluent}}$$

The percentage exchange of added radioactive ACh with osmotically sensitive ACh under given conditions was calculated from the expression:

$$\frac{\text{Sp. activity of osmotically sensitive ACh}}{\text{Sp. activity of free ACh}} \times 100$$

The specific activity of free ACh in experiments in which free ACh was added to the preparation was measured as the specific activity of the total ACh, since osmotically sensitive ACh was less than 1% of the total added ACh. The initial rate of exchange is equivalent to the turnover rate.

RESULTS

Osmotically sensitive ACh and K^+ *of synaptosomes.* The osmotically sensitive ACh and K^+ of synaptosomes were determined immediately after isolation from the density gradient (Table 1). Synaptosomes contain ACh that is released by passage through the hypo-osmotic column. Treatment with ultrasound also releases the osmotically sensitive ACh of synaptosomes.

Compared with K^+ a substantially higher proportion of the ACh of synaptosomes is not released by hypo-osmotic shock or treatment with ultrasound. The behaviour of fraction P_2 in these types of experiment was found to be similar to that of fraction *B*.

The K^+ equilibrium volume of the synaptosome preparations was measured by equilibrating the preparation with 100mM-potassium chloride and determining the osmotically sensitive K^+; from this the volume enclosed by the synaptosome membrane can be calculated (Marchbanks, 1967*b*). From the K^+ equilibrium volume for the preparation, and assuming that only 15% of the synaptosomes are from cholinergic nerve cells (Whittaker & Sheridan, 1965), the concentration of synaptosomal osmotically sensitive ACh can be calculated to be approx. 0·4mM. On standing at 5° in 0·8M-sucrose, osmotically sensitive ACh is lost from synaptosomes. Typical values are: 80% of total remaining after 30min., 65% of total remaining after 60min.

Uptake of added ACh into synaptosomes. The permeability of the synaptosome membrane to ACh was studied by adding a high concentration of ACh (50mM) to a synaptosome preparation and observing the rate of increase of osmotically sensitive ACh at 5°. The experiment is complicated by the considerable cholinesterase activity of synaptosome preparations that destroys the added ACh. In Fig. 1 the disappearance of ACh from the preparation and the appearance of osmotically sensitive ACh are shown. The plot of percentage equilibrium volume exchanged against time is obtained by dividing the osmotically sensitive ACh by the external ACh concentration at that time and expressing this volume as a percentage of the K^+ equilibrium volume for that preparation. Also plotted in Fig. 1 is the percentage exchange of radioactive ACh with the osmotically sensitive ACh of the preparation when [*acetyl*-^{14}C]ACh (50μM) was added to the preparation.

The initial rates of exchange, and the percentage equilibrium volume exchanged, are equivalent to the turnover rate. The percentage turnover rate calculated from the percentage equilibrium volume exchanged is 0·65/min., and calculated from the percentage exchange it is 0·5/min. Eserine (10μM) had no effect on the rate of turnover. It is apparent that ACh can diffuse into synaptosomes, and its turnover rate is about 0·13 that of K^+ under similar conditions (Marchbanks, 1967*b*).

Separation of vesicular ACh. The effects of ions introduced in solution in the medium used for hypo-osmotic disruption of synaptosomes were examined in an effort to determine the optimum conditions for the isolation of synaptic vesicles. The P_2 pellet was hypo-osmotically disrupted by adding for each g. wet wt. of original tissue 2·0ml. of water along with additional ions. Of the disrupted material 2·0ml. was then passed through a column (1·7cm. × 10cm.) containing 2g. of Sephadex G-50 (bead form), which was then eluted with glass-distilled water. The macromolecular phase was collected in 5·0ml. and layered over a continuous gradient and the D_1 band (containing synaptic vesicles) isolated as described in the Methods section. Passage through the Sephadex column was found necessary to remove ions such as Ca^{2+} and Mg^{2+}, which would otherwise cause clumping of material on the density gradient.

It was found that treatment with 5mM-tris–

Table 1. *Comparison of osmotically sensitive bound ACh and* K^+ *in synaptosomes*

Preparation	Substance	Total (moles) in amount of preparation derived from 1g. wet wt. of fresh cortex	% of total in void volume of Sephadex column eluted with		Osmotically sensitive constituent as % of total (column 4 − column 5)
			0·8M-Sucrose	Water	
Synaptosomes (fraction *B*)	ACh	$2{\cdot}53 \times 10^{-9}$	78	17	61
	K^+	$8{\cdot}6 \times 10^{-6}$	46	5	41
Synaptosomes ultrasonically treated for 60sec.	ACh	—	18	17	1
	K^+	—	8	0	8

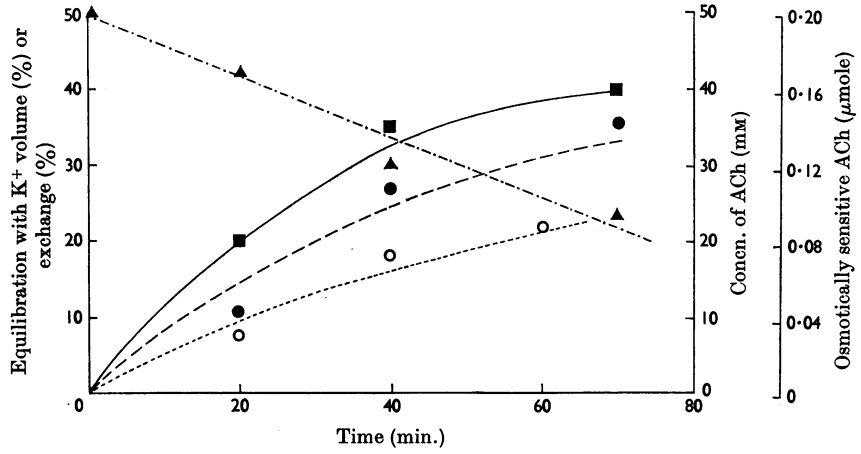


Fig. 1. Uptake of ACh into the osmotically sensitive compartment of synaptosomes on adding ACh to a synaptosome preparation at 5°. ▲, Concn. of ACh in suspension; ■, amount of osmotically sensitive ACh in that amount of preparation derived from 1g. wet wt. of cortex; ●, percentage K^+ equilibrium volume exchanged (see the text); ○, percentage radioactive exchange.

EDTA, pH 6·5, during hypo-osmotic disruption increased the amount of ACh found in the synaptic-vesicle fraction derived from 1g. wet wt. of cortex from a control value of 179 μμmoles [S.E.M. ± 105 (4)] to 760 μμmoles [S.E.M. ± 180 (4)]. This significant rise ($P < 0·05$, t test) in the amount of ACh was accompanied by smaller and less significant increases in the amounts of K^+ and protein found in the synaptic-vesicle fraction. The addition of Ca^{2+} (10mM) or Mg^{2+} (10mM) caused small decreases in the amounts of ACh, K^+ and protein found in the synaptic-vesicle fraction.

Ca^{2+} and Mg^{2+} were also found to lower the amounts of protein in the clear layer above the D_1 band (corresponding to fraction O of Whittaker *et al.* 1964). This suggests that the effect of Mg^{2+} and Ca^{2+} in decreasing the ACh amounts is connected with their ability to cause coacervation. Coacervation would result in material clumping or remaining inside the synaptosome (Salganicoff & De Robertis, 1965) and being carried further down the gradient, thus causing the low protein content in the O and D_1 fraction. The effect of EDTA is presumably to sequester endogenous Ca^{2+} and Mg^{2+}, thus preventing this action. When the P_2 fraction was subjected to ultrasonic treatment after hypo-osmotic shock the content of ACh in the synaptic-vesicle fraction was much the same as with EDTA treatment but the protein content was raised, presumably owing to increased contamination by membrane fragments.

About 98% of the ACh, 36% of the K^+, and 3·6% of the protein of the hypo-osmotically disrupted P_2 fraction after the Sephadex-column treatment were found in the isolated synaptic-vesicle fraction. The high recovery of ACh suggested that only the ACh of synaptic vesicles was found in the void volume when fraction P_2 was passed through a hypo-osmotically eluted Sephadex column. It seemed possible therefore that passage of fraction P_2 through a hypo-osmotic Sephadex column might produce a preparation of vesicular ACh relatively uncontaminated by other small-molecular-weight substances. However, the ratio of ACh to K^+ (mμmoles of ACh/μmoles of K^+) in fraction P_2 is 0·29. When fraction P_2 is hypo-osmotically disrupted in the presence of EDTA and passed through the Sephadex column the ratio in the void volume effluent was 0·62. In the synaptic-vesicle fraction isolated by density-gradient centrifugation the ratio was 3·5. This shows that the Sephadex-column treatment did not remove all the K^+ that could be removed by a further purification step, and therefore for the study of vesicular ACh isolation by density-gradient centrifugation is necessary.

Properties of vesicular ACh. In these experiments synaptic vesicles were prepared by the procedure discussed in the previous section, with 5mM-tris–EDTA, pH 6·5, in the hypo-osmotic suspension medium.

It was found that when synaptic vesicles were passed through Sephadex columns equilibrated and eluted with 0·4M-sucrose, which is iso-osmotic to the medium in which the vesicles are isolated, they retained their ACh, but when the columns were eluted with water (which is hypo-osmotic) the ACh was lost from the vesicles and retained on the

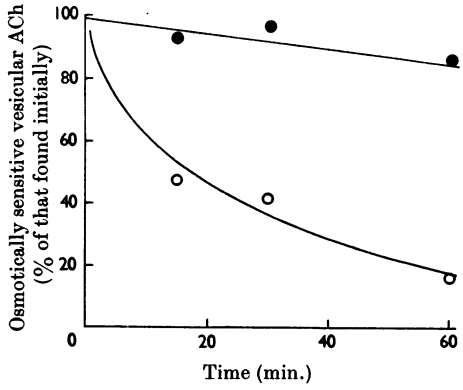


Fig. 2. Release of osmotically sensitive vesicular ACh on standing: ●, at 5°; ○, at 26°.

column. After isolation, therefore, vesicular ACh is osmotically sensitive.

Ultrasonic treatment for 1 min. had little effect in releasing ACh from vesicles. The amount of ACh recovered in the void-volume effluent of the columns eluted iso-osmotically was 75% of the total; since the recovery of protein in the same effluent was 90%, 84% of the total ACh found in the D_1 fraction was in a bound form. Osmotically sensitive vesicular ACh is not released on standing in 0·32 M-sucrose at 5°, but at 26° it is rapidly released (Fig. 2). The vesicles retain their characteristic morphology on passing through Sephadex columns (Fig. 3; compare with Plate 1c in Whittaker *et al.* 1964). Vesicular ACh is not easily sedimented; when an isolated synaptic-vesicle preparation was diluted so that the sucrose concentration was 0·2 M the pellet formed by centrifuging at 11 000*g* for 30 min. contained only 11% of the total ACh in the preparation. When the preparation was diluted and sedimented at 150 000*g* for 30 min. it contained 42% of the total ACh.

Exchangeability of vesicular ACh. The exchangeability of vesicular ACh was studied by adding radioactive ACh to a preparation of vesicles and incubating under the conditions shown in Table 3. The appearance of osmotically sensitive radioactivity was used as a criterion of exchange. Osmotically sensitive ACh and radioactivity were determined at the end of the incubation period in the usual manner. Free radioactive ACh was found to be reasonably stable when added to preparations of vesicles. The percentage remaining after 30 min. incubation at 5° varied from preparation to preparation but was usually above 50%. The results for percentage exchangeability shown in Table 3 were calculated on the assumption that all the osmotically sensitive radioactivity is ACh

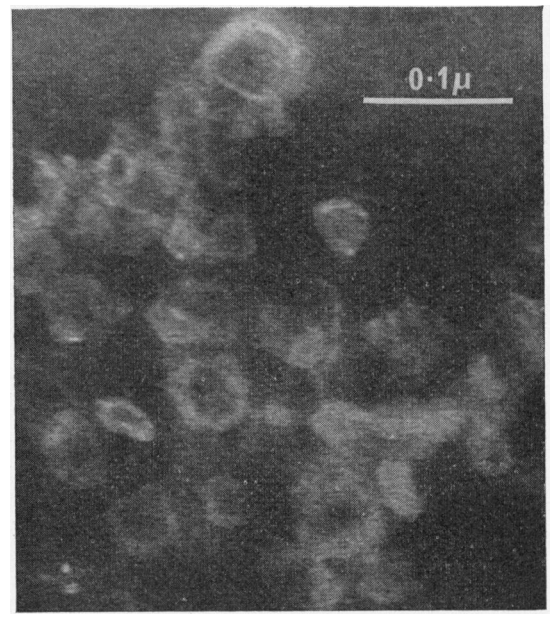


Fig. 3. Synaptic vesicles after passing through an iso-osmotic Sephadex column. Negatively stained; magnification × 195 000.

because amounts were too low to permit purification by column chromatography. They are therefore overestimates. As Table 3 shows, free ACh is hardly exchangeable with vesicular ACh either at 5° or 26°, nor is exchange stimulated by ATP and Mg^{2+} either at 5° or 26°.

An attempt was made to examine the possibility that exchange with vesicular ACh can take place at the moment of hypo-osmotic rupture of the synaptosome. [*methyl*-^{14}C]ACh was added with the hypo-osmotic resuspending medium and the preparation allowed to stand for 5 min. It was then passed through the Sephadex column and the vesicles were isolated in the usual way on the density gradient. In two experiments the average exchange was less than 0·25%.

Finally synaptosomes were loaded with radioactive ACh to test whether exchange with vesicular ACh can occur within the synaptosome. The [*methyl*-^{14}C]ACh (final concn. 10 μM) was added to intact synaptosomes (fraction P_2), which were then incubated for 30 min. at 23°. Various conditions were used: with and without the anti-cholinesterase eserine, with and without energy-producing substrates (glutamate, 10 mM; glucose, 10 mM; ATP, 10 mM; Mg^{2+}, 5 mM; Ca^{2+}, 3 mM). After incubation the P_2 suspension was spun down, the pellet was disrupted and vesicles were isolated in the usual way.

Table 2. *Osmotically sensitive ACh in synaptic vesicles*

Preparation	Substance	Total in amount of preparation derived from 1 g. wet wt. of fresh cortex	% of total in void volume of Sephadex columns eluted with		Osmotically sensitive constituent as % of total (column 4 − column 5)
			0·8 M-Sucrose	Water	
Synaptic vesicles (fraction D_1)	ACh	790 $\mu\mu$moles	75	16	59
	Protein	0·47 mg.	90	87	—
Synaptic vesicles ultrasonically treated for 60 sec.	ACh	—	74	10	64

Table 3. *Exchangeability of added radioactive ACh with vesicular ACh*

Conditions	No. of experiments	Type and amount of added ACh: Position of label	Concn. (μM)	% exchange in 30 min.
5°	3	[*methyl*-^{14}C]	5	< 2·8
26°	1	[*acetyl*-^{14}C]	20	0
5° + ATP (10 mM) + Mg^{2+} (5 mM)	1	[*acetyl*-^{14}C]	1	< 5·3
26° + ATP (10 mM) + Mg^{2+} (5 mM)	2	[*acetyl*-^{14}C]	50	< 1·3

To establish that radioactivity had entered the synaptosomes a sample of the P_2 suspension was withdrawn after the incubation and the osmotically sensitive radioactivity determined in the usual way. The specific activity of the osmotically sensitive radioactivity was not determined in these cases, but the synaptosomal volume equilibrated by the radioactivity was estimated by dividing the amount of osmotically sensitive radioactivity by the external concentration of radioactivity. The volume equilibrated was found to be greater than 80% of the K^+ equilibrium determined as described above. Even though radioactivity had penetrated the synaptosome exchange of radioactive ACh with the ACh of synaptic vesicles could not be detected (less than 0·5%).

In all these experiments osmotically sensitive vesicular ACh was found, so lack of exchange cannot be due to the destruction of the vesicular form. No increases of osmotically sensitive vesicular ACh took place as a result of the incubation with added free ACh. Though it is difficult to be certain that the conditions were optimum for the demonstration of exchange they include those used by other investigators in the study of neurotransmitters into their storage particles, e.g. by Burton (1964) and Kirshner (1962). It seems therefore that the differences in ACh exchangeability between the synaptosomal and vesicular compartment are genuine.

DISCUSSION

A substantial proportion of the synaptosomal ACh behaves as free ACh retained within the synaptosomal membrane. It is released by hypo-osmotic shock and ultrasonic treatment and is freely exchangeable with added free ACh. In this respect a proportion of synaptosomal ACh is similar to the osmotically sensitive K^+ of synaptosomes. However, some of the ACh of synaptosomes is not released by hypo-osmotic treatment, is insensitive to ultrasonic treatment and does not freely exchange with added ACh. The similarity of the rates of exchange when measured as the rate of radioactive ACh exchange, or the rate of K^+ equilibrium volume exchange by ACh, suggests that the compartment containing osmotically sensitive K^+ and that containing osmotically sensitive ACh are the same in synaptosomes.

Osmotically sensitive ACh is 60% of the total in synaptosomes. The labile bound ACh described by Whittaker *et al.* (1964) constitutes 50% of the total in synaptosomes. The osmotically sensitive ACh of synaptosomes is therefore thought to be

equivalent to the labile bound ACh of synaptosomes described by Whittaker *et al.* (1964).

In view of the failure of the cholinesterases in vesicle preparations to hydrolyse all of the free added ACh it is suggested that the ability of vesicles to carry their bound ACh through iso-osmotic Sephadex columns is a better criterion of bound ACh than the more usual one of immunity of the ACh from the action of endogenous cholinesterase.

The finding that vesicular ACh is osmotically sensitive was unexpected, since hypo-osmotic shock is used to rupture synaptosomes in the isolation procedure for synaptic vesicles.

It seems likely that during hypo-osmotic disruption of synaptosomes the synaptic vesicles inside them are protected from the suddenness of the osmotic change by the synaptosome membrane. Isolated vesicles are not protected in this way, and having re-equilibrated with their osmotic environment (0·4M-sucrose) during isolation on the density gradient they become susceptible to hypo-osmotic shock on the columns. The failure of ultrasonic treatment for 1 min. to rupture vesicles, in contrast with synaptosomes, is most likely to be due to the much greater energy required to rupture the smaller vesicles. Maynert, Levi & De Lorenzo (1964) have observed a similar difference of sensitivity between synaptosomes and noradrenaline-containing vesicles.

The rate of exchange of vesicular ACh is less than about one-fifth of the rate of exchange into the osmotically sensitive ACh of synaptosomes under similar conditions.

The exchange and uptake of the neurotransmitter noradrenaline into many types of storage particle have been observed into chromaffin granules (Kirshner, 1962; Carlsson, Hillarp & Waldeck, 1963), into amine granules from splenic nerve (Euler & Lishajko, 1963) and into a microvesicular brain fraction (Colburn & Maas, 1965). The failure in the present study to observe ACh exchange into vesicles under conditions that encompass those used by the above investigators suggests that the mechanism of ACh incorporation into vesicles is different from that for noradrenaline.

Burton (1964) has reported that synaptic vesicles take up ACh when incubated with free radioactive ACh in the presence of eserine. It seems possible that the discrepancy between his results and those reported in this investigation is due to the fact that the M_2 fraction (De Robertis, Arnaiz, Salganicoff, de Iraldi & Zieher, 1963) used in his studies is contaminated with synaptosomes (Whittaker *et al.* 1964) and that therefore the uptake observed by Burton (1964) was uptake into synaptosomes. Only 11% of the vesicular ACh could be sedimented at the centrifugal forces Burton (1964) used. This suggests that the particulate material that took up ACh was larger and heavier than synaptic vesicles.

The possibility that synaptic vesicles are small membrane fragments that encapsulate and occlude a portion of the synaptosomal cytoplasm during the hypo-osmotic treatment is ruled out by the failure of added ACh to exchange when added at the moment of hypo-osmotic disruption, or when synaptosomes are loaded with radioactive ACh before disruption.

The osmotic sensitivity of vesicles suggests that a membrane is involved in the retention of ACh. The concentration of ACh inside the vesicle has been estimated at 0·1M (MackIntosh, 1959) or at 0·15M (Whittaker & Sheridan, 1965). It has been estimated in this study that the concentration of ACh in the synaptosome cytoplasmic compartment is about 0·4mM. There are several possible explanations of the way the concentration of ACh is maintained inside the vesicle against this concentration gradient.

There may be an extremely active transport of ACh to maintain the concentration difference. However, this possibility is ruled out by the failure to observe exchange under conditions under which an active transport mechanism would be expected to be maintained. Even if the conditions used did not allow an active transport system to function some exchange should still be observable, since vesicular ACh was present in the preparation after the incubation period.

Alternatively, ACh in the vesicle may be in a bound form and thus not free to diffuse out. However, to explain the osmotic sensitivity of vesicular ACh the bound form must either be sufficiently small to penetrate the Sephadex grains in the column, or the stability of the bound form must be dependent on the integrity of the membrane such that it is irreversibly decomposed when the vesicular membrane is ruptured.

Finally, the vesicular membrane may be impermeable to ACh, thus preventing its escape, in which case the vesicular membrane is fundamentally different from the synaptosome membrane, which is permeable to ACh.

Even when synaptosomes are pre-loaded with radioactive ACh no exchange into the vesicular compartment occurs. This suggests that synaptic vesicles and vesicular ACh are already preformed in the synaptosome. Sattin (1966) has reported that ACh synthesis in brain slices influences the amounts of osmotically labile bound ACh more than those of stable bound ACh. Taken in conjunction with the results in this study this suggests that the synthesis of ACh is not necessarily accompanied by the formation of vesicular ACh.

It is concluded that the present results are con-

sistent with the existence of two distinct compartments of ACh within the synaptosome, as has already been proposed on chemical and morphological grounds. The osmotically sensitive ACh of synaptosomes is equivalent to the labile bound form described by Whittaker *et al.* (1964) and occupies the cytoplasmic compartment enclosed by the synaptosome membrane. Synaptic vesicles exist preformed in synaptosomes, and the ACh within them does not appear to exchange easily with ACh in the synaptosome cytoplasm or with added free radioactive ACh.

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