Biochemical Pharmacology, Vol. 16, pp. 921–923. Pergamon Press Ltd. 1967. Printed in Great Britain

PRELIMINARY COMMUNICATION

Compartmentation of acetylcholine in synaptosomes*

(*Received* 18 *January* 1967; *accepted* 16 *March* 1967)

WHEN brain tissue is homogenised in iso-osmotic sucrose the presynaptic nerve terminals are detached and may be isolated as discrete particles (synaptosomes) by differential and density gradient centrifugation.[1, 2] Acetylcholine is present in the preparation in a particle bound state in which it is pharmacologically inactive and immune from the action of cholinesterases until released by appropriate disruptive procedures. Disruption of synaptosomes by suspension in hypo-osmotic media, however releases only about 50 per cent of the bound acetylcholine; this fraction has been referred to as the 'labile bound' fraction and is believed to represent acetylcholine within the cytoplasm of synaptosomes derived from cholinergic neurons.[2] The remainder (the 'stable bound' fraction) is associated with synaptic vesicles which are in part released by hypo-osmotic treatment and can be isolated by sucrose density gradient fractionation of the disrupted synaptosomes.[3, 4]

The compartment enclosed by the synaptosome membrane has been investigated by gel filtration techniques using potassium as a cytoplasmic marker.[5] In this study these techniques have been used to investigate the compartmentation and exchangeability of acetylcholine within the synaptosome, and its relationship to the 'labile bound' and 'stable bound' forms described by Whittaker *et al.*[3]

Gel filtration was through small (11 × 0·8 cm) columns containing 0·5 g of G-50 Sephadex in bead form. After adding the suspension of synaptosomes or synaptic vesicles, prepared essentially as previously described[1, 4] the columns were eluted with a total of 1·8 ml of the medium with which the gel was equilibrated (sucrose or water). The effluent contained 80 per cent of the macromolecules and less than 1 per cent of the unbound small molecules of the original suspension and was taken to represent the void volume.

Acetylcholine was released from the bound state by heating samples at pH 4 and 100° for 10 min and assayed on small slips of the dorsal muscle of the leech,[6] previously sensitised with eserine sulphate (1 mg/100 ml) and mounted in a vertical organ bath of 0·1 ml capacity. The identity of the acetylcholine was checked by treating samples with cholinesterase and reassaying. The radioactivity of acetylcholine was separated from that of its hydrolysis products by chromatography on ion exchange columns.

Radioactivity was determined in an automatic scintillation counter using a dioxane-based scintillation medium. Potassium was determined by flame photometry. Protein was estimated by the method of Lowry *et al.*[7] using bovine plasma albumin as a standard.

When samples of synaptosomes equilibrated with 0·8 M sucrose during separation were submitted to gel filtration through columns previously equilibrated with 0·8M sucrose, 78 per cent of the acetyl choline of the preparation remained bound and was recovered in the void volume. When columns equilibrated with water were used 17 per cent so appeared; thus 22 per cent of the bound acetylcholine resisted osmotic stress imposed by gel filtration through a strongly hypo-osmotic column. With potassium the corresponding figure was 11 per cent. Similar experiments with isolated synaptic vesicles (*vide infra*) showed that vesicular bound acetylcholine was also partially released on passage through a hypo-osmotic column so that the osmotically sensitive bound acetylcholine of synaptosomes measured in this way probably includes some of the 'stable bound' fraction.

The penetrability of the synaptosome membrane to acetylcholine was studied by adding a high concentration (50mM) [acetyl-^{14}C] acetylcholine to a synaptosome preparation and observing the

* This work was supported in part by grant no. NB-03928 from the U.S. Public Health Service (to Dr. V. P. Whittaker) and was carried out during my tenure of a Medical Research Council Junior Fellowship. I am grateful to Dr. V. P. Whittaker for helpful discussion during all stages of this investigation, and to the able technical assistance of Miss L. Swales and Mr. N. J. Fincham.

rate of increase of osmotically sensitive bound acetylcholine and radioactivity by gel filtration. The potassium equilibrium volume of the preparation was determined as described previously.[5] The turnover rate of acetylcholine expressed as the percentage of the potassium equilibrium volume exchanged by acetylcholine per minute was 0·65 and as the percentage radioactive exchange per minute was 0·5. This is about one eighth that of potassium under similar conditions.[8] On standing at 5°, osmotically sensitive bound acetylcholine was lost from the synaptosome preparation about 60 per cent of the initial amount remaining after 60 min.

Synaptic vesicles were prepared as previously described,[4] except that EDTA (5mM) was added to the water (2 ml/g tissue) in which the synaptosomes were suspended for osmotic disruption. This increased the amounts of acetylcholine and protein recovered in the vesicle (D_1) fraction. Addition of Ca^{2+} and Mg^{2+} had the reverse effect and also caused coacervation of the vesicles; so that EDTA probably increased the yield of vesicles by sequestering endogenous Ca^{2+} and Mg^{2+} and promoting the release of vesicles in mono-disperse form.

When synaptic vesicle suspensions was passed through Sephadex columns equilibrated with sucrose of the same concentration (0·4M) as the region of the gradient in which the vesicles came to rest, 75 per cent of the acetylcholine of the preparation was recovered in the void volume. When however, columns equilibrated with water were used, about 75 per cent of the void volume acetylcholine disappeared showing that vesicle bound acetylcholine is also osmotically sensitive under these conditions. Evidently its ability to withstand osmotic stress during the initial hypo-osmotic disruption of synaptosomes is relative rather than absolute.

The ability of added radioactive acetylcholine to exchange with vesicle-bound acetylcholine was next examined. Vesicles were incubated with ^{14}C-acetylcholine at 5° and 26° with and without the addition of ATP (15mM) and Mg^{2+} (5mM) for up to 30 min and then passed through Sephadex columns as previously described. In all cases, vesicle bound acetylcholine was present at the end of the incubation period though there was never any increase over the initial endogeneous value. In no case did radioactive exchange exceed 5 per cent (ATP, Mg^{2+}, 5° for 30 min) and in most cases it was below 2 per cent. All these figures are maximum estimates since it was not possible to establish that the small amount of void-volume radioactivity was in fact from acetylcholine.

Incorporation of radioactivity into vesicular acetylcholine was not observed if radioactive acetylcholine was added when the synaptosomes were ruptured by suspension in hypo-osmotic media, nor was it found if the synaptosomes were preloaded with radioactive acetylcholine before rupture.

It seems clear from these experiments that acetylcholine exists in two compartments within the synaptosome, one readily exchangeable at a rate of about one-eighth that of potassium and one which cannot be exchanged with added acetylcholine. The exchangeable acetylcholine is probably to be identified with the labile-bound acetylcholine observed in earlier work and represents free cytoplasmic acetylcholine, whereas the non-exchangeable acetylcholine is that bound to or contained within synaptic vesicles.

Burton[9] has reported that a crude[4] (M_2) preparation of synaptic vesicles takes up radioactive acetylcholine. I found that only 11 per cent of vesicle-bound acetylcholine could be sedimented at the centrifugal speeds Burton used to separate his bound acetylcholine, which suggests that the material which took up acetylcholine was larger and heavier than synaptic vesicles and may well have been intact synaptosomes. Exchange and uptake of noradrenaline into its storage granules have been observed.[10, 11] The failure to observe acetylcholine exchange into vesicles under comparable conditions suggest that the mechanism of uptake of acetylcholine is different from that of noradrenaline.

Biochemistry Department,
Agricultural Research Council,
Institute of Animal Physiology,
Babraham Hall,
Cambridge

R. M. MARCHBANKS*

* Present address: Biochemistry Department, Tennis Court Road, University of Cambridge, Cambridge.

REFERENCES

1. E. G. GRAY and V. P. WHITTAKER, *J. Anat.* **96**, 79 (1962).
2. V. P. WHITTAKER, *Prog. Biophys. mol. Biol.* **15**, 39 (1965).
3. V. P. WHITTAKER, I. A. MICHAELSON and R. J. A. KIRKLAND, *Biochem. J.* **90**, 293 (1964).
4. V. P. WHITTAKER and M. N. SHERIDAN, *J. Neurochem.* **12**, 363 (1965).
5. R. M. MARCHBANKS, *Biochem. J.* **100**, 65P (1966).
6. J. C. SZERB, *J. Physiol.* **158**, 8P (1962).
7. D. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
8. R. M. MARCHBANKS, *Biochem. J.* in press.
9. R. M. BURTON, *Int. J. Neuropharmcc.* **3**, 13 (1964).
10. N. KIRSCHNER, *J. biol. Chem.* **237**, 2311 (1962).
11. A. CARLSSON, N. A. HILLARP and B. WALDECK, *Acta physiol. scand.* **59**, *suppl.* 215 (1963).