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The Storage and Release of Acetylcholine

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Preparations of detached presynaptic nerve terminals (synaptosomes) and synaptic vesicles from mammalian brain (Whittaker, 1965, 1969) have proved useful in unravelling the organization of the synapse, identifying the subcellular sites of transmitter synthesis and storage and characterizing the carrier-mediated uptake systems for transmitters and their precursors. For acetylcholine it has been shown that the transmitter is synthesized in the cytoplasm (Fonnum, 1967) from choline taken up by a carrier-mediated transport system in the terminal plasma membrane (Marchbanks, 1968b; Diamond & Kennedy, 1969) and is concentrated and stored in synaptic vesicles. The concentration of acetylcholine in the vesicles is estimated to be at least 200 mM (Whittaker & Sheridan, 1965) and in the cytoplasm to be no more than 3 mM and probably lower (0.4 mM; Marchbanks, 1968a). At these concentrations acetylcholine exerts negligible inhibitory effect on choline acetyltransferase, the enzyme synthesizing it (Glover & Potter, 1971; Morris *et al.*, 1971), and the regulation of acetylcholine synthesis is unlikely to be brought about, under physiological conditions, by acetylcholine inhibition of the synthetic pathway.

One of the limitations of work with synaptosomes and synaptic vesicles isolated from mammalian brain is that the preparations are derived from a mixed population of cholinergic and non-cholinergic nerve terminals in which the latter considerably outnumber the former and cannot be effectively separated from them (Whittaker, 1969). We have therefore been investigating tissues richer in cholinergic terminals than mammalian brain. Two tissues have proved particularly useful: the electric organs of elasmobranch fish belonging to the family Torpedinidae and the head ganglion of the squid (*Loligo pealii* and *Loligo forbesi*).

The electric organ of Torpedinidae has a purely cholinergic innervation, with acetylcholine concentrations of 200 nmol/g or more, i.e. over 15 times that of mammalian cerebral cortex. The tissue contains much collagen and is thus difficult to homogenize in the usual way; however, when crushed to a coarse powder after freezing in Freon 12, the portion of the

presynaptic terminal plasma membrane away from the synaptic cleft is stripped off, leaving an exposed layer of frozen terminal cytoplasm containing intact vesicles attached to morphologically well-preserved fragments of electroplaque cells (D. Soifer & V. P. Whittaker, unpublished work). The frozen cytoplasm and synaptic vesicles can then be extracted into a suitable extraction medium iso-osmotic with elasmobranch plasma (e.g. 0.2 M sucrose–0.3 M NaCl) with relatively little contamination from larger membrane fragments and separated by means of density-gradient centrifuging in a zonal rotor (Essman & Whittaker, 1970; V. P. Whittaker, W. B. Essman & G. H. C. Dowe, unpublished work). In this way milligram quantities of almost pure cholinergic synaptic vesicles may be obtained in a single experiment. Such vesicles contain up to 1300 nmol of acetylcholine/mg of protein and are recovered as a sharp band of density equivalent to that of 0.38 M sucrose–0.21 M NaCl.

Investigations, not yet complete, indicate (M. J. Dowdall, R. M. Facino & V. P. Whittaker, unpublished work) that the vesicles contain not more than four main protein components, three in the membrane and the fourth, accounting for over 50% of the total protein, in the core. After dialysis and freeze-drying, the core protein (vesiculin) is recovered as a protein of molecular weight approx. 10000 rich in acidic and hydroxy amino acids and bound to five or six nucleotide residues (Whittaker, 1971; Whittaker *et al.*, 1971). Freshly prepared vesicles contain considerable amounts of ATP and its breakdown product AMP (M. J. Dowdall, A. F. Boyne & V. P. Whittaker, unpublished work); the molar ratio of acetylcholine to ATP varies in different experiments and in different regions of the vesicle band from about 4:1 to 80:1. It is therefore probable that in the native vesicle the nucleotide bound to vesiculin is largely ATP. Vesiculin, by virtue of its content of nucleotide and acidic amino acid residues, must be strongly negatively charged, and calculations show (a) that the number of negative charges in the vesiculin–nucleotide complex is sufficient to neutralize the positive charges of the amount of acetylcholine cation (up to 24 nmol/mol of vesiculin) associated with it in the vesicle, and (b) that the vesiculin–ATP–acetylcholine complex could largely fill the vesicle core. Vesiculin is thus believed to serve as a non-diffusible polyanion whose presence in the vesicle core ensures the maintenance of the high local concentration of acetylcholine. Spontaneous or enzyme-catalysed hydrolysis of bound ATP would permit loss of vesicular acetylcholine by providing a readily diffusible small anion, i.e. P_i . This and other possible release mechanisms are being examined. The chromogranin–ATP–adrenaline complex in the chromaffin granule (for a review see Winkler, 1971) and the protein–heparin–histamine complex of

the mast-cell granule represent obvious parallels in other amine-storage granules.

Squid brain synaptosomes have been isolated (Dowdall & Whittaker, 1971) and found to contain about 60 times the concentration of acetylcholine in mammalian cortical synaptosome preparations. When incubated in sea-water with [^3H]choline (M. J. Dowdall & E. J. Simon, unpublished work) they take up the latter at about 100 times the rate of mammalian cortical synaptosomes by an Na^+ -dependent, cyanide-, dinitrophenol-, ouabain- and hemicholinium-sensitive carried-mediated uptake mechanism. Li^+ or K^+ cannot replace Na^+ . Kinetic analysis shows that two systems are apparently involved in choline uptake, a high-affinity ($K_m = 2-4 \mu\text{M}$) and a low affinity ($K_m > 24 \mu\text{M}$) system. It is thought that the high-affinity system may be that involved in the uptake of choline by cholinergic synaptosomes. Careful re-examination of published data for the uptake system in mammalian nervous tissue (Diamond & Kennedy, 1969; Adamič, 1970) suggests that here, too, two carriers may be involved, but the high-affinity system is largely masked by the low-affinity system, probably because of the low concentration of cholinergic terminals in these preparations. The rate of choline uptake declines in synaptosomes that have been stored for a few hours. Such synaptosomes show elevated cytoplasmic acetylcholine concentrations, and this suggests that choline uptake may be inhibited by acetylcholine in concentrations only a little higher than those normally found in the cytoplasm. If so, this may provide a clue to the regulation of acetylcholine synthesis in the terminal region. Externally applied acetylcholine is known to compete with choline for the choline carrier in mammalian synaptosomes (Marchbanks, 1969) and brain slices (E. Heilbronn, personal communication).

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Regulatory Significance of the Release and Action of Adenine Derivatives in Cerebral Systems

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Adenosine addition and electrical excitation are among the most effective means of augmenting the cyclic AMP (adenosine 3':5'-cyclic monophosphate) of tissues from the brain (Kakiuchi *et al.*, 1969; Sattin & Rall, 1970). The two are linked: electrical stimulation, among its other actions, releases adenosine from the tissues to extracellular fluids (McIlwain, 1971; Pull & McIlwain, 1972a,b). The present account takes for granted the involvement of cyclic AMP in metabolic regulation and queries: to what extent and in what manner may translocation of adenine derivatives play a part in conditioning the amounts and the actions of cyclic AMP, or in other aspects of metabolic regulation in the brain?

Accompanying the adenosine that appeared on excitation, and could be collected by superfusion, were adenine nucleotides, inosine and hypoxanthine (Pull & McIlwain, 1972a). Indeed, the nucleotides may be the form released, for rapid addition of AMP or of ATP during superfusion of neocortical tissues gave considerable conversion into adenosine, with some inosine and hypoxanthine though not adenine. Output of adenosine from the native or [^{14}C]adenine-labelled nucleotides increased with the frequency and duration of stimulating pulses; up to 60 nmol/g was collected as adenosine and some 160 nmol/g as adenosine plus inosine and hypoxanthine after 10 min excitation of guinea-pig cortical samples (Pull & McIlwain, 1972a, and unpublished work).

After the uptake of [^{14}C]adenine by incubated cerebral tissue, most ^{14}C is present in ATP (Santos *et al.*, 1968). The effluent adenosine and its metabolites from superfusion of such tissues were of low specific radioactivity, and increased several-fold on electrical excitation (I. Pull & H. McIlwain, unpublished work). Heterogeneity in pools of adenine derivatives in cerebral tissues has been demonstrated also by Sattin & Rall (1970) and Shimizu & Daly (1970). In particular, in such tissues, cyclic AMP was