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PRELIMINARY COMMUNICATION

The conversion of ^{14}C-choline to ^{14}C-acetylcholine in synaptosomes *in vitro**

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THE AVAILABILITY of isolated preparations of the synaptic region of the nerve cell makes it possible to study *in vitro* the biochemistry of processes associated with chemical transmission at the synapse. Pre-synaptic nerve terminals (synaptosomes) are isolated by homogenisation of cerebral cortical tissue followed by differential and density gradient centrifugation.[1] Synaptosomes as isolated, contain synaptic vesicles, sometimes mitochondria, and a sample of the pre-terminal cytoplasm enclosed within a sealed[2] membrane. The complement of cytoplasm and intra-terminal mitochondria is sufficient to maintain respiration and bring about the accumulation of high-energy phosphates.[3, 4]

When incubated with ^{14}C-choline synaptosomes take up the radioactive choline by a carrier mediated transport mechanism that is activated by sodium ions and inhibited by hemicholinium-3.[5, 6] The choline uptake process is qualitatively and quantitatively similar to the process as observed in more intact preparations such as erythrocytes[7] and cerebral cortex slices.[8]

Synaptosomes contain choline acetyltransferase[9] and it has been shown that acetylcholine is produced by the preparation if suitable substrates are added.[10] A proportion of the radioactive choline taken up by synaptosomes *in vitro* is converted to radioactive acetylcholine. This process has been studied in order to investigate acetylcholine synthesis and storage in the synaptic region without interference from other parts of the nerve cell and under conditions permitting greater experimental flexibility than is possible with more conventional physiological preparations.

A crude preparation of synaptosomes (fraction P_2 of Gray & Whittaker[11]) was generally used in these experiments, but it was shown (see below) that the acetylcholine synthesising capability of the preparation was completely due to synaptosomes rather than any contaminating material. After isolation the synaptosomes were incubated at 25° in a physiological medium (NaCl, 100 mM, KCl 5 mM, $MgCl_2$ 10 mM, $CaCl_2$ 0·6 mM, phosphate 1 mM, glucose 10 mM, succinate 10 mM, Tris-Cl pH 7·4 50 mM and small amounts of various cofactors[5]). Iso-osmolarity of the medium with the synaptosomes was maintained by the addition of sucrose when necessary. After 20 min pre-incubation ^{14}C-choline was added to give a final concentration 1–2 μc/ml and 20–40 μM. At 30 min (or other periods as necessary) after the addition of the radioactive choline the synaptosomes were separated from their incubation medium by passing them through small Sephadex columns eluted with 0·4 M sucrose.[2]

The void volume effluent containing some 80 per cent of the synaptosomal protein was adjusted with HCl to pH 4·0 and heated at 100° for 10 min to release bound acetylcholine. Acetylcholine was measured on a small strip of the dorsal muscle of the leech.[12, 13] The radioactivity due to choline and acetylcholine was separated and estimated by adding carrier amounts of choline and acetylcholine and then retaining choline bases on a small carboxylic acid ion exchange column to remove them from sucrose and other interfering substances. The bases were eluted off with 0·1 N HCl, concentrated by evaporation and acetylcholine and choline separated by thin-layer chromatography.[5, 14] The spots of separated choline and acetylcholine were identified by iodoplatinate reagent, scraped off and the radioactivity estimated in a scintillation spectrometer. Sometimes the sample was submitted to the action of acetylcholinesterase before chromatography, which resulted in the transfer of radioactivity from the acetylcholine to the choline position. The extraction and separation procedure caused 15 per cent hydrolysis (SEM $\pm$ 3, 5 experiments) of the ester and the overall recovery of authentic radioactive acetylcholine was 50 per cent (SEM $\pm$ 4, 3 experiments). The results are not corrected for these losses.

In this way the amount of radioactive choline taken up into the synaptosomes, the percentage conversion to radioactive acetylcholine and the specific activity of the acetylcholine within the synaptosome were estimated. Effects on acetylcholine synthesis can be distinguished from effects on

choline uptake by measuring the percentage conversion of ^{14}C-choline to ^{14}C-acetylcholine within the synaptosome.

At the end of a 30 min incubation the amount of ^{14}C-choline taken up by the synaptosomes in fraction P_2 was usually about 30,000 disintegrations/min per mg of protein. The percent conversion to ^{14}C-acetylcholine was 16·8 ± 1·5 (SEM, N = 20). The amount of acetylcholine found in the synaptosome was 242 ± 35 (SEM, N = 20) $\mu\mu$moles per mg of protein. The specific activity of radioactive acetylcholine after the 30 min incubation expressed as a percentage of the specific activity of the precursor ^{14}C-choline (i.e. percentage incorporation) was 14·3 ± 2 (SEM, N = 20). The percentage incorporation was about 8 per cent after 15 min incubation and 20 per cent after 60 min incubation.

The crude synaptosomal fraction was submitted to further density gradient centrifugation to yield purified myelin, synaptosomal and mitochondrial fractions, and their ability to synthesis acetylcholine was examined. The purified synaptosomal fraction accounted for 98 per cent of acetylcholine-synthesizing ability of the crude parent fraction. This suggests that synaptosomes are the only particle present in the crude fraction capable of converting ^{14}C-choline to ^{14}C-acetylcholine in this type of experiment.

The effects of various experimental conditions on the incorporation of ^{14}C-choline to ^{14}C-acetylcholine were examined. Some of the more notable effects are shown as a percentage of control values in Table 1. The presence of sodium ions was found to activate both choline transport across the

TABLE 1. EFFECTS OF IONS AND PHARMACOLOGICAL AGENTS ON ^{14}C-CHOLINE UPTAKE AND CONVERSION TO ^{14}C-ACETYLCHOLINE IN SYNAPTOSOMES

Conditions	As percentage of control values given in text	
	^{14}C-choline uptake	Conversion to ^{14}C-acetylcholine
− Na^+	*68 ± 6	*44 ± 6
− Na^+ + K^+ (100 mM)	*65 ± 3	*38 ± 8
+ K^+ (100 mM)	*58 ± 5	*81 ± 6
+ Oxo-tremorine (100 μM)	113 ± 11	*137 ± 14
+ Hemicholinium-3 (100 μM)	*45 ± 6	*53 ± 13
+ Ouabain (100 μM)	94 ± 4	118 ± 23

Means and standard errors of six experiments. * Indicates a significance of $P<0·05$ (t-test).

membrane, and also its conversion to acetylcholine. Sodium ions could not be replaced by potassium ions in order to stimulate acetylcholine synthesis. Addition of potassium ions in the presence of sodium ions inhibited choline uptake, but had a much smaller effect on acetylcholine synthesis.

Oxo-tremorine caused an increase in the conversion of choline to acetylcholine, while having little effect on choline uptake. Hemicholinium-3 inhibited both choline uptake and acetylcholine synthesis. Ouabain had little effect on either choline uptake or acetylcholine synthesis.

It was observed that several experimental conditions had marked effects on the total amounts of acetylcholine found in the synaptosomes after incubation. Potassium ions in particular caused a decrease in the amount of acetylcholine remaining after incubation. Changes in the total amounts of acetylcholine are being further investigated in order to determine whether the acetylcholine is released from the synaptosome into the incubation medium, or whether the changes are caused by other processes.

Acetylcholine exists within the synaptosome in two compartments; in the intra-synaptosomal cytoplasm and in the synaptic vesicles. The cytoplasmic acetylcholine is readily exchangeable with added acetylcholine and is lost when synaptosomes are osmotically shocked.[13, 15] The acetylcholine of synaptic vesicles survives osmotic shock of synaptosomes and is separated by a permeability barrier from the acetylcholine in the synaptosomal cytoplasm.

The compartment into which the newly synthesised acetylcholine enters was investigated by

incubating the synaptosomes for 30 min under the usual conditions. They were then rapidly spun down, submitted to hypo-osmotic "shock" and passed through a Sephadex column to remove interfering ions. Synaptic vesicles were then isolated from the hypo-osmotically shocked material by continuous density gradient centrifugation.[17]

The specific activities of acetylcholine in the parent synaptosome fraction, and in the isolated synaptic vesicle fraction were measured with the results (expressed as percentage incorporation) shown in Table 2. The amount of vesicular acetylcholine is less than that usually isolated from

TABLE 2. INCORPORATION OF ^{14}C-CHOLINE INTO THE ^{14}C-ACETYLCHOLINE OF SYNAPTOSOMES AND SYNAPTIC VESICLES AFTER 30 MIN INCUBATION

	Acetylcholine*	% Incorporation of ^{14}C into acetylcholine†
Synaptosomes	2375 ± 552	13·6 ± 4·5
Synaptic vesicles	476 ± 109	1·77 ± 0·71

Means and standard errors of seven experiments. The percent incorporation into synaptic vesicles expressed as a ratio of the incorporation into the synaptosomes in the same experiment was 0·15, SEM = ± 0·037 (n = 7, P < 0·01 *t*-test).

* Acetylcholine is expressed as $\mu\mu$moles in that amount of tissue derived from 1 gm wet wt. of cortex.

† % Incorporation is the specific activity of ^{14}C-acetylcholine expressed as a percentage of the specific activity of the precursor ^{14}C-choline.

unincubated synaptosomes presumably because of losses during the incubation, and also losses resulting from the Sephadex column treatment during the isolation. The radioactivity due to acetylcholine in the synaptic vesicle fraction was so low that the cholinesterase verification could not be carried out so the figure for specific activity is an upper estimate. It is clear that the incorporation of ^{14}C-choline into the acetylcholine of synaptic vesicles is much less than the incorporation into synaptosomes as a whole. Assuming that the amount of cytoplasmic acetylcholine is approximately equal to the amount of vesicular acetylcholine as is the case of unincubated synaptosomes,[15] it can be calculated that the rate of radioactive choline incorporation into the acetylcholine of vesicles is about 7 per cent of the rate of incorporation into the acetylcholine in the cytoplasm. However, if the figures in Table 2 represent the ratios of the amounts of acetylcholine in the cytoplasmic and vesicular compartments at the termination of the incubation period, a similar calculation shown that the rate of incorporation into vesicles is about 11 per cent of the rate into cytoplasm.

These results show that the pre-terminal region of the nerve cell has the capability to synthesise acetylcholine and that the synaptosome preparation is metabolically competent to do so. The necessity for sodium ions for synthesis of acetylcholine in the perfused superior cervical ganglion has been observed by Birks.[18] Sodium ions do not specifically activate choline acetyltransferase,[19, 20] so the effect of sodium must be related to aspects of the cellular organisation. Oxo-tremorine has been shown to raise acetylcholine levels when injected *in vivo*.[21] However this effect cannot be accounted for by any actions on isolated choline acetyltransferase or acetylcholinesterase[21] so presumably the activation effect of oxo-tremorine on acetylcholine synthesis also depends on the retention of some degree of cellular organisation. The existence of these effects on acetylcholine synthesis in synaptosomes *in vitro* indicates that the preparation has retained these aspects of cellular organisation and is behaving physiologically by these criteria.

The failure of synaptic vesicles to incorporate ^{14}C-acetylcholine may be due to a deficiency in the experimental arrangements, but this seems unlikely considering the facility with which synaptosomes synthesise acetylcholine, and the similarity of the process to that observed in more intact preparations. Reports that vesicular acetylcholine can be formed from ^{14}C-choline in synaptosomes,[6] or by uptake of ^{14}C-acetylcholine by isolated synaptic vesicles do not[22, 23] include measurements of the specific radioactivity of the vesicular acetylcholine and are therefore difficult to compare with the results in this investigation.

It is possible that synaptic vesicles containing recently formed acetylcholine are extremely unstable such that they do not survive the isolation procedure. This is not a very satisfactory explanation but it cannot be rigorously excluded. Alternatively it may be that acetylcholine is only very slowly or not at all incorporated into synaptic vesicles in the pre-terminal region of the nerve cell. These possibilities are under further investigation.

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