Compartmentation and regulation of acetylcholine synthesis at the synapse

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Acetylcholine and choline release was measured by using an automated and modified version of the chemiluminescence technique of Israel & Lesbats [(1981) *Neurochem. Int.* 3, 81–90]. A comparison of acetylcholine and choline release from synaptosomes demonstrated that acetylcholine release was K⁺-stimulated and inhibited by the Ca²⁺ ionophore A23187 and cyanide. Choline release, however, did not vary markedly under different conditions, suggesting that it is not associated with acetylcholine release at the nerve ending. Total acetylcholine synthesis in synaptosomal preparations was measured concurrently with the incorporation of [¹⁴C]acetyl and [³H]choline moieties by using the chemiluminescence method. Under sub-optimal glucose concentrations or in the absence of treatment of the synaptosomes with the acetylcholinesterase inhibitor phospholine, the incorporation of radioactivity exceeded total synthesis, indicating that cycling between acetylcholine and its precursors may occur. After treatment with phospholine, acetyl-group incorporation from D-[U-¹⁴C]glucose occurred without dilution of the precursor at optimal (1.0 mM) and low (0.1 mM) glucose concentrations; however, at very low (0.01 mM) glucose concentrations, dilution by a small endogenous pool occurred. [¹⁴C]Acetyl incorporation into acetylcholine was compared with various metabolic parameters. A closer correlation was observed between [¹⁴C]acetyl-group incorporation into acetylcholine and the calculated acetyl-carrier efflux from the mitochondria than with the calculated pyruvate-dehydrogenase-complex flux. The results are discussed with respect to the regulation of acetylcholine concentrations at the synapse and the mechanism whereby turnover occurs.

INTRODUCTION

The use of synaptosomes (nerve-ending particles) to study molecular events at the synapse is now well established. They may be considered as model nerve-ending cells that may be depolarized by electrical or ionic means and the subsequent biochemical events such as neurotransmitter release, inactivation and resynthesis studied (Whittaker, 1984).

Cholinergic neurons synthesize acetylcholine from choline and acetyl-CoA by means of the enzyme choline acetyltransferase (EC 2.3.1.6) located in the cytosolic compartment of the nerve ending (Tuček, 1978). Glucose and pyruvate are the main precursors of acetyl-CoA in adult mammalian brain (Browning & Shulman, 1968; see Clark *et al.*, 1982, for review), although ketone bodies (3-hydroxybutyrate, acetoacetate) may also contribute in the neonatal brain (Gibson & Blass, 1979; Patel & Clark, 1981; Sterling *et al.*, 1981). The precise mechanism whereby the acetyl moiety for acetylcholine synthesis is transported from the mitochondrial compartment to the cytosol is still unclear, given that the mitochondrial membrane is impermeable to acetyl-CoA. Current evidence suggests that citrate probably acts as the main carrier for the 'acetyl' moiety of acetylcholine across the mitochondrial membrane. However, a number of other possible candidates have been proposed (Tuček, 1978;

Benjamin & Quastel, 1981; Dolezal & Tuček, 1981; Clark *et al.*, 1982).

Choline is also required for acetylcholine synthesis, and is thought to enter the synaptosomes by a high-affinity carrier (see Kuhar & Murrin, 1978, for review). It has been suggested, however, that the intrasynaptosomal choline pool may be functionally compartmented, and the relationship between high-affinity choline uptake and acetylcholine synthesis *de novo* has been the subject of considerable controversy (Barker & Mittag, 1975; Kessler & Marchbanks, 1979; Marchbanks & Kessler, 1982; for review, see Barker *et al.*, 1978, and Jope, 1979b). The actual regulation of the synthesis of new acetylcholine after depolarization and release is thought to be controlled by mass-action effects on the choline acetyltransferase equilibrium (Tuček, 1985). This concept is, however, complicated by the relationship between free (cytosolic) acetylcholine and that which is vesicularized (Whittaker, 1984) and uncertainty as to which of these two sources actually provides the acetylcholine that is released (Suszkiw & O'Leary, 1982).

Studies in this laboratory have centred around the effects that decreased glucose and oxygen availability have on brain function, particularly acetylcholine synthesis, using the synaptosomal model. In previous studies, acetylcholine synthesis was estimated by incorporation of [¹⁴C]acetyl groups derived from glucose into

Abbreviations used: SRA, specific radioactivity; PDHc, pyruvate dehydrogenase complex; GABA, γ -aminobutyric acid

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acetylcholine without measurement of the total acetylcholine content. It was found that the incorporation of [^{14}C]acetyl groups from glucose into acetylcholine in non-depolarized synaptosomes showed a low apparent K_m for glucose (Harvey *et al.*, 1982) and that at glucose concentrations optimal (1 mM) for acetylcholine synthesis, vulnerability of synaptosomes to anoxia is a function of their state of polarization (Harvey *et al.*, 1983).

In the present study, both the incorporation of [^{14}C]acetyl groups (derived from glucose) into acetylcholine and the absolute acetylcholine levels have been assessed at both optimal and sub-optimal glucose concentrations in depolarized and non-depolarized synaptosomes. This has permitted a clearer understanding of the rate of synthesis of acetylcholine and its turnover under conditions of depolarization and restricted glucose availability. Furthermore, by utilizing glucose labelled in different positions, an attempt has been made to rationalize changes in acetyl-group incorporation into acetylcholine with other metabolic fluxes and parameters.

In order to measure absolute levels of acetylcholine at the same time as precursor incorporation, the chemiluminescence technique of measuring acetylcholine (choline) first described by Israel & Lesbats (1981) has been modified and automated to measure both the content and rate of release of acetylcholine (choline) from synaptosomes. Since there have been some recent reports expressing difficulties in using this method (Alberch *et al.*, 1985), the modifications are described in detail. At the same time as measuring acetyl-group incorporation into acetylcholine, the rate of [^3H]choline incorporation, as well as endogenous choline production by synaptosomal preparations, have also been assessed.

MATERIALS AND METHODS

Chemicals

Acetylcholine chloride, choline chloride, neostigmine methyl sulphate, choline oxidase (*Alcaligenes* spp.), horseradish peroxidase (type II) and acetylcholinesterase (electric-eel type V1-S) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. The acetylcholinesterase was purified by the method detailed in Israel & Lesbats (1982). Acetyl [*methyl- ^3H*] choline chloride (70–90 mCi/mmol) used for recovery estimations in the tissue-extraction methods was purchased from Amersham International, Amersham, Bucks., U.K., and was purified as described by Fonnum (1969). KIO_3 was purchased from BDH, Poole, Dorset, U.K. Luminol (3-aminophthalhydrazide), tetraphenylboron and heptan-3-one were purchased from Aldrich, Gillingham, Dorset, U.K. All other chemicals were of the highest grade available and all solutions were made up in double-glass-distilled water. Phospholine iodide was generously given by Dr. A. P. Kapadia of Ayerst Laboratories, Andover, Hants, U.K.

Experimental protocols

Male rats (50–60 days old) of the Wistar strain were used for all experiments and were permitted free access to food and water until 30 min before being killed.

Synaptosomes were prepared by the method of Booth & Clark (1978a), using the whole forebrain, and were suspended in a modified Krebs–Henseleit saline [136 mM-NaCl/5.6 mM-KCl/1.2 mM- MgCl_2 /16.2 mM- NaHCO_3 /1.2 mM- NaH_2PO_4 /10 mM-sodium Hepes (all final

concns.), pH 7.4] at an approximate concentration of 10 mg of protein/ml. For most experiments the synaptosomes were incubated for 15 min at 4 °C in the saline medium containing 12.5 μM -(final concn.) phospholine and then were washed by centrifugation and resuspension three times in saline (0.9% NaCl) without phospholine to remove excess phospholine. Untreated synaptosomes were incubated and washed in the same way in saline without the added phospholine. Phospholine treatment of synaptosomal preparations completely inhibited endogenous acetylcholinesterase activity (results not shown), but after the washing procedure, endogenously added acetylcholinesterase was still active.

The synaptosomes were then incubated in the following way: 0.25 ml aliquots of the final synaptosomal suspension (2–3 mg of protein) were added to 1 ml of Krebs–Henseleit/Hepes medium (KHH medium) in a conical flask. The KHH medium contained additions to provide (final concns. in mM): Ca^{2+} , 2.2; glucose, 1, 0.1 or 0.01; choline, 0.1 or 0.01; veratrine, 0 or 0.4. All flasks were incubated with shaking at 37 °C for various times and 0.25 ml of 20% (w/v) trichloroacetic acid was injected to stop the reaction. After centrifugation (9800 g , 15 min) of the acid-quenched suspension, the clear supernatant was washed with water-saturated diethyl ether until its pH rose to 5, and excess diethyl ether was removed under vacuum. Samples were then removed for either chemiluminescence assay or radiochemical analysis. Proteins were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Statistical analysis was by the Student's two-way unpaired t test.

Chemiluminescence assay

Aliquots (100 μl) of the synaptosomal incubation mixture for chemiluminescence assay were oxidized by the addition of 10 μl of 0.5% (w/v) KIO_3 for 20 min before the addition of 900 μl of chemiluminescence buffer cocktail [0.2 M-sodium phosphate (pH 8.6)/16 μM -luminol/peroxidase (0.3 unit/ml)/choline oxidase (1 unit/ml)(all final concns.)]. The excess KIO_3 , which interacts directly with the luminol, and H_2O_2 generated from the added choline present in the incubation medium caused a protracted burst of chemiluminescence, which was allowed to die out before further additions.

To measure acetylcholine, acetylcholinesterase was added to the sample to give a final concentration of 0.6 unit/ml. If choline was to be measured before measurements of acetylcholine, then the choline oxidase was omitted from the buffer cocktail and an equivalent activity injected separately. When this reaction had been completed, the acetylcholinesterase was added for acetylcholine measurement. All assays in the luminometer were at 25 °C.

The LKB 1251 luminometer (LKB Wallac, Turku, Finland), which holds up to 25 samples in a temperature-controlled carousel, was used for the experiments described. The sample, when located in the photomultiplier chamber, may be stirred, and additions made to it from any of three automatic dispensers. The machine's onboard memory can store simple manipulative programs. For greater flexibility the machine was controlled by an Apple microcomputer, which was programmed to evaluate and store the data in real time and initiate, continue or terminate a particular reaction according to

Scheme 1. Experimental protocol

Abbreviations used: CHOX, choline oxidase; ACHE, acetylcholinesterase; CH, choline; ACH, acetylcholine.

programmed mathematical constraints. Thus the Apple IIe/LKB 1251 system was able to search for a sample whose initial light output had fallen to a stable baseline and automatically make sequential injections of reagents to that sample before passing to another. Alternatively, the machine could be programmed to measure the light output from more than one sample at timed intervals, and in this way construct a progress curve for each sample.

It should be noted that the amplified voltage measured with this technique is proportional to the current (rate of electron flow) through the photomultiplier tube and thus the rate of photon emission by the sample. The peak voltage signal (Israel & Lesbats, 1981) reflects the maximum rate of photon emission and thus, within certain limits, the concentration of the substance to be assayed. However the total amount is more correctly proportional in all experiments to the time-integrated voltage signal and its value has been used in all experiments. Acetylcholine (choline) was determined by peak integration and quantified by subsequent injection of an authentic standard into the same cuvette.

Radiochemistry

Radiochemical analysis was undertaken by using exactly the same buffer and enzyme (choline oxidase, acetylcholinesterase) concentrations as those used for chemiluminescence. Three types of radioactive incubation were carried out by using (a) [U-¹⁴C]glucose (5 μ Ci/ μ mol), (b) [U-¹⁴C]glucose (5 μ Ci/ μ mol) and [methyl-³H]choline (0.5 μ Ci/ μ mol) or (c) specifically labelled glucose (0.5 μ Ci/ μ mol). After incubation and treatment with trichloroacetic acid and diethyl ether as described above,

aliquots were neutralized by the addition of a 10-fold excess of 200 mM-sodium phosphate buffer, pH 8.6 (see Scheme 1). When [U-¹⁴C]glucose alone had been used as substrate, extracts were divided in two: one half was untreated (estimate of [¹⁴C]acetylcholine) and the other treated with acetylcholinesterase (radioactive blank). When [methyl-³H]choline was present as an additional substrate, the extract was divided into three parts: one was treated with acetylcholinesterase alone (total choline estimate and hence free choline recovery), one was treated with choline oxidase alone (acetylcholine estimate) and the third treated with choline oxidase and acetylcholinesterase (radioactive blank) (see Scheme 1). After enzyme treatments, which were for 1 h at room temperature, each aqueous phase was extracted with 1 ml of heptan-3-one, containing 15 mg of tetraphenylboron, and 0.7 ml of the organic phase was counted for radioactivity with a Phillips PW 4700 liquid-scintillation counter. Where [³H]acetylcholine (20nCi in trichloroacetic acid extract) had been injected, i.e. radioactive incubation (a), it was used as a recovery standard for [¹⁴C]acetylcholine. Where [³H]choline was present, it was used as a recovery standard on the basis that, under these conditions, it is extracted with only 40% the efficiency of acetylcholine (Fonnum, 1969). Recovery of authentic radioactive standard in the extraction process confirmed this ratio. Routinely, > 93% of the [methyl-³H]choline treated with choline oxidase was not extracted by the procedure of Fonnum (1969), as the product ([³H]betaine) has no net charge at neutral pH. Choline oxidase treatment is therefore equivalent to the use of choline kinase and ATP to separate [³H]choline from [acetyl-³H]choline (Vocci *et al.*, 1979), but is technically easier to perform.

RESULTS

Preliminary studies

The chemiluminescence method was validated by repeating the measurement of acetylcholine content in regions of the brain. Striatal and cerebellar measurements of acetylcholine of 28.4 and 2.1 nmol/g wet wt. in our hands were essentially the same as values determined by Israel & Lesbats (1982) of 30 and 1.6 nmol/g wet wt. respectively. There is also a good correlation between our regional acetylcholine content and those of Spector *et al.* (1978) [$r = 0.99$, $P = 0.0004$]. Continuous release of acetylcholine from striatal slices was also measured and found to be K^+ -stimulated and Ca^{2+} -dependent as described by Israel & Lesbats (1982) (results not shown). Continuous monitoring of acetylcholine release from synaptosomes could not be reliably performed owing to choline release from the preparation, which was apparent even in the presence of phospholine. A modified procedure was therefore used which involved taking samples of the synaptosomal incubation mixture at timed intervals.

Choline release

Figs. 1(a) and 1(b) show the release of acetylcholine and choline in different incubation conditions. Fig. 1(a) shows the release of acetylcholine from synaptosomes, demonstrating the characteristic stimulation by high KCl (35.6 mM) compared with the resting condition at low KCl (5.6 mM) and also the effect of KCN (1 mM), a potent metabolic inhibitor and the Ca^{2+} ionophore A23187 (5 μ g/mg of protein). The results with KCN show a complete inhibition of acetylcholine release between 1 and 15 min and a negligible release between 15 and 30 min. The effect of ionophore A23187 at the concentration used was to inhibit completely acetylcholine release, indicating its Ca^{2+} -dependence. In contrast with this, the choline release from synaptosomes shown in Fig. 1(b) is not affected to any great extent by any of the conditions that affect acetylcholine release, i.e. plus added KCN or ionophore A23187. These results, coupled with the order-of-magnitude difference between the choline and acetylcholine released, imply that the choline efflux is not

Fig. 1. Acetylcholine and choline release from synaptosomes under different conditions

Preincubations of phospholine-treated synaptosomes were carried out in the modified Krebs–Henseleit medium containing (final concns.) $CaCl_2$ (2.2 mM) and glucose (1 mM) at 37 °C in a shaking water bath. The incubation conditions were varied; the synaptosomes were incubated with either a high KCl concentration (35.6 mM) (○), a low KCl concentration (5.6 mM) (●) or in the presence of KCN (1 mM) (△) or the ionophore A23187 (5 μ g/mg of protein) and 5 mM-EGTA (□). Aliquots of the synaptosomal incubation were taken at timed intervals and the synaptosomes were separated from the medium by rapid centrifugation (9800 g for 30 s). Samples of the supernatant were then added to the chemiluminescence cocktail for assay. Results are expressed as pmol·mg of synaptosomal protein⁻¹ and are means $\pm$ S.E.M. (bars) for three to five assays. (a) Acetylcholine release; (b), choline release.

Fig. 2. Total acetylcholine synthesis and [^{14}C]acetyl incorporation from [$\text{U-}^{14}\text{C}$]glucose of synaptosomes incubated at optimal glucose and choline concentrations

Synaptosomes were prepared from rat forebrains as described in Materials and methods section and either phospholine-treated or control-washed. The synaptosomal preparations were then incubated in the presence of 1.0 mM-glucose and 0.1 mM-choline under conditions of basal ($\bullet$) (control) and maximal ($\blacktriangle$) (veratrine added) Na^+ conductance for times ranging between 0 and 40 min. Total acetylcholine content and [^{14}C]acetyl incorporation were determined as described in the Materials and methods section. (a) and (b) show the relationship between total acetylcholine content (expressed as pmol/mg of protein) and [^{14}C]acetyl incorporation (expressed as pmol/min per mg of protein) into acetylcholine. (a) Shows synaptosomes that have been phospholine-treated. Regression analysis of (a) gives the following values: control ($\bullet$): $m = 0.725 \pm 0.194$, $r = 0.60$, $c = 136 \pm 45$, $p = 0.12\%$ and $n = 27$; veratrine ($\blacktriangle$) added: $m = 0.755 \pm 0.252$, $r = 0.56$, $c = 122 \pm 19$, $p = 0.9\%$ and $n = 22$ (m = slope, r = correlation, c = intercept of y -axis coefficient, p = probability factor and n = number of determinations). (b) shows synaptosomes that have not been phospholine-treated. Regression analysis of (b) gives the following values: control ($\bullet$): $m = 0.327 \pm 0.161$, $r = 0.41$, $c = 80 \pm 21$, $p = 6.2\%$ and $n = 22$; veratrine ($\blacktriangle$) added: $m = 0.620 \pm 0.206$, $r = -0.54$, $c = 137 \pm 18$, $p = 0.8\%$ and $n = 24$. (c) Shows the relationship between net acetylcholine synthesis and incorporation of [^{14}C]acetyl groups into acetylcholine under veratrine-stimulated and control conditions in the presence and absence of phospholine treatment. The results are derived from (a) and (b) and additional data from experiments where total acetylcholine and [^{14}C]acetyl incorporation into acetylcholine were determined individually and are expressed as means with s.e.m. (bars) for n values between 21 and 24. V, veratrine- and non-phospholine treated, PV, phospholine- and veratrine-treated, C, untreated by either veratrine or phospholine; and P, phospholine-treated only.

associated with neurotransmitter release and may be due to degradation of choline-containing compounds such as phospholipids.

Optimal acetylcholine synthesis

Synaptosomes were incubated under the optimal metabolic conditions (1 mM-glucose, 100 μM -choline and 2.2 mM- Ca^{2+}) for the synthesis of acetylcholine. Measurements were made under conditions reflecting the maximum (phospholine-treated) and physiological (untreated) rates of synthesis, at both basal and maximal (veratrine added) rates of Na^+ conductance.

Figs. 2(a) and 2(b) show the regression analysis of simultaneous determinations of [^{14}C]acetyl incorporation and net acetylcholine synthesis after different incubation times. Phospholine-treated synaptosomes (Fig. 2a) show similar correlation coefficients and regression equations whether or not veratrine is present; in both cases the regression equations suggest that the rate of [^{14}C]acetyl incorporation slightly exceeds the rate of acetylcholine synthesis. Synaptosomes untreated with phospholine (Fig. 2b) show a variable acetylcholine synthesis. The released transmitter will be hydrolysed and will not be detectable. However, it is apparent that the addition of veratrine gives a considerably different regression equation, with [^{14}C]acetyl-group incorporation occurring despite a net decrease in the acetylcholine content.

From the data in Figs. 2(a) and 2(b) and additional data from experiments where [^{14}C]acetyl incorporation or net synthesis was measured individually, rates were calculated and plotted in Fig. 2(c). This shows the stoichiometry of incorporation and synthesis under different conditions. In the presence of phospholine (points P and PV in Fig. 2c) the results agree with those of Browning & Schulman (1968) and Lefresne *et al.* (1973, 1977) in that they showed that acetyl groups derived from glucose do not undergo dilution by endogenous pools before incorporation into acetylcholine. That in the absence of phospholine (points U and V in Fig. 2c) the rates of incorporation exceed those of synthesis does not conflict with this conclusion.

The incorporation of [^3H]choline under similar conditions to those in Fig. 2 is qualitatively similar (Fig. 3) to that of the acetyl moiety, with the exception that noticeable dilution occurs when phospholine alone is present (Fig. 3c). Fig. 4 illustrates more clearly the relationship between [^{14}C]acetyl and [^3H]choline incorporation under optimal conditions.

Sub-optimal acetylcholine synthesis

In this separate series of experiments, all synaptosomes were treated with phospholine, so that the total (intra-plus extra-synaptosomal) acetylcholine was measured. Glucose was decreased from 1.0 mM to 0.1 or 0.01 mM

Fig. 3. Total acetylcholine synthesis and $[^3\text{H}]$ choline incorporation of synaptosomes incubated at optimal glucose and choline concentrations

All conditions were as in Fig. 2, except that $[^3\text{H}]$ choline was used instead of $[^{14}\text{C}]$ glucose. (a) and (b) show the relationship between total acetylcholine content (expressed as pmol/mg of synaptosomal protein) and $[^3\text{H}]$ choline incorporation into acetylcholine (expressed as pmol/min per mg of synaptosomal protein). Synaptosomes were incubated under conditions of basal (●) (control) and maximal (▲) (veratrine added) Na^+ conductance at time points ranging between 0 and 40 min. (a) Shows synaptosomes that have been phospholine-treated. Regression analysis of (a) gives the following values: control (●): $m = 1.87 \pm 0.424$, $r = 0.68$, $c = 67 \pm 49$, $p = 0.03\%$ and $n = 25$; veratrine added (▲): $m = 1.486 \pm 0.494$, $r = 0.59$, $c = 63 \pm 33$, $p = 1.0\%$ and $n = 19$ (the parameters being defined in Fig. 2). (b) Shows synaptosomes that have not been phospholine-treated. Regression analysis of (b) gives the following values: control (●): $m = 0.186 \pm 0.278$, $r = 0.15$, $c = 107 \pm 23$, $p = 52.3\%$ and $n = 21$; veratrine added (▲): $m = 0.065 \pm 0.074$, $r = -0.22$, $c = 62 \pm 8$, $p = 41\%$, $n = 16$. (c) Shows the relationship between net acetylcholine synthesis and incorporation of $[^3\text{H}]$ choline groups into acetylcholine under veratrine-stimulated and control conditions in the presence and absence of phospholine treatment. The results are derived from (a) and (b) and additional data where total acetylcholine and $[^3\text{H}]$ choline were determined individually and expressed as means with S.E.M. (bars) for n values between 15 and 24. V, veratrine- and non-phospholine-treated; PV, veratrine- and phospholine-treated; U, untreated with phospholine or veratrine; P, phospholine-treated only.

Fig. 4. Relationship between $[^{14}\text{C}]$ acetyl and $[^3\text{H}]$ choline incorporation in synaptosomes

The Figure shows the relationship between $[^3\text{H}]$ choline and $[^{14}\text{C}]$ acetyl incorporation into acetylcholine using values derived from Figs. 2 and 3. The results are expressed as means with S.E.M. (bars) for n values between 15 and 24. V,

and in some experiments choline was reduced from 0.1 to 0.01 mM. At a given glucose concentration, decrease in choline concentration was found not to affect total acetylcholine synthesis significantly.

Table 1 shows the $[^{14}\text{C}]$ acetyl incorporation and total synthesis at different glucose concentrations. Results are expressed as percentages of the values obtained when using phospholine-treated synaptosomes metabolizing 1.0 mM-glucose (see Fig. 2a). In the absence of veratrine, significant ($P < 0.01$) parallel decreases occur in the rates of both $[^{14}\text{C}]$ acetyl incorporation and total synthesis, firstly when glucose is decreased from 1.0 mM to 0.1 mM, and again when it is decreased from 0.1 mM to 0.01 mM. The addition of veratrine causes significant ($P < 0.02$) decrease in $[^{14}\text{C}]$ acetyl incorporation and total synthesis at 1.0 mM- and 0.1 mM-glucose, and significant decrease in $[^{14}\text{C}]$ incorporation at 0.01 mM glucose. In the absence of veratrine, there are no significant differences seen in $[^3\text{H}]$ choline incorporation as the concentration of glucose is decreased. Whereas veratrine significantly ($P < 0.03$) decreases $[^3\text{H}]$ choline incorporation at 1.0 mM- and 0.1 mM-glucose, there are no significant differences between the 'veratrine-added' incorporation values at

veratrine-treated and non-phospholine-treated; PV, veratrine- and phospholine-treated; U, untreated with phospholine and veratrine; and P, phospholine-treated only.

Table 1. Total acetylcholine synthesis and [^{14}C]acetyl incorporation from [^{14}C]glucose of synaptosomes incubated at sub-optimal glucose concentrations

Synaptosomes were prepared from rat forebrains, phospholine-treated, and incubated with [^{14}C]glucose at sub-optimal glucose concentrations for acetylcholine synthesis in the presence and absence of veratrine. All other conditions were as in Fig. 2. Values are expressed as percentages ($\pm$ S.E.M.) of the mean value at 1.0 mM-glucose in the absence of veratrine (the numbers of independent determinations are given in parentheses).

[Glucose] (mM)	[Veratrine] (μM)	[^{14}C]Glucose incorporation	[^3H]Choline incorporation	Total acetylcholine synthesis
1.0	0	100 $\pm$ 0.9(21)	100 $\pm$ 0.6(23)	100 $\pm$ 1.1(24)
1.0	400	33.7 $\pm$ 3.9(24)	59 $\pm$ 10.9(19)	29.9 $\pm$ 5.5(24)
0.1	0	58.8 $\pm$ 10.3(10)	112 $\pm$ 15.6(6)	61.2 $\pm$ 7.1(6)
0.1	400	7.3 $\pm$ 1.7(10)	49.2 $\pm$ 20.3(8)	4.9 $\pm$ 2.3(6)
0.01	0	5.4 $\pm$ 0.9(9)	59.1 $\pm$ 23(8)	-16.6 $\pm$ 10.1(6)
0.01	400	0.2 $\pm$ 0.1(9)	24.2 $\pm$ 21(8)	-13.5 $\pm$ 11.6(6)

different glucose concentrations. The absence of significance of some changes at 0.01 mM-glucose is due to low absolute mean values at this substrate concentration.

Optimal and sub-optimal glucose oxidation

Synaptosomes were incubated with glucose labelled at different positions in the molecule. The amount of $^{14}\text{CO}_2$ generated from each form is found by calculating the amount of glucose oxidized using the SRA of the substrate, and then multiplying by 6 for the U-labelled form and 1 for all others. Since carbon atoms 2 and 5 are considered to be equivalent, the $^{14}\text{CO}_2$ output from the C-2-labelled glucose was multiplied by 2 to allow for the generation of unlabelled CO_2 from C-5 position.

From the data obtained (Table 2) the apparent fluxes in Table 3 were calculated. The flux through PDHc was calculated as the difference between $^{14}\text{CO}_2$ from [$\text{U-}^{14}\text{C}$]glucose output and the sum of outputs from [$1\text{-}^{14}\text{C}$]-, [$2\text{-}^{14}\text{C}$]- and [$6\text{-}^{14}\text{C}$]glucose. PDHc flux can be measured by using [$3,4\text{-}^{14}\text{C}$]glucose, but the procedure used here is more economical. From the data of Hotherhall *et al.* (1981) it can be shown that, in brain slices, PDHc flux calculated by difference (as used in the present paper) represents $97 \pm 5\%$ (mean $\pm$ S.E.M., $n = 4$) of PDHc measured with [$3,4\text{-}^{14}\text{C}$]glucose. Ksiezak & Gibson (1981) suggested that the difference between PDHc flux ([$3,4\text{-}^{14}\text{C}$]glucose $\rightarrow$ $^{14}\text{CO}_2$) and the rate of citric-acid-cycle activity ([$2\text{-}^{14}\text{C}$]glucose $\rightarrow$ $^{14}\text{CO}_2$) may be used as an approximation of the rate of export of acetyl groups to the cytosol in the form of citrate, isocitrate or 2-oxoglutarate. In addition, Hotherhall *et al.* (1981), who used similar preparations, suggested that the difference between the $^{14}\text{CO}_2$ output from [$2\text{-}^{14}\text{C}$]glucose and [$6\text{-}^{14}\text{C}$]glucose represents an approximation to the flux through the GABA-shunt pathway. The 'GABA-glutamate' pathway was calculated as the difference between the $^{14}\text{CO}_2$ output from the [$2\text{-}^{14}\text{C}$]glucose and the sum of the [$1\text{-}^{14}\text{C}$]- and [$6\text{-}^{14}\text{C}$]glucose, since a high flux through this pathway will cause a preferential release of $^{14}\text{CO}_2$ from [$2\text{-}^{14}\text{C}$]glucose (Hotherhall *et al.*, 1981).

From the data in Table 2, a decrease in glucose from 1.0 mM to 0.1 mM or the addition of veratrine at a given glucose concentration significantly ($P < 0.005$) changes the $^{14}\text{CO}_2$ output from a given position in the molecule. The exception is C-1-labelled glucose, where a decrease in substrate concentration has no effect, nor does veratrine

Table 2. $^{14}\text{CO}_2$ release from synaptosomes incubated with glucose labelled at different positions

Synaptosomes were prepared from cerebral cortex and incubated as described in the Materials and methods section without phospholine treatment. Results are expressed in pmol/min per mg of synaptosomal protein and are means ($\pm$ S.E.M.) of duplicate or triplicate determinations in at least three separate experiments (no. of expts. in parentheses).

Position of label	[Glucose] (mM)	$^{14}\text{CO}_2$ output (pmol/min per mg of protein)	
		Control	+ 400 μM - Veratrine
Uniform	1.0	915 $\pm$ 289(18)	2077 $\pm$ 656(14)
	0.1	637 $\pm$ 191(17)	480 $\pm$ 155(11)
	0.01	146 $\pm$ 13(4)	46 $\pm$ 6(3)
C-2	1.0	141 $\pm$ 23(11)	829 $\pm$ 170(10)
	0.1	91 $\pm$ 50(15)	143 $\pm$ 30(30)
	0.01	13 $\pm$ 3(4)	8 $\pm$ 1(4)
C-6	1.0	14.6 $\pm$ 4.9(17)	165 $\pm$ 52(14)
	0.1	7.4 $\pm$ 4.2(14)	26.1 $\pm$ 8(13)
	0.01	1.8 $\pm$ 0.8(4)	1.6 $\pm$ 0.6(4)
C-1	1.0	35.3 $\pm$ 13.6(10)	151 $\pm$ 40(9)
	0.1	26.7 $\pm$ 6.8(8)	36.6 $\pm$ 9.7(7)

addition, at 0.1 mM-glucose. Further significant reductions ($P < 0.02$) in $^{14}\text{CO}_2$ output are seen when glucose is decreased from 0.1 mM to 0.01 mM. At this concentration veratrine affects only $^{14}\text{CO}_2$ from U- ^{14}C -labelled glucose.

At the optimal glucose concentration, flux through the PDHc step (Table 3) under conditions of basal Na^+ conductance is 78% of the flux at maximal (veratrine added) Na^+ conductance. This agrees with the data of Schaffer & Olson (1980), who showed that PDHc flux increased 27% on depolarization of synaptosomes, and also with the native/maximal ratio of activity of PDHc in isolated brain mitochondria (Booth *et al.*, 1978b). At sub-optimal glucose concentrations, however, PDHc flux decreases on addition of veratrine. Oxidative reactions of the citric acid cycle ($^{14}\text{CO}_2$ from 2- or 6-positions) (Table 2) behave in a similar fashion to PDHc. At 1.0 mM-glucose these fluxes are greatly increased by veratrine,

Table 3. Apparent fluxes through various pathways in synaptosomes

The fluxes through various pathways were determined from data derived from Table 2. Flux through PDHc 4-carbon output (GABA-glutamate shunt) and hexose-monophosphate-pathway fluxes were calculated by using methods derived from Hothershall *et al.* (1981) (see the text). Acetyl-carrier export was calculated from a method derived from Ksiezak & Gibson (1981) (see the text). $[U-(2+1+6)] = {}^{14}\text{CO}_2$ output from $[U-{}^{14}\text{C}]\text{glucose} - \Sigma({}^{14}\text{CO}_2 \text{ output from } [1-{}^{14}\text{C}]\text{glucose} + [2-{}^{14}\text{C}]\text{glucose} + [6-{}^{14}\text{C}]\text{glucose})$; $\text{PDHc}-2 = {}^{14}\text{C}$ flux through $\text{PDHc}-2 \times ({}^{14}\text{CO}_2 \text{ output from } [2-{}^{14}\text{C}]\text{glucose})$; $[2-(1+6)] = ({}^{14}\text{CO}_2 \text{ output from } [2-{}^{14}\text{C}]\text{glucose}) \times 2 - \Sigma({}^{14}\text{CO}_2 \text{ output from } [1-{}^{14}\text{C}]\text{glucose} + [6-{}^{14}\text{C}]\text{glucose})$; $[1-6] = {}^{14}\text{CO}_2$ output from $[1-{}^{14}\text{C}]\text{glucose} - {}^{14}\text{CO}_2$ output from $[6-{}^{14}\text{C}]\text{glucose}$.

Pathway	[Glucose] (mM)	Flux (pmol of ${}^{14}\text{C}$ /min per mg of protein)	
		Control	+400 μM -Veratrine
PDHc $[U-(2+1+6)]$	1.0	725	931
	0.1	512	274
	0.01	129	36
Acetyl-carrier export $[\text{PDHc}-2]$	1.0	584	102
	0.1	421	131
	0.01	116	28
4-Carbon output 'GABA shunt' $[2-(1+6)]$	1.0	91	513
	0.1	57	80
	0.01	11	6
Hexose monophosphate pathway $[1-6]$	1.0	21	0
	0.1	19	0

whereas at low substrate concentrations smaller increases, or decreases, are seen. The consequences of this are (Table 3) that decreasing the glucose concentrations appears to diminish the rate of acetyl-carrier export, and that veratrine depolarization at any given glucose concentration further diminishes that export. The addition of veratrine at 1.0 mM- or 0.1 mM-glucose was consistent with an apparent increase in flux through the 'GABA shunt' as estimated by these experiments. The percentage of $[{}^{14}\text{C}]\text{acetyl}$ incorporation into acetylcholine (Table 1) was correlated with the apparent fluxes calculated in Table 3. Correlation coefficients (r) and probability values (P) with $n = 6$ in each case were as follows: $r = 0.71$, $P = 0.19$ (PDH); $r = 0.95$, $P = 0.01$ (acetyl-carrier export); and $r = 0.13$, $P = 0.84$ (GABA shunt).

DISCUSSION

In these experiments, choline concentrations (1 μM) approximating to the K_m of the high-affinity carrier have not been used since, under those conditions, added choline would be subject by choline efflux to considerable dilution before uptake (see Fig. 1b). At high external concentrations of choline, as used in these experiments, non-specific choline uptake will occur in addition to that mediated by the high-affinity system. Uptake of choline (approx. 30 pmol/min per mg of protein; Marchbanks & Kessler, 1982) will approximately equal the production of

choline from the preparation (37 pmol/min per mg of protein; J. Willoughby, unpublished work). Thus fractional turnover of the endogenous pool (1169 pmol/min per mg of protein; Jope 1979a; 1427 pmol/min per mg of protein, J. Willoughby, unpublished work) would be $2.4\% \cdot \text{min}^{-1}$. It can be calculated that, after 30 min (a typical incubation time), the relative SRA of the intrasynaptosomal choline pool will be 0.52. If cholineacetyltransferase catalyses a bi-directional reaction, then the pools of acetylcholine and choline would be at equilibrium, permitting a continuous updating of the specific radioactivity of the choline moiety of acetylcholine. Under these conditions it would be expected that the calculated relative SRA of the choline pool of 0.52 would be equal to the apparent dilution of the $[{}^3\text{H}]\text{choline}$ moiety of acetylcholine (Fig. 3c). If, however, choline acetyltransferase were unidirectional, a continuous updating would not occur and these values would not agree, since acetylcholine synthesized early in the incubation will contain little $[{}^3\text{H}]\text{acetylcholine}$. The fact that there is good agreement between the apparent dilution of both the choline pool and the acetylcholine pool is consistent with the choline acetyltransferase reaction being bidirectional. The apparent rate of $[{}^3\text{H}]\text{choline}$ incorporation will depend solely on the final SRA of cytosolic choline (a function of the passive rates of exchange of choline; see above) and the relative sizes of the cytosolic choline and acetylcholine pools. Conditions that decrease cytosolic acetylcholine, such as treatment with veratrine (Figs. 2 and 3) and/or 0.01 mM-glucose, will therefore decrease the apparent rate of incorporation of $[{}^3\text{H}]\text{choline}$, since the percentage of total internal choline moieties in acetylcholine will be diminished.

Veratrine depolarizes the synaptosome (Ohta *et al.*, 1973), and this might be expected to increase acetylcholine release and resynthesis. However, in brain slices, the total content of acetylcholine is decreased by treatment with (proto)veratrine (Grewaal & Quastel, 1973). The data in Fig. 2(c) indicate decreases in both total acetylcholine synthesis and $[{}^{14}\text{C}]\text{acetyl-group}$ incorporation in the presence of veratrine when synaptosomes that had been treated with phospholine were used. These results are consistent with the decrease in apparent acetyl-group efflux shown in Table 3, and suggest that veratrine also acts to limit acetyl-group supply for acetylcholine efflux. A similar relative effect of veratrine on $[{}^{14}\text{C}]\text{acetyl-group}$ incorporation into acetylcholine and total acetylcholine synthesis is also apparent in synaptosomal preparations that were not treated with phospholine (Fig. 2c). In addition, rates of incorporation of $[{}^{14}\text{C}]\text{acetyl}$ and $[{}^3\text{H}]\text{choline}$ moieties respond in the same way to phospholine treatment and/or veratrine addition (Fig. 4).

It is consistently apparent from studies both in slices (Browning & Shulman, 1968; Lefresne *et al.*, 1973) and synaptosomes (Lefresne *et al.*, 1977) that radioactive precursors contribute to the acetyl moiety of acetylcholine without dilution. Further, in all these reports the SRA of acetylcholine slightly exceeds that of the exogenous glucose or pyruvate substrates. Browning & Shulman (1968) attributed this to a factor in extracts of brain slices that inhibited their bioassay of acetylcholine. An alternative explanation is that reaction cycling causes the incorporation of radioactivity into acetylcholine to be greater than net synthesis.

There are two possibilities to explain this cycling; the

first is that cycling might occur through reversal of the choline acetyltransferase equilibrium (whose K_{eq} is small enough to permit this; Glover & Potter, 1971; Pielik & Guynn, 1975; Marchbanks & Kessler, 1982); the second explanation is that cycling occurs via an internal cholinesterase activity, if the choline acetyltransferase reaction is considered to be effectively unidirectional (Carrol & Benishin, 1984). The second alternative differs in that cycling will generate acetate, which cannot be used to resynthesize acetylcholine (Tuček & Cheng, 1974) and also that cycling will only occur in the absence of an anti-cholinesterase such as phospholine. The conditions in our experiments are such that there is no acetylcholinesterase activity after phospholine treatment but measurable activity in the absence of phospholine treatment. Our results show that, at optimal (1 mM) glucose concentrations, insignificant cycling occurs in the presence of phospholine, but that marked cycling occurs in its absence (Fig. 2c). This suggests that, at optimal glucose concentrations, cycling is mainly due to the activity of an internal cholinesterase, although the possibility of a limited amount of cycling by the choline acetyltransferase equilibrium cannot be excluded. However, at sub-optimal glucose concentrations (0.1 and 0.01 mM; see Table 1), cycling does occur in the presence of phospholine, suggesting cycling due to the choline acetyltransferase equilibrium (Table 1). It is possible that, *in vivo*, both mechanisms may be involved and will be dependent upon glucose concentrations and the systems such as the blood barrier and the hexokinase activity, which regulate glucose levels in the brain.

Early work in this field (Gibson *et al.*, 1975) showed that, under certain conditions, acetylcholine synthesis was directly correlated with flux through PDHc, a control point in oxidative metabolism. However, treatments *in vitro*, such as decreasing glucose (Harvey *et al.*, 1982), low pH (Harvey *et al.*, 1983) or the addition of an uncoupler (Ksiezak & Gibson, 1981) or veratrine (Harvey *et al.*, 1982), were found to affect acetylcholine synthesis and oxidative metabolism disproportionately. The suggestion (Ksiezak & Gibson, 1981) that this might be due to a decreased ability of the synaptic mitochondrion to export acetyl units is strongly supported by the present data. Studies utilizing the present method for concurrent assay of total synthesis and precursor incorporation and rapid methods of separating the mitochondrial/cytosolic compartments of the synaptosomes (Booth & Clark, 1979) should lead to a fuller understanding of acetylcholine compartments at pathologically low glucose concentrations.

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REFERENCES

- Alberch, J., Marsal, J. & Solsona, C. (1985) *Brain Res.* **346**, 353–356
- Barker, L. A. & Mittag, T. W. (1975) *J. Pharmacol. Exp. Ther.* **192**, 86–94
- Barker, L. A., Mittag, T. W. & Krespan, B. (1978) *Cholinergic Mechanisms and Psychopharmacology*, pp. 465–480, Plenum Press, New York
- Benjamin, A. & Quastel, J. (1981) *Science* **213**, 1495–1497
- Booth, R. F. G. & Clark, J. B. (1978a) *Biochem. J.* **176**, 365–370
- Booth, R. F. G. & Clark, J. B. (1978b) *J. Neurochem.* **30**, 1003–1008
- Booth, R. F. G. & Clark, J. B. (1979) *FEBS Lett.* **107**, 387–392
- Browning, E. T. & Schulman, M. P. (1968) *J. Neurochem.* **15**, 1391–1405
- Carrol, P. T. & Benishin, C. G. (1984) *Brain Res.* **291**, 261–272
- Clark, J. B., Booth, R. F. G., Harvey, S. A. K., Leong, S. F. & Patel, T. B. (1982) in *Neurotransmitter Interaction and Compartmentation* (Bradford, H. F., ed.) (NATO Advanced Study Institutes Series, A48), pp. 431–460, Plenum Press, New York
- Dolezal, D. & Tuček, S. (1981) *J. Neurochem.* **36**, 1323–1330
- Fonnum, F. (1969) *Biochem. J.* **113**, 291–298
- Gibson, G. E. & Blass, J. P. (1979) *Biochem. Pharmacol.* **28**, 133–140
- Gibson, G. E., Jope, R. & Blass, J. P. (1975) *Biochem. J.* **148**, 17–23
- Glover, V. A. S. & Potter, L. T. (1971) *J. Neurochem.* **18**, 571–580
- Grewaal, D. S. & Quastel, J. H. (1973) *Biochem. J.* **132**, 1–14
- Harvey, S. A. K., Leong, S. F. & Clark, J. B. (1982) *J. Neurochem.* **39**, 1481–1484
- Harvey, S. A. K., Booth, R. F. G. & Clark, J. B. (1983) *Biochem. J.* **212**, 289–295
- Hothershall, J. S., Zubairu, S., McLean, P. & Greenbaum, A. L. (1981) *J. Neurochem.* **37**, 1484–1496
- Israel, M. & Lesbats, B. (1981) *Neurochem. Int.* **3**, 81–90
- Israel, M. & Lesbats, B. (1982) *J. Neurochem.* **39**, 248–250
- Jope, R. S. (1979a) *J. Neurochem.* **33**, 487–495
- Jope, R. S. (1979b) *Brain Res. Rev.* **1**, 313–344
- Kessler, P. & Marchbanks, R. M. (1979) *Nature (London)* **279**, 542–544
- Ksiezak, H. & Gibson, G. E. (1981) *J. Neurochem.* **37**, 88–94
- Kuhar, M. J. & Murrin, L. C. (1978) *J. Neurochem.* **30**, 15–21
- Lefresne, P., Guyenet, P. & Glowinski, J. (1973) *J. Neurochem.* **20**, 1083–1097
- Lefresne, P., Hamon, M., Beaujouan, J. C. & Glowinski, J. (1977) *Biochimie* **59**, 197–215
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marchbanks, R. M. & Kessler, P. D. (1982) *J. Neurochem.* **39**, 1424–1433
- Patel, T. B. & Clark, J. B. (1981) *Biochem. Biophys. Acta.* **677**, 373–380
- Pielik, J. R. & Guynn, R. W. (1975) *J. Biol. Chem.* **250**, 4445–4450
- Ohta, M., Narahashi, T. & Keeler, R. F. (1973) *J. Pharmacol. Exp. Ther.* **184**, 143–154
- Schaffer, W. T. & Olson, M. S. (1980) *Biochem. J.* **192**, 741–751
- Spector, S., Felix, A., Seminuk, G. & Findberg, J. P. M. (1978) *J. Neurochem.* **30**, 685–689
- Sterling, G. H., McCafferty, M. J. & O'Neill, J. J. (1981) *J. Neurochem.* **37**, 1250–1259
- Susziw, J. B. & O'Leary, M. E. (1982) *J. Neurochem.* **38**, 1668–1676
- Tuček, S. (1978) *Acetylcholine Synthesis in Neurons*, Chapman and Hall, London
- Tuček, S. (1985) *J. Neurochem.* **44**, 11–24
- Tuček, S. & Cheng, S. C. (1974) *J. Neurochem.* **22**, 893–914
- Vocci, F. J. (Jr), Karbowski, M. J. & Dewey, W. L. (1979) *J. Neurochem.* **32**, 1417–1422
- Whittaker, V. P. (1984) *Handb. Neurochem.* (2nd Ed.) **7**, 1–40