The Uptake of [^{14}C]Choline into Synaptosomes *in vitro*

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1. The uptake of [^{14}C]choline into synaptosomes *in vitro* was investigated by a gel-filtration method. Synaptosomes incubated in a medium fortified with glucose and succinate rapidly take up [^{14}C]choline. 2. A substantial proportion of the radioactivity taken up can be released by osmotic shock, and is recoverable as choline on a thin-layer chromatogram. This suggests that choline is taken up across the limiting membrane into the cytoplasmic compartment of the synaptosome. 3. The concentration of choline in the synaptosome has a dependence on the external concentration of choline that is similar to that in erythrocytes and mouse cerebral-cortex slices. The choline influx has two components, one that is linear and one that is saturable with increasing choline concentration. 4. Omission of Na^+ from the incubation medium, or addition of 100mM-K^+ , inhibits choline uptake. Hemicholinium no. 3 is a powerful inhibitor of the choline uptake. 5. The similarity of the choline-uptake process in synaptosomes to that in erythrocytes and cortex slices indicates that the synaptosome limiting membrane is functionally competent in this respect.

Synaptosomes are presynaptic nerve terminals that have been detached by homogenization of brain cerebral-cortical tissue and isolated by differential and density-gradient centrifugation (see review by Whittaker, 1965). The limiting membrane of the synaptosome appears to be completely sealed (Marchbanks, 1967). By utilizing the osmotic sensitivity of synaptosomes a gel-filtration procedure has been devised to measure the volume (Marchbanks, 1966) and contents enclosed by the synaptosome limiting membrane. It is thus possible to study *in vitro* the processes taking place across the membrane of this part of the nerve cell.

Synaptosomes usually contain one or more mitochondria, and since they can respire with glucose as sole substrate (Marchbanks & Whittaker, 1967) they presumably contain a full complement of the glycolytic enzymes. On incubation with glucose and succinate the intrasynaptosomal concentration of ATP and phosphocreatine rises (R. M. Marchbanks, unpublished work). Choline is concentrated by incubated mouse cerebral-cortex slices (Schuberth, Sundwall, Sörbo & Lindell, 1966) and taken up by a carrier system in the squid axon (Hodgkin & Martin, 1965). Active uptake of choline has also been demonstrated in the kidney (Sung & Johnstone, 1965) and across the erythrocyte membrane (Martin, 1968). To examine a functional property of the synaptosome membrane, the uptake of [^{14}C]choline into synaptosomes was investigated.

An additional reason for interest in choline uptake in the synaptosome is that choline is a precursor of the chemical transmitter acetylcholine.

EXPERIMENTAL

Preparation and treatment of subcellular fractions

Preparation of subcellular fractions. For experimental convenience a crude synaptosomal preparation (contaminated with some mitochondria and myelin) was used in these experiments. This was fraction P_2 of Gray & Whittaker (1962). That the choline uptake found in this preparation was due to synaptosomes is shown in the Results section. Guinea-pig cerebral cortex scraped to remove white matter was homogenized in 0.32M-sucrose (brought to pH 6.5 with NaOH) in a pestle-and-mortar-type homogenizer to give a 10% (w/v) homogenate. The clearance between pestle (diam. 29.5 mm.) and mortar was 0.25 mm. and the pestle was rotated at 850 rev./min. The homogenate was centrifuged at $1000g$ for 10 min., the pellet was resuspended in a small volume of 0.32M-sucrose and centrifuged again at $1000g$, and the supernatant fluids from both spins were combined. These were centrifuged at $10000g$ for 20 min., and the pellet was resuspended with 2 ml. of 0.32M-sucrose/g. of original tissue. The suspension was then centrifuged at $10000g$ for 30 min. The pellet (fraction P_2) derived from 1 g. wet wt. of original tissue was resuspended in 2–5 ml. of the appropriate medium and used for experiments immediately.

Further purification of synaptosomes was carried out by discontinuous density-gradient centrifugation. A portion (6–8 ml.) of fraction P_2 resuspended in 0.32M-sucrose or otherwise treated was layered on top of a sucrose density gradient composed of 10 ml. of 1.2M-sucrose and 10 ml. of

0.8M-sucrose. The gradient was spun for 2 hr. at 53000g. The purified synaptosome layer (fraction B; Gray & Whittaker, 1962) was separated from the myelin layer (fraction A) and the mitochondrial pellet (fraction C) by sectioning the centrifuge tube.

Incubation medium. Unless otherwise stated synaptosomes were incubated in the following medium (final concns.): NaCl, 100mM; KCl, 5mM; tris-HCl buffer, pH 7.4, 50mM; MgCl₂, 10mM; CaCl₂, 1mM; tris dihydrogen phosphate, 1mM; glucose, 10mM; sodium succinate, 10mM; ADP, 0.3mM; pyridoxal phosphate, 0.02mM; CoA, 0.01mM; NAD, 0.01mM; NADH₂, 0.03mM; GSH, 0.5mM; lipoic acid, 0.01mM; thiamine phosphate, 0.01mM. A medium of this composition had been found to give optimum respiration rates. Sucrose was added if necessary to maintain the osmolarity of the medium at 320–420 milliosmolar. All incubations were carried out at 25° rather than 37° to minimize the rupturing of synaptosomes that takes place at the higher temperature. The synaptosomes were usually preincubated for 20 min. in the medium (with additions as specified) before [¹⁴C]choline was added.

Gel filtration of synaptosomes. The content of small-molecular-weight substance in synaptosomes was determined by using small columns (0.8cm. diam. × 11cm.) of Sephadex G-50 (bead form). The column was previously equilibrated with 0.4M-sucrose, which is approximately iso-osmotic to the incubation medium. A portion (0.2–0.5ml.) of the suspension was applied to the column and allowed to drain down; 1.0ml. of 0.4M-sucrose was then added and allowed to drain down. A further 1.0ml. of eluting solution was added and 1.0ml. of the effluent was collected. This effluent contained 80% of the protein and less than 1% of the unbound small molecules applied to the column, and is taken to represent the void volume.

The amount of small-molecular-weight substances released by osmotic shock was determined by an identical procedure except that the column was equilibrated and eluted with water. In this case the osmotic shock disrupts the synaptosomes, thus releasing their content of small-molecular-weight substances, which are retained in the column. The difference between the amount of small-molecular-weight substance present in the void-volume effluent from the iso-osmotically eluted column and that in the effluent from the hypo-osmotic column is termed the osmotically sensitive content of the synaptosome preparation.

The osmotically sensitive volume of the synaptosomes was determined by allowing the preparation to equilibrate with 100mM-KCl for 3–4 hr. at 5° and then measuring the osmotically sensitive K⁺ within the synaptosomes. The amount (in moles/mg. of protein in the column effluent) of osmotically sensitive K⁺ divided by the external concentration in moles/l. is termed the potassium equilibrium volume of the synaptosomes (μl./mg. of protein) and is taken to represent the volume enclosed by the synaptosome limiting membranes (Marchbanks, 1966, 1967). The concentration of [¹⁴C]choline within the synaptosome was determined by dividing the amount of osmotically sensitive [¹⁴C]choline (measured as disintegrations/min./mg. of protein) by the potassium equilibrium volume. By dividing this figure by the external concentration of [¹⁴C]choline (disintegrations/min./μl.) the concentration ratio of choline between the inside and outside of the synaptosome membrane was calculated.

Analytical procedures

Radioactivity measurements. [¹⁴C]choline (specific radioactivity 32μC/μmole) was purchased from The Radiochemical Centre, Amersham, Bucks. The radioactivity of [¹⁴C]choline was determined in the scintillation medium described by Bray (1960), with a Nuclear-Chicago Unilux scintillation spectrometer. Counting efficiencies were always checked and corrected for by using the channels-ratio method, and in addition internal standards were used to ensure the validity of this procedure.

The purity of the radioactive choline was checked at intervals by thin-layer chromatography (see below) and found to be greater than 96%. The value for the specific radioactivity of the radioactive choline used in the calculations of flux etc. was corrected for the presence of endogenous choline by assuming that all the endogenous choline in the preparation was available to mix with the added radioactive choline.

Purification of radioactive choline. Separation of the radioactivity of [¹⁴C]choline from that of its metabolites was carried out by a modification of the thin-layer chromatographic procedure of Hemsworth & Morris (1964). This procedure also separates acetylcholine from choline, a separation that was required for studies on the incorporation of [¹⁴C]choline into acetylcholine carried out in conjunction with these experiments (R. M. Marchbanks, unpublished work). To concentrate the radioactivity from a comparatively large volume of 0.4M-sucrose, an ion-exchange column was used to separate the choline bases from sucrose and other interfering materials before concentration by evaporation. The exact procedure was as follows. Samples of synaptosome suspensions (0.5–4.0ml.) were brought to pH 4.0 with HCl and heated at 100° for 10 min. to denature the protein and release the choline. Non-radioactive choline (2.5μmoles) (and an equal amount of acetylcholine when a separation of this substance was to be carried out) was added as carrier, and the protein was removed by centrifugation. A small column (0.8cm. diam.) was filled with 1.5ml. (wet volume) of XE-97 carboxylic acid ion-exchange resin (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), which had been washed alternately with 50ml. of 0.1N-HCl and 50ml. of water for three cycles. The column was then washed with water until the effluent was at pH 4.0. The deproteinized sample at pH 4.0 was added to the column, and allowed to drain down. The column was then eluted with water (6ml. minus the volume of the original sample). After this, the column was eluted with 7ml. of 0.1N-HCl and the effluent was collected. The column was recycled by passing through it 50ml. of 0.1N-HCl and then water until the effluent was at pH 4.0.

The effluent was evaporated to dryness at 18° and taken up in 25μl. of methanol. Portions (10μl.) in duplicate were applied to a thin-layer chromatographic plate with a layer of cellulose powder (without CaSO₄ binder) MN 300 H (Macherey, Nagel and Co., 516 Düren, Germany). The eluting solvent was butan-1-ol-ethanol-acetic acid-water (8:2:1:3, by vol.). The choline spot was identified by spraying the dried plate with iodoplatinate reagent [1 part of aq. 5% (w/v) chloroplatinic acid added to 20 parts of 4.5% (w/v) KI in ethanol-water (1:1, v/v)]. Choline and acetylcholine appear as blue spots on a red background, the *R_F* of choline being 0.45 and that of acetylcholine 0.56. The spots were scraped off the plate and dispersed in the

scintillation-counting vial with 1 ml. of 0.1 N- NH_3 in ethanol. A portion (0.1 ml.) of 5% (w/v) $\text{Na}_2\text{S}_2\text{O}_3$ in ethanol-water (1:1, v/v) was added to decolorize the mixture. The powder was prevented from settling in the scintillation suspension by adding the thixotropic material Cab-O-Sil (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.) at a final concentration of 3% (w/v) to the counting vial. The counting efficiency for ^{14}C in the scintillation medium was 30%. Settling of the cellulose powder was found to cause self-absorption losses unless Cab-O-Sil was added. Cellulose powder with CaSO_4 binder was found to be unsatisfactory because it was difficult to disperse and caused larger self-absorption losses.

The weakly acidic resin column used in the first stage retains free choline and acetylcholine but allows sucrose, phospholipids and phosphorylcholine to pass through into the first 6 ml., which is discarded. High concentrations of other cations interfere with the retention of choline on the resin column and thus cause losses in the overall recovery. In these experiments the passage of a sample over the Sephadex column removed most of the free salt from the incubation medium, thus avoiding the possibility of losses of this kind.

The overall recovery of authentic previously chromatographed [^{14}C]choline put through this procedure was $70 \pm 4\%$ (mean $\pm$ S.E.M. of four experiments), the losses being about equal at each stage of the procedure.

Choline. Choline was measured by the procedure of Bligh (1952). After treatment of the sample with 10% (final concn.) trichloroacetic acid at 5° for 30 min. the denatured protein was removed by centrifugation. The trichloroacetic acid was removed by repeated ether extraction until the sample was at pH 4.0. It was then passed over an XE-97 resin column exactly as described above for the purification of [^{14}C]choline. Choline bases were removed from the resin column with 0.1 N-HCl and the solution was evaporated to dryness. It was then acetylated as described by Bligh (1952) and the acetylcholine thus formed was assayed on a slip of the dorsal muscle of a leech as described by Szerb (1962).

Endogenous acetylcholine contributes to the amounts of choline found by this method. When allowance is made for this, an amount of 0.22 μmole of choline/g. wet wt. of guinea-pig cortex was found. This is to be compared with a value of 0.165 μmole /g. wet wt. of rat forebrain (Dr C. O. Hebb, personal communication) and 0.17 μmole /g. wet wt. of rat brain (Dr S. G. Spanner, personal communication).

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

Potassium ions. K^+ was determined on an EEL Mark II flame photometer.

Materials

Hemicholinium no. 3 (HC-3; Schueler, 1955) was obtained from Eli Lilly Co. (Indianapolis, Ind., U.S.A.) and oxotremorine [1-(2-oxopyrrolidin-1-yl)-4-pyrrolidin-1'-ylbut-2-yne] was kindly given by May and Baker Ltd., Dagenham, Essex. Sarin (isopropyl methylphosphonofluoridate) was supplied by Dr D. R. Davies of the Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts. Ouabain (Strophanthin-G) was obtained from British Drug Houses Ltd., Poole, Dorset.

RESULTS

Fig. 1 shows the typical uptake curve of [^{14}C]choline at 25° when radioactive choline (1 $\mu\text{C}/\text{ml}$., 30 μM) was added to a synaptosome preparation that had been preincubated for 20 min. with the medium containing glucose, succinate etc. (see the Experimental section). The rate of uptake and final equilibrium content were decreased if Na^+ was omitted from the incubation medium, or if potassium cyanide was added. Hemicholinium no. 3 severely inhibited the uptake. It was necessary to establish that the radioactivity in the void-volume effluent was principally due to choline, and not to a metabolite. Samples of the void-volume effluent were usually taken at between 30 and 45 min. after the addition of the radioactivity and submitted to the procedure for purification of [^{14}C]choline described in the Experimental section. In 14 experiments the average recovery of radioactivity in the choline position of the chromatogram of the radioactivity in the void-volume effluent was 47%. A varying proportion (5–20%) of the radioactivity was found in the acetylcholine position of the chromatogram. By applying the recovery factor found for authentic choline it was calculated that 67% of the radioactivity in the void-volume effluent after 30–45 min. incubation was due to choline and not to any of its metabolic products.

The experiments were performed on a crude synaptosomal preparation contaminated with some myelin fragments and mitochondria. It was therefore necessary to show that the [^{14}C]choline uptake was into synaptosomes and not into contaminating material. After 30–45 min. of

Fig. 1. Uptake of [^{14}C]choline into metabolizing synaptosomes with the medium described in the Experimental section. ●, Control; ▲, Na^+ omitted from medium; ■, + KCN (5 mM); ○, + hemicholinium no. 3 (100 μM).

incubation, $78 \pm 2\%$ (mean $\pm$ S.E.M. of 21 experiments) of the radioactivity was released by passing the synaptosome preparation through a hypo-osmotic column. Osmotic sensitivity measured in this way is a characteristic of the constituents of synaptosomes rather than myelin fragments or mitochondria (Marchbanks, 1967).

The distribution in a sucrose density gradient of the radioactivity from [14 C]choline taken up by the crude synaptosomal fraction was next examined. The crude preparation was incubated and allowed to take up [14 C]choline in the usual way. After

45 min. at 25° , 3.0 ml. was passed through a column containing 3.0 g. of Sephadex G-50 (bead form), eluted with 0.4 M-sucrose, and the void-volume effluent was collected. This was submitted to sucrose-density-gradient centrifugation as described in the Experimental section, and pure myelin, synaptosomal and mitochondrial fractions were collected. The bound radioactivity was estimated by passing samples through iso-osmotic Sephadex columns. Of the bound radioactivity found in the gradient 71% was found in the purified synaptosome fraction, and 14% and 15% were respectively in the myelin and mitochondrial fractions.

Finally the ability of the purified fractions to take up [14 C]choline was examined. The incubation conditions were exactly as usual except that the incubation media were made iso-osmotic with the subfractions by the addition of sucrose. In Fig. 2 logarithmic plots (see the next section) of the uptake in the crude synaptosomal fraction and in the purified synaptosomal and mitochondrial fractions derived from it are shown. The kinetics of uptake were approximately the same for parent fraction and subfractions. This suggests that only one component with a characteristic flux was present in the crude parent fraction and the subfractions. In Table 1 the ability of the subfractions to concentrate [14 C]choline is shown. The concentration ratio achieved and fluxes were higher than usual in this experiment, but they illustrate the fact that the purified synaptosome fraction (fraction B) contains 79% of the choline-uptake ability as judged by the amount of radioactivity taken up at equilibrium. Except for the myelin fraction the concentration ratios of [14 C]choline achieved by the subfractions were approximately the same. Hyper-osmotic conditions did not affect choline uptake ability, since the purified synaptosome fraction that had been exposed to 1.0 M-sucrose during the course

Fig. 2. Logarithmic plot of the uptake of [14 C]choline into: ●, crude synaptosome fraction; □, purified synaptosome fraction; ○, purified mitochondrial fraction. The slope (see the text) equals the flux (μ moles/min./mg. of protein), from which the flux/cm.² of membrane (given in Table 1) was calculated as described in the text.

Table 1. *Ability of purified subcellular brain fractions to take up [14 C]choline*

For experimental details and definitions of terms, see the text.

Fraction	Morphology	*Potassium equilibrium volume (μ l.)	*†Radioactivity taken up at equilibrium (disintegrations/min.)	Radioactivity released by osmotic shock (%)	Concentration ratio	Flux (μ moles min. ⁻¹ cm. ⁻²)
P ₂	Crude parent fraction	34	530 000	81	4.2	0.186
A	Myelin	9	30 000	82	0.9	0.02
B	Synaptosomes	24	615 000	80	6.8	0.136
C	Mitochondria	4	140 000	60	7.0	0.154

* These values refer to that amount of tissue derived from 1 g. wet wt. of cerebral cortex.

† The radioactivity in the incubation medium was 3.0×10^6 disintegrations/min./ml.; the choline concentration was 45μ M.

of density-gradient centrifugation was just as active in this respect as the crude parent fraction (fraction P_2), which had not been exposed to hyperosmotic conditions.

Effect of choline concentration on uptake. Before the investigation of the effects of choline concentration on uptake, the endogenous choline content of the brain fractions was analysed so that its contribution to the concentration and specific radioactivity of the added choline could be calculated. The amount of choline found in the amount of sample derived from 1 g. wet wt. of cortex was in fraction P_2 (crude synaptosomes) $8.3\mu\text{moles}$, in fraction A (myelin) $4.1\mu\text{moles}$, in fraction B (synaptosomes) $0.8\mu\text{mole}$, and in fraction C (mitochondria) $1.2\mu\text{moles}$. These values were used to calculate the true choline concentration and specific radioactivity.

In these experiments the 20 min. preincubation was carried out in the presence of various added concentrations of non-radioactive choline. Then [^{14}C]choline was added, and at timed intervals portions of the synaptosome suspension were passed through the Sephadex columns and the radioactivity (disintegrations/min./mg. of protein) in the void-volume effluent was measured. The potassium equilibrium volume was measured on the same preparation. The synaptosomes were kept at 5° during the measurement of the potassium equilibrium volume, so the estimate of volume was probably slightly higher than the volume in the choline-uptake experiments conducted at 25° . This is because there is a slight tendency for synaptosomes to rupture at the higher temperature (Marchbanks, 1967).

The concentration ratio of [^{14}C]choline, which is therefore slightly underestimated, was determined from the amount of radioactivity in the void-volume effluent 40 min. after the addition of [^{14}C]choline.

Fig. 3. Concentration ratio of [^{14}C]choline taken up by synaptosomes as a function of the external choline concentration.

Fig. 3 shows the concentration ratio as a function of choline concentration. The dependence of the concentration ratio on choline concentration is very similar to that found in mouse cerebral-cortex slices by Schuberth *et al.* (1966).

The uptake of [^{14}C]choline is fast, and because of the difficulty of manipulating the columns at time-intervals of less than 5 min. it is not easy to get an accurate estimate of the initial rate of choline uptake. Accordingly, the choline flux was estimated from results gathered throughout the time-course of uptake. The treatment that follows is essentially that proposed by Cohn & Brues (1945), except that a steady-state assumption is introduced to allow the calculation of the concentration of choline inside the synaptosomes. After preincubation the system is assumed to be in a steady state with respect to choline concentration inside and outside the synaptosome, so that $\text{influx} = \text{efflux} = F$ (moles min^{-1}).

It is assumed that the addition of a small amount of radioactive choline does not disturb the steady state. The rate of appearance of radioactivity (C_i , in disintegrations/min./mg. of protein) inside the synaptosome is then:

$$dC_i/dt = F(S_o - S_i)$$

where S_o and S_i are the specific radioactivities of radioactive choline outside and inside the synaptosome respectively. Given the steady-state assumption:

$$S_i = S_o C_i / (C_i)_e$$

where $(C_i)_e$ is the amount of radioactivity inside the synaptosome after equilibrium with respect to radioactivity has been reached (i.e. after about 45 min.), substituting this expression in the differential equation and integrating yields:

$$2.303 \log \{[(C_i)_e - (C_i)_0] / [(C_i)_e - (C_i)_t]\} = tFS_o / (C_i)_e$$

where $(C_i)_0$ and $(C_i)_t$ are the amounts of radioactivity inside the synaptosome at time zero and at time t respectively. Thus the choline flux, F (in moles $\text{min}^{-1}/\text{mg. of synaptosomal protein}$), can be determined from a linear plot (see Fig. 2). The flux per unit area of synaptosome membrane (moles $\text{min}^{-1}\text{cm}^{-2}$) can be determined by dividing the flux/mg. of protein by the potassium equilibrium volume ($\text{cm}^3/\text{mg. of protein}$) and multiplying by one-third of the mean synaptosomal radius. A value of 0.28μ is taken for the mean synaptosomal radius (Clementi, Whittaker & Sheridan, 1966).

An objection to this method of treating the results is that a perturbation to the steady state achieved during preincubation always occurs when the radioactive choline is added. This perturbation can be minimized by ensuring that the absolute amount

(in moles) of added radioactive choline is as small as possible compared with the amount present during the preincubation period. It was not always possible to achieve minimal perturbation, but where this was particularly important, i.e. in the examination of the flux as a function of choline concentration, the concentration of added radioactive choline was kept low compared with that present during the preincubation period. In addition, the plot of the logarithmic function against t was always found to be linear (see Fig. 2), suggesting that the perturbation was not large enough to cause a serious deviation from the assumptions used.

The flux as a function of choline concentration is shown in Fig. 4. The rate of increase of flux with concentration increase falls off as the concentration rises. The concentration ratios and fluxes were found to vary quite widely with different preparations. The concentration ratio never fell below 1.7 but was sometimes as high as 10. The flux at a choline concentration of $30\mu\text{M}$ varied between 0.03 and $0.25\mu\text{moles min.}^{-1}\text{cm.}^{-2}$. Most of this variability is thought to derive from the variability of the potassium equilibrium volume between different preparations.

The concentration ratios achieved under usual conditions (choline concentration $30\mu\text{M}$) measured in the eight different preparations on which the effects of ions and drugs etc. were examined ranged between 1.5 and 7 and averaged 3.1 ± 0.7 (mean $\pm$ S.E.M.). The fluxes ($\mu\text{moles min.}^{-1}\text{cm.}^{-2}$) in the same experiments ranged between 0.031 and 0.089 and averaged 0.052 ± 0.008 (mean $\pm$ S.E.M. of eight experiments).

Effects of ions. The effects of changes in the concentrations of ions in the medium on choline uptake were examined. The results, as percentages of a control run under normal conditions (choline concentration $30\mu\text{M}$), are shown in Table 2. Omission of Na^+ from the medium decreased both the flux and the concentration ratio. K^+ does not

replace Na^+ in maintaining the flux and concentration ratio; indeed, addition of K^+ in the presence of Na^+ depressed the flux and the concentration ratio. Changes in the Mg^{2+} and Ca^{2+} concentrations had little effect.

Effects of metabolic inhibition. The effects of some inhibitors of metabolism are shown in Table 3. 2,4-Dinitrophenol ($100\mu\text{M}$) had surprisingly little effect on the flux and concentration ratio. Small decreases in flux and concentration ratio were observed when cyanide was added to the incubation medium, or when glucose and succinate were omitted from the medium, but in general the uptake was less affected by changes in the metabolic state than was expected.

Effect of pharmacological agents. The effects on choline uptake of a number of pharmacological agents that affect the physiological actions of choline and acetylcholine are shown in Table 4.

Table 2. *Effect of ions on the uptake of choline into synaptosomes*

For experimental details, see the text. The values are expressed as percentages of a control run under usual conditions, and are means of two determinations except when otherwise stated in parentheses, in which cases the mean $\pm$ S.E.M. is given.

Conditions	Flux (% of control)	Concentration ratio (% of control)
— Na^+ *	58	61 ± 3 (4)
+ K^+ (100mM)	63	51 ± 4 (4)
— Na^+ + K^+ (100mM)	55	53
— Mg^{2+} + EDTA (2mM)	—	102
+ Ca^{2+} (10mM)	—	105 ± 8 (3)

* Constant ionic strength and osmolarity of the medium were maintained by adjusting the concentration of the tris buffer.

Fig. 4. Choline flux into synaptosomes as a function of the external concentration of choline.

Table 3. *Effects of metabolic state on choline uptake into synaptosomes*

For experimental details, see the text. The values are expressed as percentages of a control run under usual conditions, and are means of two determinations, except when otherwise stated in parentheses, in which cases the mean $\pm$ S.E.M. is given.

Conditions	Flux (% of control)	Concentration ratio (% of control)
Omission of glucose and succinate from incubation medium	69	80
+ KCN (5mM)	88	62 ± 12 (4)
+ 2,4-Dinitrophenol ($100\mu\text{M}$)	86 ± 12 (3)	105 ± 12 (4)

Table 4. *Effects of some drugs on choline uptake into synaptosomes*

For experimental details, see the text. The values are expressed as percentages of a control run under usual conditions, and are means of two determinations, except when otherwise stated in parentheses, in which cases the mean $\pm$ S.E.M. is given.

Drug	Concentration of drug (μM)	Flux (% of control)	Concentration ratio (% of control)
Hemicholinium no. 3	100	16 ± 8 (3)	38 ± 5 (6)
Ouabain	100	103	88 ± 10 (3)
Oxotremorine	100	120	98 ± 5 (4)
Atropine	100	—	89
Sarin	1.0	120	100

Hemicholinium no. 3 was extremely effective in decreasing the flux and concentration ratio. Oxotremorine, ouabain and Sarin did not appear to affect choline uptake very significantly.

DISCUSSION

The fact that a substantial proportion of the radioactivity of [^{14}C]choline taken up into synaptosomes behaves as choline on chromatographic separation suggests that a genuine uptake of choline into synaptosomes, rather than its metabolism by synaptosomes, is being measured. This conclusion is reinforced by the finding that the radioactivity is readily released by hypo-osmotic shock. It is believed that the uptake of [^{14}C]choline is mostly into synaptosomes rather than other contaminating particles because the radioactivity can be isolated in the purified synaptosome fraction after further density-gradient centrifugation. Further, purified synaptosome preparations can take up [^{14}C]choline just as well as the less pure preparation, and most of choline-uptake activity of the crude preparation can be recovered in the purified synaptosome fraction. It is possible that mitochondria also take up choline with approximately the same kinetics as synaptosomes. However, since the greatest capacity to take up choline exists in the synaptosome fraction, the properties of the choline-uptake process must be ascribed principally to synaptosomes, and it is concluded that the experiments are measuring the uptake of [^{14}C]choline across the synaptosome membrane.

The relationship between the uptake of choline into synaptosomes and the external choline concentration is strikingly similar to that observed in more organized tissues such as cerebral-cortex slices (Schuberth *et al.* 1966), erythrocytes (Martin, 1968), or squid axon (Hodgkin & Martin, 1965). Schuberth *et al.* (1966) proposed an expression that relates the concentration ratio to the choline concentration by assuming an 'active' transport component inwards which is saturable, and a passive leak operating in both directions. From this expression they deter-

mined the concentration of choline at which half-saturation of the 'active' transport component occurs; this is $226 \mu\text{M}$ in mouse cerebral-cortex slices. By using this expression on the results from the present experiments on synaptosomes a value of $200 \mu\text{M}$ is obtained.

The relationship between choline influx and external choline concentration is qualitatively similar to that described by Glynn (1956) for K^+ entry into erythrocytes. There is a saturable component, and a component linear with choline concentration. By applying the equation proposed by Glynn, which relates flux and concentration, to the results shown in Fig. 4, the rate constant for the passive leak is found to be 0.0143 min.^{-1} . The choline concentration for half-saturation of the saturable component is $282 \mu\text{M}$ and the limiting value of the flux due to this component is $0.32 \mu\text{mole min.}^{-1} \text{ cm.}^{-2}$. The half-saturating concentration of choline influx into erythrocytes is $30 \mu\text{M}$ (Martin, 1968), and that into squid axon is about $100 \mu\text{M}$ (Hodgkin & Martin, 1965).

The turnover rate due to passive diffusion of [^{14}C]choline at 25° into synaptosomes is $1.43\%/ \text{min.}$, whereas that of acetylcholine at 5° is $0.65\%/ \text{min.}$ (Marchbanks, 1968), and that of K^+ at 5° is $4.5\%/ \text{min.}$ (Marchbanks, 1967).

The fact that the choline flux shows saturation kinetics indicates that a facilitated-diffusion transport mechanism is involved in the choline-uptake process (Stein, 1967; cf. chapter 4). Current theories (reviewed by Stein, 1967) propose that facilitated-diffusion mechanisms involve the existence of a 'carrier' in the membrane, which has the property of forming a transient complex with the transported molecule and then transferring it across the membrane. Inhibition of choline transport in synaptosomes by structural analogues such as hemicholinium is also indicative of a facilitated-diffusion, or carrier-mediated, transport mechanism.

The possibility that choline uptake in synaptosomes could be caused by the presence of a macromolecule with a high binding capacity for choline

is implausible since, although it could account for concentration ratios of choline greater than 1, such a mechanism would not impose saturation kinetics on the flux. Also, the fact that much of the choline can be released by osmotic shock suggests that it is retained within synaptosomes by a semi-permeable membrane. It seems reasonable to suppose that this is the synaptosome membrane itself since, as previously discussed, intraterminal mitochondria (unless they are very different from other brain mitochondria) would not have sufficient capacity to account for the choline uptake observed. If synaptic vesicles were the choline-accumulating particle within the synaptosome it would not be expected that they would release the choline on osmotic shock, since intrasynaptosomal synaptic vesicles do not release their acetylcholine when passed through hypo-osmotically eluted Sephadex columns (Marchbanks, 1968).

The existence of facilitated-diffusion, or carrier-mediated, transport systems does not necessarily imply that active transport is taking place, and it is not claimed that the uptake of choline into synaptosomes is an active transport process. Since the electrochemical gradient of choline in synaptosomes is not known it is not possible to decide whether the transport is thermodynamically 'uphill'. Further, the requirement for metabolic energy for choline uptake into synaptosomes is not clearly demonstrable, but possible other reasons for this are discussed below.

As in erythrocytes (Martin, 1968) and cerebral-cortex slices (Schubert, Sundwall & Sörbo, 1967), choline uptake into synaptosomes is decreased if Na^+ is omitted from the incubation medium or if extra K^+ is added. External Na^+ is a common requirement for the transport of many substances across biological membranes: for instance, amino acids (Rosenberg, Coleman & Rosenberg, 1965) and glucose (see review by Crane, 1965). A significant effect of 2,4-dinitrophenol in inhibiting choline uptake into synaptosomes could not be demonstrated. This is surprising, but it has been found in this Laboratory that incubation for 30 min. with 2,4-dinitrophenol does not completely decrease amounts of phosphocreatine inside the synaptosome to zero (R. M. Marchbanks, unpublished work). Presumably the energy resulting from glycolysis and the endogenous energy stores are sufficient, if they are required, to maintain choline transport. Omission of glucose and succinate from the incubation medium, or addition of cyanide, decreased choline transport only slightly, as would be expected by the same reasoning. Hemicholinium inhibition of choline uptake is a characteristic feature of the choline uptake in cortex slices and erythrocytes as well as synaptosomes. Oxo-tremorine had little effect on the uptake of choline

into synaptosomes. Ouabain also had little effect on the uptake; in this respect the synaptosome preparation is similar to erythrocytes, in which no inhibition of choline uptake by ouabain was observed (Martin, 1968), whereas in cerebral-cortex slices ouabain had an inhibitory effect (Schubert *et al.* 1967).

It cannot be determined from these experiments whether carrier-mediated choline transport is a property of all synaptosomes, or of only a limited proportion perhaps derived from cholinergic endings.

The uptake of choline in synaptosomes is qualitatively and quantitatively very similar to the transport of choline seen in more organized tissues. While this paper was being prepared a preliminary report (Potter, 1968) of a study of choline uptake into synaptosomes from rat cerebral cortex appeared, in which essentially the same conclusion was reached. Although somewhat different methods were used, choline uptake was found to take place by a carrier-mediated mechanism that was dependent on the presence of Na^+ and was inhibited by hemicholinium no. 3. The synaptosome limiting membrane therefore appears to be functionally competent for choline transport. This reinforces the view that the synaptosome may be regarded as a fairly intact non-nucleated portion of the nerve cell. The functional competence of the synaptosome for other physiological systems, notably active transport of K^+ and Na^+ , remains to be established. Nevertheless, for choline metabolism the synaptosome preparation makes possible the study *in vitro* of the interaction of metabolic and membrane processes at the synapse, without interference from other parts of the nerve cell.

In this connexion the role of choline as a precursor of the chemical transmitter acetylcholine is of particular interest. By the methods outlined in this investigation it may be possible to study the synthesis and storage of acetylcholine *in vitro* by using synaptosomes as a relatively pure and intact preparation of the presynaptic nerve terminal.

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