Biochimica et Biophysica Acta 815 (1985) 109–127
Elsevier

BBA 72547

High-affinity, sodium-gradient-dependent transport of choline into vesiculated presynaptic plasma membrane fragments from the electric organ of *Torpedo marmorata* and reconstitution of the solubilized transporter into liposomes

Ilze Ducis * and Victor P. Whittaker **

Abteilung Neurochemie, Max-Planck-Institut für biophysikalische Chemie, Postfach 2841, D-3400 Göttingen (F.R.G.)

(Received October 10th, 1984)

Key words: Choline transport; Transport protein; Presynaptic plasma membrane fragment; Membrane reconstitution; Electric organ; (*T. marmorata*)

Vesiculated fragments of presynaptic plasma membranes have been isolated from the purely cholinergic electromotor nerve terminals of *Torpedo marmorata*. Synaptosomes, generated from the terminals by homogenization, were separated on a discontinuous Ficoll gradient and then lysed by osmotic shock at 2°C, pH 8.5 in the presence of 0.1 mM $MgCl_2$. These conditions for lysis were optimal for choline transport. Electron micrographs of lysed synaptosomes showed vesiculated membranes with diameters smaller than those of synaptosomes; occasionally, synaptic vesicles were observed attached to them. Intact mitochondria or synaptosomes and basal laminae were not present. High-affinity ($K_T = 1.7\ \mu M$) uptake of choline into these vesiculated membrane fragments showed: (1) an absolute dependence on the Na^+ gradient (outside > inside), (2) a transient Na^+-gradient-dependent accumulation of choline over the equilibrium concentration (overshoot), (3) electrogenicity and rheogenicity, since the uptake was further stimulated in the presence of a Na^+ gradient by valinomycin, (4) dependence on the presence of external Cl^-, and partial dependence on a Cl^- gradient (outside > inside), (5) high-affinity ($K_i = 25$ nM) inhibition by hemicholinium-3 and (6) temperature sensitivity. The plasma membranes were further purified by centrifugal density gradient fractionation on a 4–12% Ficoll gradient. Several enzymes and polypeptides copurified with the specific binding sites for choline present in the membranes. The fraction with the most binding sites was one denser than 12% Ficoll. This was also the fraction richest in acetylcholinesterase, 5′-nucleotidase and polypeptides of relative molecular mass, M_r ($\times 10^{-3}$) of > 200, 140, 68 (doublet), 57, 54 and 28. Acetylcholinesterase was positively identified as a M_r 68000 component by immune blot. By contrast the ouabain-sensitive ATPase showed a negative correlation with choline binding sites. When the solubilized proteins of the vesiculated membranes were transferred to liposomes, they conferred on the latter the capacity to take up choline in a manner closely resembling its transport in natural membranes but with an initial (one minute) rate of uptake approximately 10-times greater per mg of protein. Several proteins were selectively transferred to the liposomes including ones of M_r ($\times 10^{-3}$) 34, 42, 47, 54, 60, 68, 92, 160 and > 200. The polypeptides of M_r ($\times 10^{-3}$) 140, 57

* Present address: Section of Biochemistry, Molecular and Cell Biology, Cornell University, Division of Biological Sciences, Wing Hall, Ithaca NY 14853, U.S.A.

** To whom reprint requests should be addressed.

Abbreviations: Taps, 3-([tris(hydroxymethyl)methyl]amino)propanesulphonic acid; Tris, tris(hydroxymethyl)aminomethane.

Enzymes: cytochrome oxidase (EC 1.9.3.1, ferrocytochrome *c*: oxygen oxidoreductase); ($Na^+ + K^+$)-ATPase (EC 3.6.1.3, ATP phosphohydrolase); 5′-nucleotidase (EC 3.1.3.5, 5′-ribonucleotide phosphohydrolase); lactate dehydrogenase (EC 1.1.1.27, L-lactate-: NAD^+ oxidoreductase); fumarase (EC 4.2.1.2, L-malate hydrolase); acetylcholinesterase (EC 3.1.1.7, acetylcholine acetylhydrolase); butyrylcholinesterase (EC 3.1.1.8, acetylcholine acylhydrolase).

and 28 were lost in the transfer. When a correlation was made between transport rates observed in the various liposome preparations tried and the presence of particular polypeptides, only three proteins correlated with the ability of liposomes to transport choline: the M_r ($\times 10^{-3}$) > 200, 54 and 34 components.

Introduction

Neurotransmitter uptake into presynaptic terminals or neighbouring glial elements serves to terminate the signal to postsynaptic receptors generated by transmitter release [1,2]. In the case of cholinergic terminals, however, released acetylcholine is first hydrolysed to acetate and choline and the latter is subsequently reutilized for transmitter synthesis by being transported back into the presynaptic terminal by a specific high-affinity process [3–5]. Neurotransmitter (or neurotransmitter precursor) uptake has been studied in brain slices [6–8], synaptosomes (Refs. 3–5, Refs. 9–17 and references cited therein) and in resealed presynaptic plasma membrane fragments [18–23] obtained by the osmotic lysis of synaptosomes. Such fragments are useful for studying neuronal uptake mechanisms since there is no interference from subcellular storage and metabolism and ion gradients and energy sources can be readily defined. However, all reported studies with this preparation, when derived from mammalian brain, have been concerned only with the transport of putative amino acid transmitters [18–21]; the paucity of studies with choline, with the exception of two reconstitution studies [24,25], could be due to the low content of synaptosomes derived from cholinergic terminals in a mammalian brain synaptosome preparation [26].

For this reason we have turned to the purely cholinergic [27,28] electromotor nerve terminals of *Torpedo marmorata*. Although membranous sacs derived from *Torpedo* electromotor synaptosomes have been used to study the release of acetylcholine [29] and different membrane fractions isolated from such synaptosomes after disruption have been characterized by measuring marker enzyme activities [30,31], no transport studies have been attempted so far with resealed presynaptic membrane fragments from this source. This has now been achieved; optimum conditions for uptake have been defined and some of the characteristics of the system are described. Fractionation of lysed synaptosomes on a Ficoll gradient resulted in the separation of membrane fractions with an enhanced capacity for choline transport and a simplified polypeptide content. Several of these same polypeptides were selectively incorporated into liposomes that had acquired a capacity for choline uptake after reconstitution of solubilized membrane proteins. In this way, it has been possible tentatively to identify the choline transporter as one of a limited number of polypeptides.

Materials and Methods

Materials

Animals. Live *Torpedo marmorata* were obtained from the Institut de Biologie Marine, Arcachon, France, and were kept in tanks of circulating artificial sea water at 16°C until required.

Chemicals. [^{3}H]Choline chloride (80 Ci/mmol) and [^{3}H]acetylcoenzyme A (705 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Valinomycin and all specific reagents needed for enzyme assays were obtained from Sigma (Munich, F.R.G.). All other chemicals used were of the highest obtainable grade and were from Merck (Darmstadt, F.R.G.). Sodium cholate and cholesterol were purchased from Sigma (Munich, F.R.G.). The lipids used in the reconstitution experiments were all grade 1 from Nutfield Nurseries (South Nutfield, Redhill, Surrey RH1 5PG, U.K.); bovine sphingomyelin and phosphatidylcholine were both from egg yolk; ethanolamine phosphatides and phosphatidylserine (monosodium salt) were from bovine spinal cord; phosphatidylinositol was as the sodium salt from wheat germ.

Membrane preparations

Preparation of synaptosomes. Synaptosomes were prepared from the electric tissue at 0–4°C as described in Ref. 32 and by the following modification. Aliquots of the homogenate from 60–75 g of tissue were layered onto discontinuous density gradients formed in six tubes of the Beckman SW

27 rotor and each consisting of 12 ml of 12%, 14 ml of 8% and 8 ml of 2% (w/v) Ficoll 400 (Pharmacia, Uppsala, Sweden) in *Torpedo* Ringer's solution. Material equilibrating at the 2–8% interface after centrifuging at 23000 rev · min^{-1} for 45 min (Fraction F_1) and consisting mainly of synaptosomes was separated, diluted 1 : 4 with *Torpedo* Ringer's solution and centrifuged at $17000 \times g_{av}$ for 20 min. The pellet so obtained was resuspended in a small volume of ice-cold buffer comprising 20 mM imidazole-HCl (pH 8.5 at 2°C) and 0.1 mM $MgCl_2$ (buffer A) to which was also added 0.7 M sucrose (buffer A + sucrose). When the effect of different buffers, Mg^{2+} or H^+ concentration on lysis was being investigated the composition of buffer A was adjusted accordingly. In some experiments material collecting between 0 and 2% Ficoll (Fraction F_0) 8 and 12% Ficoll (Fraction F_2) and that sedimenting to the bottom of the tubes (Fraction F_3) were also collected. The F_0 fraction was small and was not always present.

Lysis of synaptosomes. The synaptosomal (F_1) suspension was lysed at 2°C by diluting it with buffer A to 0.1 mg of protein per ml and gently stirring for 90 min; the suspension was further stirred for 20 min after the addition of 0.1 vol. of 3 M KCl in buffer A giving a final KCl concentration of 0.3 M. The lysate so obtained was centrifuged at $27000 \times g_{av}$ for 20 min and the pellet suspended in 0.5–1.0 ml of an ice-cold buffer comprising 20 mM imidazole-HCl (pH 7.4 at 25°C), 300 mM KCl and 0.1 mM $MgCl_2$ (buffer B), and dialysed for 16 h against 1.5 litre of buffer B at 2°C. This had the effect of loading the resealed vesiculated fragments of the presynaptic plasma membrane which were formed during lysis with K^+. When the effect of loading with other ions was being investigated, the pellet was suspended in a buffer containing the appropriate 'loading' ions and similarly dialysed against suspension buffer.

To subfractionate the synaptosomal lysate, the suspension in buffer B was layered (3 ml per tube) onto a Ficoll density gradient consisting of layers of 12, 10, 8, 6 and 4% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) in buffer B (7 ml of each) formed in each of two tubes of the Beckman SW 27 rotor and centrifuged at 20000 rev · min^{-1} for 90 min. Material equilibrating at each of the interfaces and at the bottom of the tube was removed, diluted 1 : 4 with buffer B and centrifuged at $27000 \times g_{av}$ for 20 min. The pellets so obtained (designated A–F, from top to bottom of the gradient) were suspended in 0.5–1.5 ml of buffer B and either used immediately or refrigerated at 0–4°C and used for uptake, binding or biochemical characterization studies the following day.

Morphological characteristics of fractions. Samples of subcellular fractions were fixed in suspension for electron microscopy by the addition of ice-cold 5% (v/v) glutaraldehyde in 0.38 M sodium cacodylate buffer (pH 7.4) for 2 h. The fixed material was then centrifuged for 10 min in a desk-top Eppendorf centrifuge. All fractions were rinsed in fixation buffer for 10 min and then post-fixed with 1% OsO_4 in 0.38 M cacodylate buffer (pH 7.4) for 1 h at 4°C. The fixed material was again centrifuged for 10 min and subsequently washed in buffer. Each suspension was then filtered under vacuum through a Millipore filter (HAWP, 0.45 μm pore size). The particles trapped on the filter were overlaid with warm Agar solution (3% in water) and upon hardening small blocks of Agar with the membrane sample and filter adhering to one surface were cut away, dehydrated in ethanol, transferred to propylene oxide and embedded in Epon. Blocks were sectioned and stained with uranyl acetate and lead citrate in the usual way for electron microscopic examination.

Measurements of choline uptake. Unless otherwise stated synaptosomes or the vesiculated presynaptic plasma membrane fragments derived from them were preincubated for 20 min at 25°C; valinomycin (2.5 μM) was present where noted. Uptake was initiated by adding 50 μl of synaptosome or membrane suspension to 400 μl of incubation medium containing [^{3}H]choline (2 μM, 10 μCi · μmol^{-1}). In experiments with synaptosomes the medium consisted of *Torpedo* Ringer's solution containing 10 mM glucose; in those with vesiculated membrane fragments it was buffer B, for experiments at K^+ equilibrium, or 20 mM imidazole-HCl (pH 7.4 at 25°C), 300 mM NaCl and 0.1 mM $MgCl_2$ (buffer C) when Na^+ and K^+ gradients were being imposed. Hemicholinium-3 (10 μM) was added where indicated. Where indicated, samples were diluted into medium containing 0.1% Triton X-100 after uptake had occurred

at 25°C. When the effect of other ionic gradients was being studied the composition of either buffer B or C was modified as described. At specified time intervals a 50 μl sample of the incubation mixture was diluted with 1 ml of ice-cold *Torpedo* Ringer's solution, buffer B or buffer C as appropriate, mixed and filtered through a Millipore filter of 0.65 μm pore size (synaptosomes) or 0.45 μm pore size (vesicles); the particles retained on the filter were washed with 4 ml of appropriate medium, the filters were removed and solubilized in Bray's solution [33] and their radioactivity determined in a Berthold LB 5004 liquid scintillation spectrometer. The uptake measurements were performed in triplicate or quadruplicate; errors of the mean are indicated in the figures if they exceed the symbol size. When uptake measurements were made under hyperosmotic conditions (see below), membranes were preincubated for 45 min at 25°C in buffers B or C to which appropriate amounts of sucrose had been added. Ice-cold buffers similarly modified were used in the dilution and washing steps used to terminate the reaction. Values obtained at K^+ equilibrium were deducted from those measured in the presence of an inwardly directed Na^+ gradient to arrive at the net uptake.

Specific binding of choline. To obtain a rough estimate of the relative number of specific binding sites for choline use was made of the finding that choline uptake is reduced when the osmotic pressure of the suspension medium is raised. Uptake was measured at several osmolarities in the range 0.6 to 1.0 osmol $\cdot l^{-1}$; the values were plotted as a function of the reciprocal of osmolarity, a straight line fitted to the data points and extrapolated to infinite osmotic pressure. The y intercept was taken as a measure of the number of binding sites.

Reconstitution of the choline transporter in liposomes

Solubilization of the choline transporter. Synaptosomes (Fraction F_1) were prepared from 3- to 4-times the usual amount of electric tissue (i.e. 210–240 g) distributed over 12 Ficoll gradients and lysed in buffer A. Before solubilization, the synaptosomal lysate (in buffer B) was washed twice with a buffer containing 10 mM triethanolamine-HCl (pH 7.4, 25°C), 5 mM Na_2-EDTA and 300 mM NaCl (buffer D) by resuspension and sedimentation in an Eppendorf bench-top centrifuge and then solubilized (1.5 mg of membrane protein per ml) by stirring for 1 h at 2°C in buffer D to which had been added 20 mM choline and 0.1% sodium cholate (final concentrations). Undissolved material was removed by centrifuging at $10^5 \times g_{av}$ for 1 h and the supernatant was concentrated to 1 ml by ultrafiltration through a YM 10 Amicon filter.

Transfer to liposomes. Transfer of solubilized protein to liposomes was achieved as follows. Lipids stored in 5% (v/v) methanol in chloroform under N_2 at −20°C were mixed in a round-bottom flask in the following proportions (mol%) to give a total of 10.4 mg of mixed lipids: phosphatidylcholine, 33; phosphatidylethanolamine, 21; phosphatidylserine, 9; phosphatidylinositol, 3.5; sphingomyelin, 3.5; cholesterol, 30. After the solvent was evaporated in a stream of dry N_2, 1 ml of buffer D containing 0.1% sodium cholate was added and the flask was shaken for 3 h under N_2 at room temperature (20°C). The suspension so formed was added to 1 ml of concentrated supernatant. The mixture was dialysed for 20 h against 600 ml of 0.1 mM $MgCl_2$/20 mM K_2HPO_4 (pH 7.4, 25°C)/260 mM KCl (buffer E) with three changes of buffer at 2, 4 and 16 h. At 4 h only, buffer volume was increased to 1.2 litre. The liposomes were sedimented at $1.5 \cdot 10^5 \times g_{av}$ for 30 min, resuspended in 600 μl of buffer E, then centrifuged for 30 s in an Eppendorf bench-top centrifuge. The pellet was discarded and the supernatant provided the liposomal suspension for uptake studies.

Choline uptake into liposomes. This was measured as already described for plasma membrane fragments except that the incubation medium consisted of buffer E (for experiments at K^+ equilibrium) or 0.1 mM $MgCl_2$, 20 mM Na_2HPO_4 (pH 7.4, 25°C) and 260 mM NaCl (buffer F) when an inwardly-directed Na^+ gradient was being imposed. When the effect of anions on the uptake was being investigated, the liposomes were dialysed for 24 h against 2 litres of a buffer consisting of 130 mM K_2SO_4/150 mM mannitol/20 mM K_2HPO_4 (pH 7.4, 25°C)/0.1 mM $MgSO_4$ (buffer G). Dilution and washing of the sample were as described using the appropriate incubation buffer. Filtration was carried out using 0.22 μm pore-size cellulose acetate filters (Millipore GSWP).

Enzyme assays

Cytochrome oxidase. Activity was followed spectrophotometrically by measuring the rate of decrease in extinction at 550 nm after mixing 2.5 ml of 75 mM sodium phosphate buffer (pH 7.5), 5–100 μl of tissue sample and 100 μl of cytochrome *c* solution. Horse-heart cytochrome *c* (grade III, Sigma, 20 mg) in phosphate buffer was reduced by adding dithionite, excess of which was removed by passage through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden). The reduced cytochrome *c* solution was adjusted to 7 ml with phosphate buffer before use.

Ouabain-sensitive ($Na^+ + K^+$)-ATPase. Activity was assayed by a modification of a previously described method [34]. 10–50 μl of sample were pipetted into tubes on ice. An appropriate amount of a stock solution of 30 mM $MgCl_2$, 200 mM KCl and 900 mM NaCl was premixed with distilled water and added to the tissue samples, resulting in a total volume of 250 μl. 100 μl of a stock solution (250 mM) of imidazole-HCl (pH 7.0) and 100 μl of a 0.05% saponin solution were next added. To appropriate tubes, 25 μl of water or a 2 mM ouabain solution were added. The sample tubes, after mixing, were preincubated at 25°C in a shaking water bath. After 15 min, the tubes were placed on ice, allowed to cool and 25 μl of an ATP stock solution (60 mM) were added. After mixing, the samples were incubated for 40 min at 25°C in a shaking water bath. Final volume in each reaction tube was 500 μl. Final concentrations of all components were (mM): $MgCl_2$, 3; KCl, 20; NaCl, 90; ATP, 3; imidazole-HCl, 50; ouabain, 0.1 mM; saponin, 0.01%.

To stop the reaction, samples were placed on ice and 335 μl of ice-cold 1.2 M $HClO_4$ was added. After 10 min, the samples were centrifuged at $10\,000 \times g_{av}$ for 10 min, 700 μl of the protein-free supernatant were removed and 700 μl of a freshly prepared solution of 144 mM $FeSO_4$ in 8.15 mM ammonium molybdate/0.58 M H_2SO_4 were added, mixed and left at room temperature for 20 min. Absorbance at 700 nm was determined against distilled water blanks.

Standards (K_2HPO_4) in the range from 2 to 30 μg P_i, standard blanks, tissue blanks and ATP blanks were also included. Blanks comprised the following: standard, water plus final $FeSO_4$-molybdate-H_2SO_4 solution; tissue, all components except ATP and ouabain; ATP, all components except tissue and ouabain. Ouabain-sensitive ATPase activity was calculated by subtracting ouabain-insensitive activity from total ATPase activity after subtraction of appropriate blanks.

5'-Nucleotidase (adenosine 5'-[α,β-methylene]diphosphate-sensitive phosphatase). Activity was assayed as for ouabain-sensitive ($Na^+ + K^+$)-ATPase except that saponin was not present and the final concentrations of the components in the incubation medium were (mM): $MgCl_2$, 5; KCl, 5; NaCl, 250; Tris-HCl, 100 (pH 7.4); AMP, 1.5; Ado*P*[CH_2]*P*, 0.375.

Other enzymes. Several other marker enzymes were measured by standard methods at 25°C in the presence of 0.1% Triton X-100 in order to characterize the various subcellular fractions further. These were: lactate dehydrogenase [35], fumarase [36] acetylcholinesterase [37] and butyrylcholinesterase; this was assayed exactly like acetylcholinesterase except that 50 μM 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284C51 dibromide) was added to inhibit acetylcholinesterase and butyrylthiocholine was used as a substrate. Choline acetyltransferase was measured as described [38] at 37°C.

Other methods

Polyacrylamide gel electrophoresis and immune blotting. These were carried out exactly as described in Refs. 39 and 40. The anti-acetylcholinesterase and anti-vesicle-specific proteoglycan sera were gifts from Dr G.P. Richardson and Dr J.H. Walker, respectively.

Protein. This was determined as previously described [41].

Results

Characterization of synaptosomal fraction

Synaptosomes were initially isolated exactly as described [32]. However, when further fractionation of the synaptosomal lysate was carried out on a sucrose step-gradient no enrichment of choline transport activity relative to that of the parent fraction was obtained in any of the subfractions. By contrast, when membranes were fractionated on a Ficoll gradient, enrichment of choline trans-

TABLE I

CHARACTERIZATION OF FRACTIONS OF AN ELECTRIC ORGAN HOMOGENATE SEPARATED ON A FICOLL GRADIENT BY MEANS OF MARKER ENZYMES

Values are means ± S.E. of the number of experiments given in column 3 unless otherwise stated in parentheses. Recoveries were all over 70% except for the soluble enzyme lactate dehydrogenase (51%). This low recovery may have been due to losses in the non-particulate supernatant at the top of the gradient, which was not assayed.

Enzyme	Units of substrate transformed per min	No. of expts.	Specific activity (units·(mg of protein)$^{-1}$·min^{-1})				
			Homogenate	F_1	F_2	F_3	Total synaptosomal (F_1) lysate
Fumarase	nmol	4	21.5 ± 2.4	11.1 ± 2.6	11.0 ± 2.6	45.7 ± 8.5	0
Lactate dehydrogenase	nmol	5	392 ± 51 (4)	645 ± 92	248 ± 32	66.1 ± 9.8	329 ± 47
Choline acetyltransferase	nmol	4	6.6 ± 1.7 (3)	15.8 ± 3.7	4.1 ± 0.6	0.42 ± 0.12	11.5 ± 2.0
Acetylcholinesterase	μmol	7	19.1 ± 2.9	22.3 ± 2.5	30.5 ± 4.2	3.7 ± 0.6	22.5 ± 2.7
Butyrylcholinesterase	nmol	5	7.8 ± 1.2	15.8 ± 1.5	8.4 ± 1.5	3.5 ± 0.4	12.3 ± 1.6
5′-Nucleotidase	nmol	4	38 ± 9	62 ± 11	41 ± 25	12 ± 3	74 ± 7
Ouabain-sensitive ATPase	nmol	4	15 ± 1	27 ± 2	13 ± 2	22 ± 7	42 ± 5
Cytochrome oxidase	ΔA_{550}	5	0.43 ± 0.03	0.18 ± 0.03	0.2 ± 0.02	0.33 ± 0.04	0.17 ± 0.03

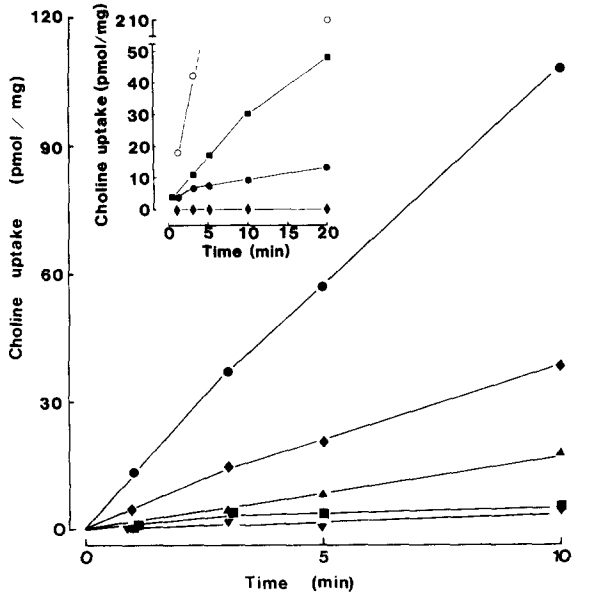


Fig. 1. Effect of gradient solute on uptake of choline by synaptosomes. The uptake of [^{3}H]choline into synaptosomes prepared on a sucrose (◆) or Ficoll (●) gradient is shown; the latter (fraction F_1) were isolated at the 2–8% Ficoll interface. Uptake into the F_3 mitochondrion-rich pellet (below 12%) (▼), F_2 mixed (8–12%) (■) and F_0 (0–2%) (▲) Ficoll fractions is shown for comparison. Values are means of differences in uptake at 25°C and 0°C. Inset: uptake by fraction F_1 at 2°C (●), or at 25°C (○■◆), in the absence (○), or presence (■◆), of 10 mM hemicholinium-3 (■), or Triton (◆) (for details see Methods).

port did occur. This suggested that prolonged exposure to hyper-osmotic sucrose is deleterious to choline uptake. Accordingly a comparison was made of choline uptake into synaptosomes isolated in parallel on sucrose and Ficoll gradients. As seen in Fig. 1, the specific uptake of choline by synaptosomes isolated on a Ficoll gradient was approximately 3-times greater than that by those isolated on a sucrose gradient. The yield of synaptosomes was 9-times greater in Ficoll than in sucrose in the one experiment in which this was quantified, and a significantly greater yield in Ficoll was regularly obtained as judged by the greater width and opacity of the band at the 2–8% Ficoll interface compared to the corresponding band in sucrose. The uptake was hemicholinium-3 sensitive and the radioactivity accumulated was completely released after treatment with Triton X-100 (Fig. 1, inset).

Electron microscopic examination of fraction F_1 (Fig. 2a) showed that most structures were synaptosomes, though some contamination with membranes of unknown origin and with mitochondria was apparent. This latter probably derived mainly from electrocytes since electromotor terminals contain very few mitochondria. A statistical analysis of several electron micrographs showed that 60–70% of the particles are synaptosomes. Fraction F_2 was similar in composition to F_1, but had a

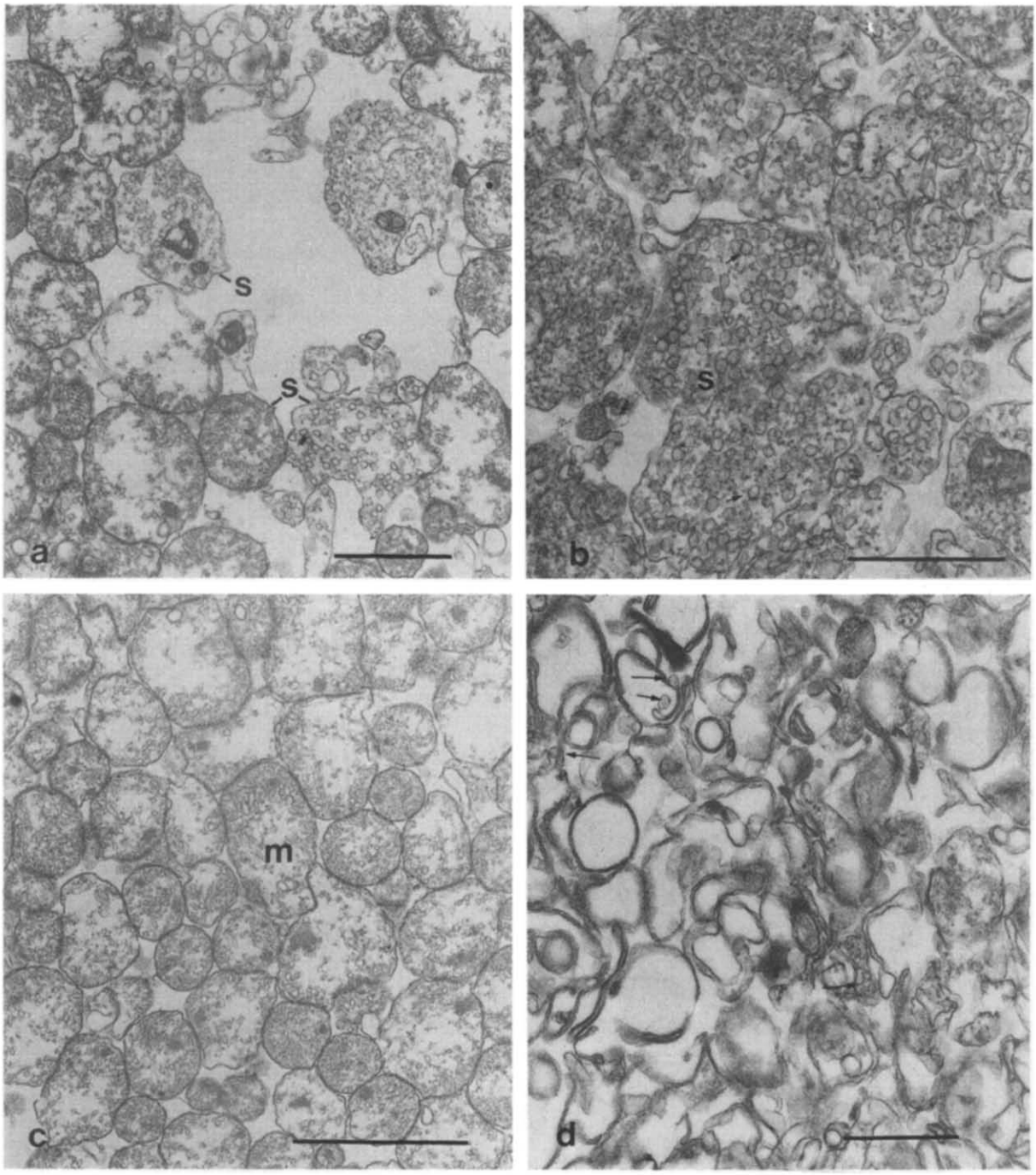


Fig. 2. Morphology of fractions. Electron micrographs of (a) fraction F_1, (b) fraction F_2, (c) fraction F_3, (d) lysate of fraction F_1 (arrows indicate synaptic vesicles); m, mitochondrion; s, synaptosome; bar 1 μm.

greater mitochondrial contamination (Fig. 2b); however, no choline uptake was observed in this fraction (Fig. 1) or in F_3, the mitochondrial pellet (Fig. 2c).

The activity of the marker enzymes in the various fractions is shown in Table I. Fraction F_1, the

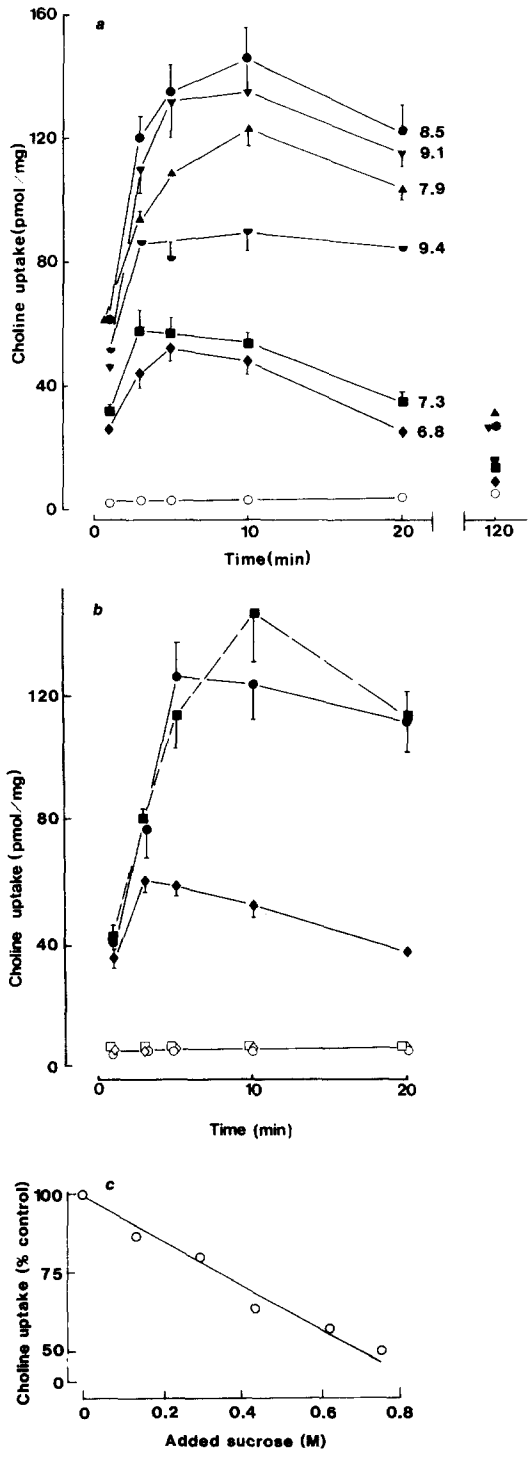


Fig. 3. Effect of lysis conditions on subsequent choline uptake into K^+-loaded vesiculated presynaptic plasma membrane fragments under the influence of an imposed sodium gradient. (a) Effect of pH. The synaptosomes isolated in Ficoll (Fraction F_1) were lysed in buffer A adjusted to the pH values shown by the numbers in the right of the curves, dialysed against buffer B and then transferred to buffer C containing [^{3}H]choline (black symbols), thereby imposing an inwardly directed Na^+ (and outwardly directed K^+) gradient on the vesicles, or buffer B containing [^{3}H]choline (open circles), thus imposing K^+ equilibrium across the membrane. There is a rapid choline uptake with overshoot and fall (in 120 min) to values close to those obtained under conditions of K^+ equilibrium; this uptake is maximal when lysis was performed at pH 8.5. (b) Effect of composition of lysis buffer at pH 8.5: 5 or 20 mM imidazole (■□); 5 mM Taps (●○); 5 mM Tris (◆◇); filled symbols, Na^+/K^+ gradient; open symbols, K^+ equilibrium. (c) Effect of osmotic pressure: the medium in which the vesiculated membrane fragments were suspended was made hyper-osmotic by the addition of sucrose to the concentrations given by the abscissae. Uptake was calculated as the difference between the initial uptakes in 40 s in buffers C and B to which sucrose had been added, expressed as a percentage of the corresponding differences in sucrose-free buffers. Points in (a) and (b) are the means of 4–8 values and bars represent S.E. values.

synaptosome fraction, shows an enrichment in lactate dehydrogenase and choline acetyltransferase due to occluded cytosol and the plasma membrane-bound enzymes butyrylcholinesterase, 5′-nucleotidase and ouabain-sensitive ($Na^+ + K^+$)-ATPase. F_3, the mitochondrial pellet, showed enrichment relative to the other fractions only in the two mitochondrial markers, fumarase and cytochrome oxidase.

Optimum conditions for the preparation of presynaptic plasma membranes

Various conditions for lysing synaptosomes (fraction F_1) were tested so as to obtain vesiculated synaptosomal (i.e. presynaptic) membrane fragments with a maximum ability to take up choline. It was found that the temperature, pH, Mg^{2+} concentration and the composition of the buffer used during hypo-osmotic lysis all influenced subsequent choline uptake in buffer C. Lysis was best performed at 2°C. Fig. 3a shows the effect of varying the pH and Fig. 3b that of varying the composition of the buffer used for lysis on the uptake of choline in the presence of an imposed, inwardly directed Na^+ gradient. Using the 20 mM imidazole buffer (buffer A) at different pH values in the range 6.8–9.4, maximum uptake was found to occur when lysis was performed at pH 8.5. Lowering the concentration of imidazole to 5 mM or replacing it by 5 mM TAPS has no

effect; replacing by 5 mM Tris-HCl had no effect on uptake at K^+ equilibrium or on the initial (one minute) rate of uptake in the presence of an inwardly directed Na^+ gradient, but the overshoot was reduced by 67%. Optimum Mg^{2+} concentration for lysis was 50–100 μM; above 200 μM the initial uptake of choline was significantly reduced (results not shown).

Morphology of the lysate. Fig. 2d shows that the main structures present after lysis are vesiculated membrane fragments somewhat smaller in diameter than the synaptosomes from which they were prepared. Occasionally, synaptic vesicles can be seen sticking to them.

Evidence for sealing. Raising the osmotic pressure of the uptake medium by the addition of sucrose reduced uptake (Fig. 3c); exposure of the resealed membrane fragments to 0.1% Triton X-100 (Fig. 4) after uptake had occurred resulted in the release of all radioactivity. This, together with the 'overshoot' (see below) indicates that [^{3}H]choline is not simply being bound, but is being taken up into a sealed, osmotically sensitive space bounded by a detergent-soluble membrane.

Characteristics of choline uptake into the vesiculated membrane fragments

Optimum temperature. Fig. 5 shows the temperature dependence of choline uptake into vesiculated presynaptic membrane fragments under standard conditions, i.e. imposed inwardly directed sodium gradient at pH 7.4 and 0.1 mM $MgCl_2$ in 20 mM imidazole buffer. Optimum uptake was obtained between 25°C and 30°C.

Sodium dependence. Fig. 4 (filled triangles) shows that choline uptake in the presence of a Na^+ gradient ceases after 5 min and is then followed by a slower loss of choline from the membrane fragments as the various ionic gradients are dissipated. This 'overshoot' phenomenon is characteristic of an uptake system driven by an imposed ion gradient. Significantly, veratridine, a veratrum alkaloid that selectively increases the permeability of the nerve membrane to Na^+ [42], decrease the uptake of choline in the presence of the sodium gradient at alkaloid concentrations of 25 and 50 μM (Fig. 4, inset), presumably by dissipating the Na^+ gradient more rapidly.

Electrogenic nature of the uptake. The uptake of

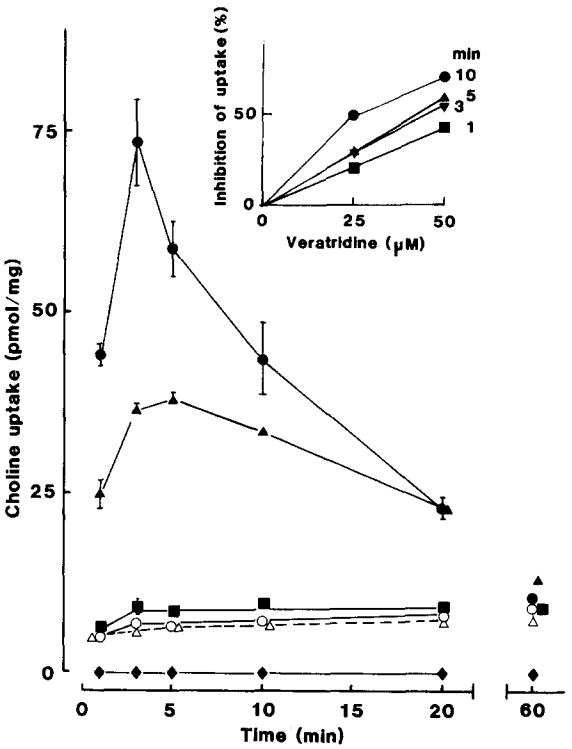


Fig. 4. Sodium dependence of choline uptake into vesiculated membrane fragments. Uptake of [^{3}H]choline by vesiculated fragments of *Torpedo* electromotor presynaptic plasma membranes loaded with buffer B and incubated in either buffer C (Na^+ gradient conditions) containing [^{3}H]choline in the absence of additions (▲), or in the presence of 2.5 μM valinomycin (●), or 10 μM hemicholinium-3 (■), or in buffer B (K^+ equilibrium conditions) containing [^{3}H]choline in the presence (○), or absence (△), of 2.5 valinomycin is shown. Samples were also diluted into buffer containing 0.1% Triton X-100 after maximum uptake had occurred (◆). Points are means of triplicates or quadruplicates; bars, S.E. values. Inset: the effect of veratridine on Na^+-gradient-stimulated choline uptake at various time intervals (min).

choline into vesiculated membrane fragments preloaded with K^+ and in the presence of an inwardly directed sodium gradient was enhanced by the addition of 2.5 μM valinomycin, an ionophore that specifically enhances the permeability of membranes to K^+ (Fig. 4). This effect was not seen at K^+ equilibrium. This suggests that the increased choline uptake is caused by the enhancement by valinomycin of an inside-negative K^+ diffusion potential, thus increasing the driving force for choline uptake.

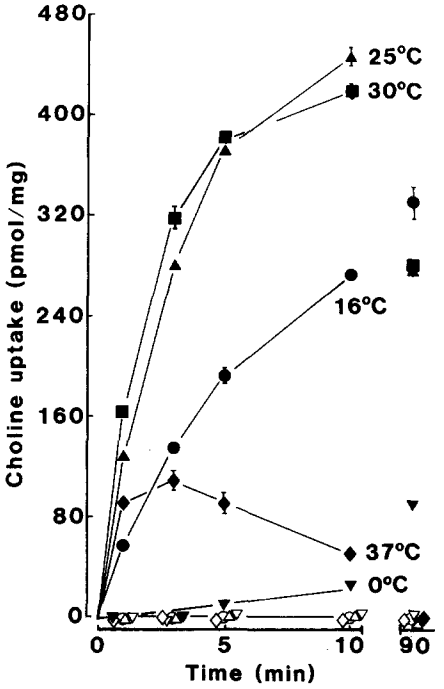
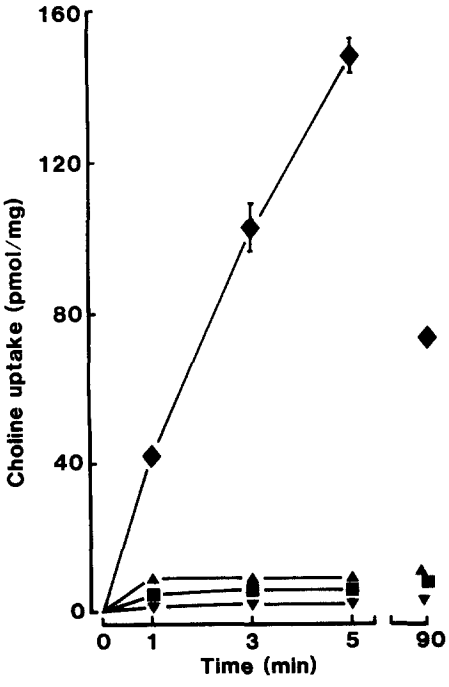


Fig. 5. Effect of temperature on the NaCl-gradient-stimulated uptake of choline. Vesiculated membrane fragments were loaded with 130 mM K_2SO_4, 150 mM mannitol, 20 mM K_2HPO_4 (pH 7.4, 25°C), 0.1 mM $MgSO_4$ and either incubated in the same buffer (open symbols, K^+ equilibrium) or in 260 mM NaCl, 20 mM mannitol, 20 mM Na_2HPO_4 (pH 7.4, 25°C), 0.1 mM $MgSO_4$ (closed symbols, inwardly directed NaCl gradient).



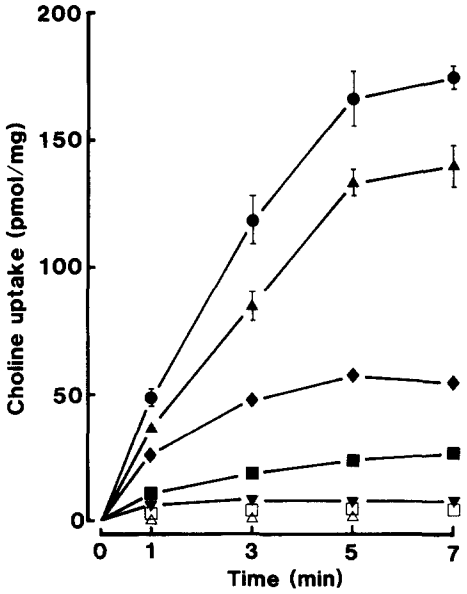


Fig. 7. Replaceability of K^+ for uptake. Filled symbols, Na^+ gradient: control (KCl-loaded) (●); loading with RbCl (▲); NH_4Cl (◆), CsCl (■), LiCl (▼). Open symbols, K^+ gradient with RbCl- (△) or CsCl-loading (□).

Cation specificity. Figs. 6 and 7 show that the uptake mechanism has an absolute dependence on a Na^+ gradient; other monovalent ions (Li^+, K^+, Rb^+ or Cs^+) cannot replace Na^+. Fig. 6 shows that vesiculated membranes loaded with KCl avidly took up choline in the presence of a Na^+ gradient (diamonds) but not when NaCl was substituted by LiCl, RbCl or CsCl (squares). At KCl, NaCl, K_2SO_4 or Na_2SO_4 equilibrium uptake was also essentially absent. Fig. 7 shows that K^+ cannot substitute for Na^+. Vesiculated membranes loaded with either RbCl (triangles) or CsCl (filled squares) took up choline in the presence of a Na^+ gradient,

Fig. 6. Absolute dependence of choline uptake on Na^+ gradient. Standard conditions (KCl loading, Cl^- equilibrium Na^+ gradient) (◆); KCl-loading with KCl equilibrium or Li^+, Rb^+ or Cs^+ gradients replacing Na^+ gradient (■ representing four sets of points all superimposable); NaCl-loaded under conditions of NaCl equilibrium (▲); K_2SO_4- or Na_2SO_4-loaded under equilibrium conditions (▼ representing two superimposable sets of points), when loading buffer was 150 mM mannitol, 20 mM imidazole-H_2SO_4 (pH 7.4, 25°C), 0.1 mM $MgSO_4$ and either 150 mM K_2SO_4 or Na_2SO_4. See Fig. 7 for comparison of an inwardly directed K^+ and Na^+ gradient on choline uptake.

but not in the presence of a K^+ gradient (open squares).

Univalent cations could, however, replace K^+ to varying extents as the loading ion (Fig. 7) in the presence of a Na^+ gradient. Ions were effective in the order $K^+ > Rb^+ > NH_4^+ > Cs^+ > Li^+$, the last being completely ineffective.

Anion specificity. In Fig. 8, the effect of replacing Cl^- by Br^-, NO_3^-, SCN^- or SO_4^{2-} is shown. Uptake was markedly reduced in the absence of Cl^-, though Br^- could partially replace Cl^-. Although uptake is absolutely dependent on an inwardly directed Na^+ gradient, it is only partially dependent on an inwardly directed Cl^- gradient (Fig. 8, compare open squares and filled circles).

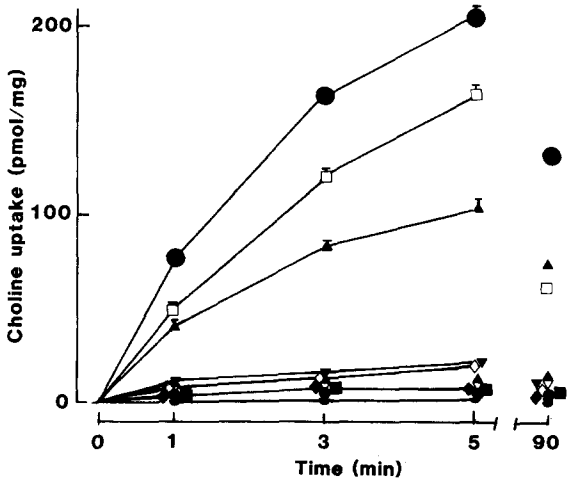


Fig. 8. Anion dependence of uptake. Filled symbols indicate loading with 150 mM K_2SO_4, 150 mM mannitol, 20 mM imidazole-H_2SO_4 buffer (pH 7.4), 0.1 mM $MgSO_4$. Incubation media were buffered with 20 mM imidazole-H_2SO_4 (pH 7.4), 0.1 mM $MgSO_4$ and contained in addition: (●) 300 mM NaCl (Na^+ and Cl^- gradients); (▲) 300 mM NaBr (Na^+ and Br^- gradients); (▼) 300 mM $NaNO_3$ (Na^+ and NO_3^- gradients); (■) 300 mM NaSCN (Na^+ and SCN^- gradients); (•) 300 mM KCl (Cl^- gradient); (◆) 150 mM Na_2SO_4, 150 mM mannitol (Na^+ gradient); (◒) 150 mM K_2SO_4, 150 mM mannitol (K_2SO_4 equilibrium). Open symbols indicate loading with 300 mM KCl, 20 mM imidazole-H_2SO_4 (pH 7.4), 0.1 mM $MgSO_4$. Incubation media were buffered as above and contained (◇) 150 mM Na_2SO_4, 150 mM mannitol (inwardly directed Na^+ gradient, outwardly directed Cl^- gradient); (□) 300 mM NaCl (Na^+ gradient, no Cl^- gradient, but Cl^- present). The partly filled symbol (◇) indicates loading with 150 mM Na_2SO_4, 150 mM mannitol, 20 mM imidazole-H_2SO_4 (pH 7.4), 0.1 mM $MgSO_4$ and incubation medium buffered as before and containing 300 mM NaCl (Cl^- gradient, no Na^+ gradient). Points are means of three or four determinations; bars are S.E. values.

Kinetic properties. The time-course of choline uptake was found to be linear for at least 60 s at external choline concentrations of 1 and 10 μM (data not shown). For determinations of the dissociation constant of the putative choline-transporter complex (K_T), the maximum velocity of choline uptake (V_m) and the inhibitor constant K_i for hemicholinium-3, incubation times of 40 s and choline concentrations of 1–10 μM were used. The specific uptake of choline was taken as the difference between uptake in the presence of an inwardly directed Na^+ gradient and that at K^+ equilibrium. The radioactivity associated with membranes at K^+ equilibrium was probably due to nonspecific binding since it was not displaced by hemicholinium-3, whereas that part of uptake attributable to the imposed Na^+ gradient was completely blocked by low concentrations of the compound (Fig. 4). Inhibition by hemicholinium-3 was competitive (Fig. 9a) with a K_i of 25 nM.

The kinetics of the transport system were those of a simple, saturable system (Fig. 9b) with K_T of 1.7 μM and a V_m of 96 pmol · mg^{-1} · min^{-1} at 25°C which are in accordance with previous values for *Torpedo* electromotor synaptosomes [44].

Enrichment of membranes containing the choline transporter and identification of polypeptides copurifying with it

In attempting to purify membrane vesicles by using uptake of a low molecular mass substance as a reference parameter one must be aware of the possibility that membrane fractions rich in transporter could escape detection because the procedure had damaged the capacity of the membranes to form well-sealed vesicles. Thus, membranes able to form such vesicles might show a higher uptake and therefore higher transporter activity than poorly sealed vesiculated membrane fragments in another fraction that were richer in transporter molecules. We have checked this by measuring radiolabelled choline uptake by each fraction at several different osmolarities; radioactivity associated with membranes at K^+ equilibrium was subtracted. The results are illustrated in Fig. 10. It will be seen that when uptake is plotted as a function of the reciprocal of osmolarity the points can be empirically fitted to straight lines. When these are extrapolated to infinite osmolarity, they intersect

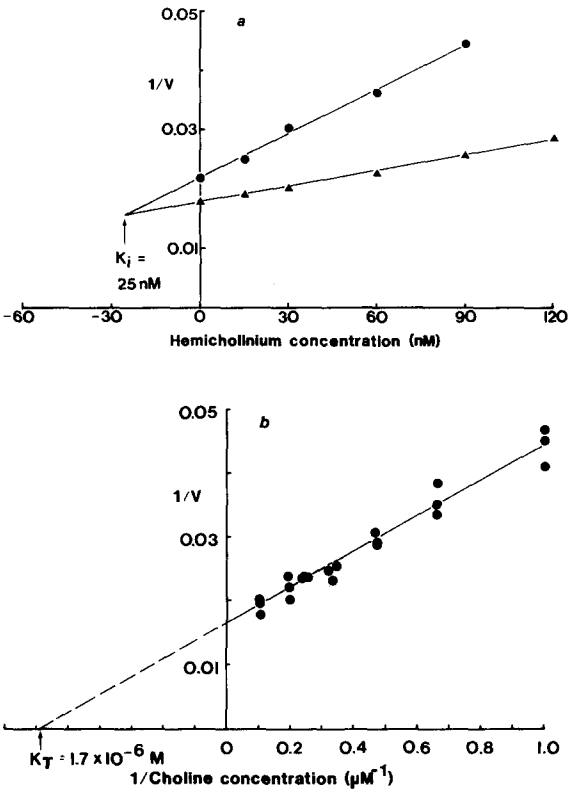


Fig. 9. Kinetics of choline uptake and its inhibition by hemicholinium-3. (a) Determination of K_i for hemicholinium-3 using a Dixon plot [43]. Velocity of upake of choline was expressed as pmol of [^{3}H]choline accumulated per mg of protein in 40 s in buffer C less those accumulated in buffer B. Values were obtained at (●) 2.1 μM and (▲) 5.5 μM [^{3}H]choline concentration and straight lines were fitted by the method of least squares. (b) Double reciprocal plot of the initial uptake of [^{3}H]choline over the range 1–10 μM into resealed membrane vesicles in the presence of an inwardly directed Na^+ gradient. Units and line-fitting as in (a).

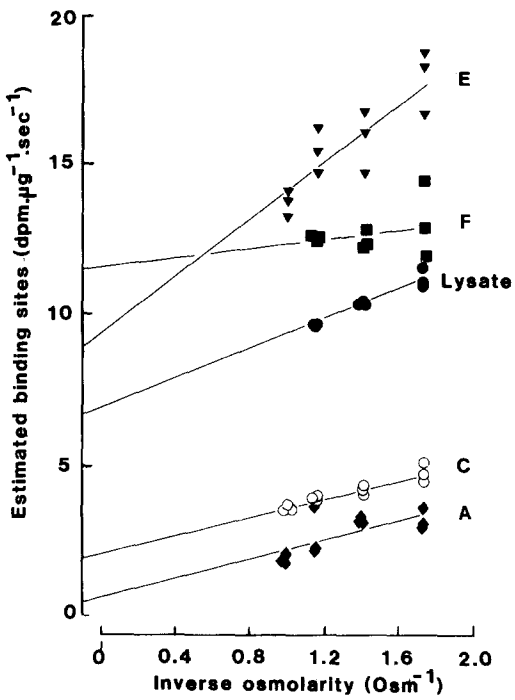


Fig. 10. Uptake of choline as a function of osmotic pressure. The initial rate (40 s) of uptake of [^{3}H]choline by the various fractions of Ficoll-prepared lysed synaptosomes separated on a Ficoll density gradient is plotted as a function of the reciprocal of osmotic pressure. The straight lines were fitted by the method of least squares and extrapolated back to the y axis to give a measure of the number of binding sites per μg of protein. These were: fraction A (◆) 0.4, B (not shown) 1.3, C (○) 1.9, D (not shown) 3.0, E (▼) 8.9, F (■) 11.5, parent fraction (●) 6.7.

the ordinate at various points above the origin. These intercepts may be regarded as a rough measure of the relative number of specific binding sites for choline per μg of membrane protein. It will be seen that only in the case of fraction F was the uptake of choline noticeably less sensitive to changes in osmotic pressure than that of the other fractions, suggesting that it forms less tightly sealed vesicles than the others; this may in part explain its lower activity relative to fraction E, since on extrapolation, the ordinate intercept for fraction F is actually higher than that for fraction E. Electron-microscopic examination (not shown) indeed confirmed that fraction F had a greater proportion of unsealed, open membrane fragments.

Correlation with enzyme activity. The activity of three marker enzymes (Table II) which are enriched in the synaptosome fraction and again in the synaptosomal lysate, namely acetylcholinesterase, 5′-nucleotidase and ouabain-sensitive ATPase, was measured in the various subfractions of the lysate and compared with their content of specific choline binding sites. The best positive correlation ($r^2 > 0.993$) was with acetylcholinesterase activity; a good positive correlation was also found with 5′-nucleotidase ($r^2 > 0.987$) but ouabain-sensitive ATPase showed a negative correlation ($r^2 > -0.915$). Also shown in Table II are measure-

TABLE II

CHARACTERIZATION OF SUBFRACTIONS OF SYNAPTOSOMAL LYSATES SEPARATED ON A 4–12% FICOLL GRADIENT BY MEANS OF MARKER ENZYMES

Enzyme	Units of substrate transformed per min	No. of expts.	Specific activity (units·(mg of protein)$^{-1}$·min^{-1})						
			Synaptosomal lysate (P)	A	B	C	D	E	F
Lactate dehydrogenase	nmol	5	329.1 ±47.4	220.4 ±52.8	309.2 ±78.2	189.7 ±35.9	250.7 ±65.1	404.0 ±99.8	344.0 ±74.4
Choline acetyltransferase	nmol	4	11.5 ±2.0	7.4 ±3.6	11.1 ±4.7	5.1 ±1.7	9.5 ±3.4	16.0 ±4.6	7.5 ±1.0
Acetylcholinesterase	μmol	7	22.5 ±2.7	10.3 ±1.0	13.2 ±1.2	14.7 ±1.3	18.6 ±2.5	28.5 ±4.2	36.3 ±8.6
Butyrylcholinesterase	nmol	5	12.3 ±1.6	13.2 ±0.9	14.3 ±1.4	11.8 ±1.1	10.8 ±1.6	10.7 ±1.0	15.1 ±1.9
5′-Nucleotidase	μg	4	7.0 ±0.6	2.3 ±0.5	3.1 ±0.6	2.2 ±0.5	5.7 ±1.1	13.4 ±3.0	15.0 ±3.4
Ouabain-sensitive ATPase	μg	4	40.1 ±4.6	42.7 ±3.0	40.6 ±5.3	36.4 ±4.6	15.2 ±3.4	5.2 ±2.3	2.5 ±1.8

ments of the cytoplasmic enzymes lactate dehydrogenase and choline acetyltransferase, and the putative glial membrane marker butyrylcholinesterase. Cytoplasmic markers were not completely eliminated and in contrast to acetylcholinesterase, butyrylcholinesterase did not copurify with the choline binding sites.

Polypeptide composition. The various membrane fractions were submitted to gel electrophoresis in sodium dodecyl sulphate. A representative gel is shown in Fig. 11. A comparison of the patterns showed that at least six or seven components copurified with the transporter; several of these had relative molecular masses (M_r) of > 200 000; others had M_r values ($\times 10^{-3}$) of 140, 68 (doublet), 57, 54 and 28. Other components of M_r ($\times 10^{-3}$) 160, 100, 60, 47 and 42 did not copurify. A 68 000 component reacted with an anticholinesterase antiserum in a blot test (Fig. 12); the second component of the 68 000 doublet is probably absorbed choline acetyltransferase since this enzyme was present in all the fractions. This left the 140 000, 57 000, 54 000 and 28 000 components as the most likely candidates for the transporter; the other components may be rejected since they failed to co-purify with the transporter.

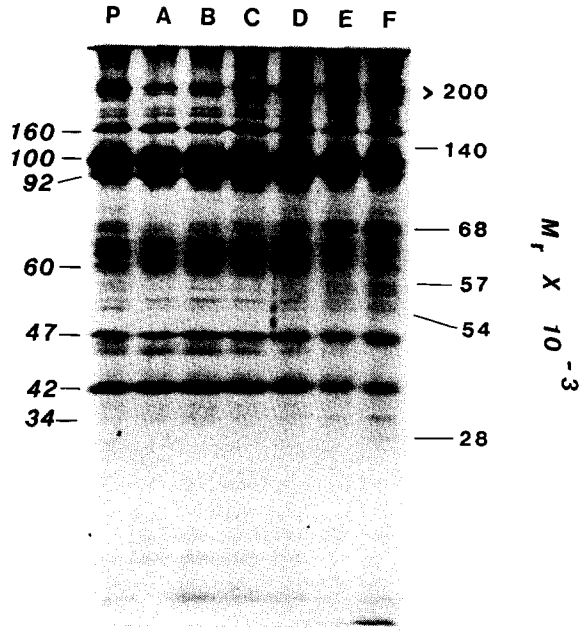


Fig. 11. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of proteins present in the various synaptosomal membrane fractions. The positions and M_r values of polypeptides that copurified with the choline transporter (M_r ($\times 10^{-3}$) > 200, 140, 68, 57, 54 and 28) are indicated on the right, and other polypeptides on the left. Each lane received 76 μg of protein. P is parent fraction.

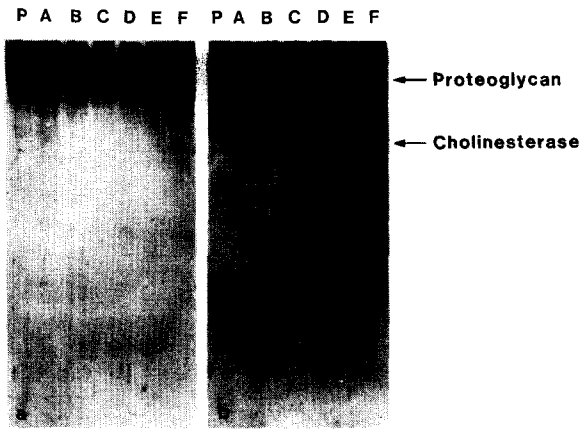


Fig. 12. Presence of vesicle-specific proteoglycan and cholinesterase in synaptosomal membrane fractions demonstrated immunochemically. Immune blots of the polyacrylamide gels obtained from electrophoretic separation of the proteins of the various membrane fractions were stained with (a) anti-vesicle proteoglycan serum followed by (b) anti-acetylcholinesterase serum. The 68 000 band is recognized as acetylcholinesterase by the anti-cholinesterase serum and the broad band of M_r approx. 200 000 reveals the presence of a specific vesicle component copurifying with the transporter.

The pattern of polypeptides seen in the parent fraction and its subfractions agreed fairly well with those previously published [30,40,45] for synaptosomal membranes purified on a sucrose gradient, except that there were more minor bands. Among the components which did not copurify with the transporter, that of M_r 100 000 is tentatively identified as the ouabain-sensitive ATPase, and another of M_r 42 000 is probably actin [30,46].

A proteoglycan specific for synaptic vesicles was detected in all the fractions but most intensely in fraction F by the immunoblot technique using an antiserum raised to it in this department [39,40] (Fig. 12).

Reconstitution experiments with proteins solubilized from lysates of Ficoll-purified synaptosomes

Characterization of the reconstituted system. Lipid suspensions mixed with solubilized proteins from concentrated detergent extracts of synaptosomal plasma membranes (derived from lysates of fraction F_1) and then dialysed acquired the ability to take up choline in a Na^+-dependent manner in the presence of an inwardly directed Na^+ gradient (Figs. 13–15). The properties of the system closely resembled those of the intact membranes, except that the initial (one minute) rate of choline uptake per mg of protein was about 10-times faster and the peak of the overshoot was attained sooner. The decrease in time to attain the peak of the overshoot is not due to an average decreased vesicle (liposome vs. intact membrane) size, because the height of the overshoot was also increased. This suggests that reconstitution brought about a partial purification of the transporter as has been observed in other systems [41] or, perhaps, an activation of the reconstituted carriers.

Another factor that might have contributed to enhanced activity, namely lower permeability of the artificial membrane to Na^+ compared to the natural membrane, seems to be ruled out by the characteristics of the 'overshoot' (see Discussion). As seen in Fig. 13 the uptake of choline showed the typical overshoot, was augmented by 250 nM valinomycin and inhibited by 10 μM hemicholinium-3. Under conditions of K^+ equilibrium and no Na^+ gradient, uptake was neglible. When Li^+ replaced Na^+ (Fig. 14), uptake was greatly reduced; Cs^+ and Rb^+ were ineffective. Although an inwardly directed Cl^- gradient was

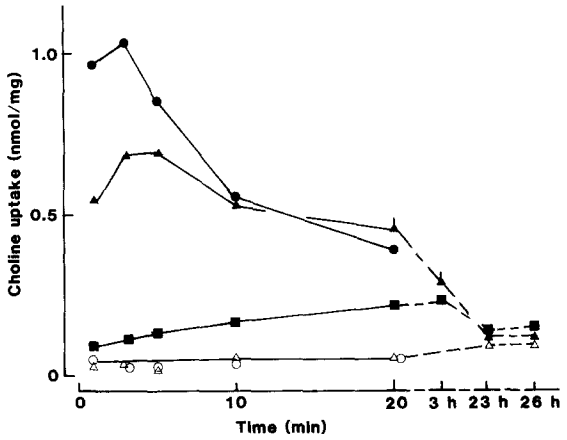


Fig. 13. Choline uptake by proteoliposomes containing membrane proteins of synaptosomal lysates. The proteoliposomes were loaded with KCl. The uptake in the presence of an inwardly directed Na^+ gradient alone (▲), or with addition of 250 nM valinomycin (●), or 10 μM hemicholinium-3 (■) is shown, also at K^+ equilibrium with (○) or without (△) valinomycin. Points are means of triplicates or quadruplicates; bars are S.E. values.

necessary for maximum choline uptake (Fig. 15) Br^- could partially replace Cl^- but NO_3^-, SCN^- and a Cl^- gradient in the absence of Na^+ were all ineffective. The optimum temperature for uptake was again 25°C (Fig. 16).

If exogenous lipids were not added to the concentrated supernatant of solubilized membrane proteins no choline uptake was subsequently obtained. When liposomes were tested without incorporated solubilized membrane proteins, no Na^+-gradient specific uptake of choline was observed. Values obtained at K^+ equilibrium and in the presence of Na^+ were not different and did not change significantly over a period of 20 min. The average radioactivity associated with liposomes was approximately 14 fmol. The use of cholate concentrations greater than 0.1% (0.3, 0.7 and 2.0% were tested) or a phosphatidylserine/cholesterol system [41] in combination with 0.2% deoxycholate or 0.7% cholate to solubilize membrane pro-

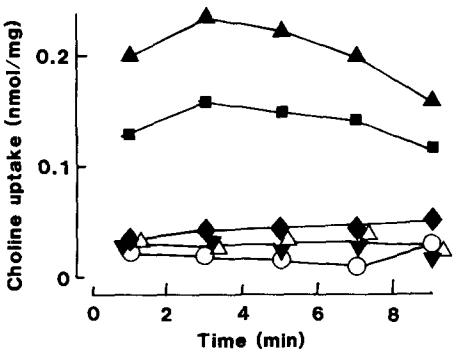


Fig. 15. Anion dependence of choline uptake into K_2SO_4-loaded liposomes. Proteoliposomes were suspended in buffer G and dialysed for 24 h. Media during uptake studies were buffered with 20 mM imidazole-H_2SO_4 (pH 7.4), 0.1 mM $MgSO_4$ and contained in addition: 300 mM NaCl (Na^+ and Cl^- gradients) (▲); 300 mM NaBr (Na^+ and Br^- gradients) (■); 300 mM $NaNO_3$ (Na^+ and NO_3^- gradients) (◆); 300 mM NaSCN (Na^+ and SCN^- gradients) (▼); 300 mM KCl (K^+ equilibrium, Cl^- gradient) (△); 150 mM K_2SO_4, 150 mM mannitol (K^+ equilibrium, no Cl^- present) (○).

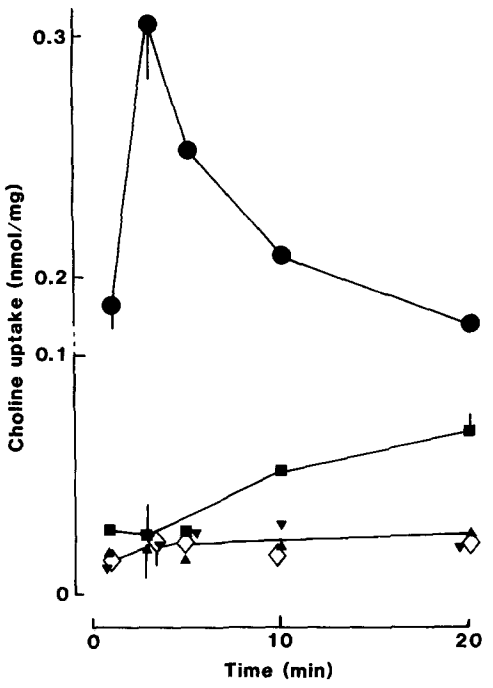


Fig. 14. Na^+ dependence of choline uptake into KCl-loaded proteoliposomes. Inwardly directed gradients: Na^+ (●), Li^+ (■), Cs^+ (▼), Rb^+ (▲). K^+ equilibrium (◇). Liposomes were loaded with buffer E and incubated in buffer B (K^+ equilibrium) of buffer C (containing either NaCl, LiCl, CsCl or RbCl) during transport studies.

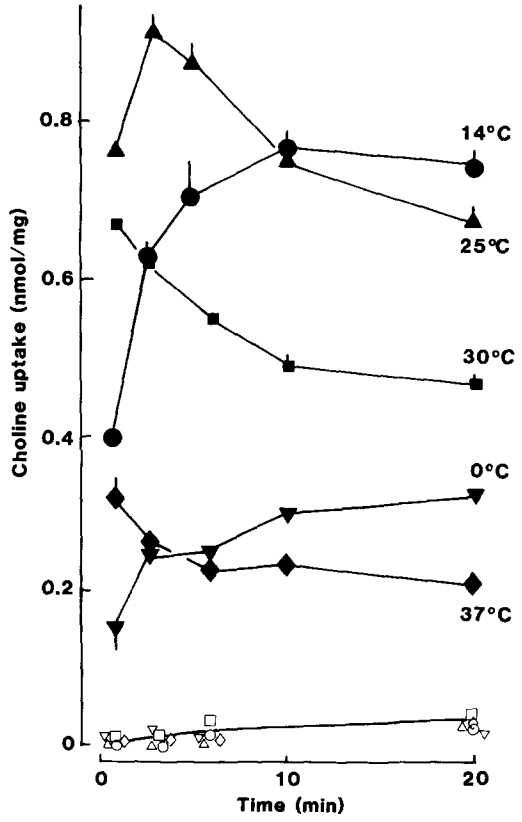


Fig. 16. Temperature dependence of choline uptake into proteoliposomes loaded with KCl. Filled symbols, Na^+ gradient; empty symbols, K^+ equilibrium.

teins gave lower rates of uptake than those obtained with the present system.

Characterization of transferred proteins. The polypeptide content of the proteoliposomes showing optimal choline uptake was analysed by gel electrophoresis in the presence of sodium dodecyl sulphate and compared with that of the synaptosomal lysate, the washed membranes of the synaptosomal lysate and the solubilized membrane proteins (Fig. 17). Solubilization simplified the polypeptide pattern and a protein of M_r 54000 previously noticed as one of those copurifying with the transporter when synaptosomal lysates were subfractionated on Ficoll gradients became prominent. The gel pattern further simplified on transfer to liposomes and the M_r 54000 protein was still present as a prominent component. The main partially lost in the transfer were that tentatively identified as the ouabain-sensitive ATPase (M_r 100000) and ones of M_r 92000 and 160000. The 140, 57 and 28 kDa components were largely or wholly lost in the transfer. When a correlation was made between transport rates observed in the various liposome preparations tried (see previous section) and the presence of particular polypeptides, only three proteins correlated with the ability of liposomes to transport choline: the > 200, 54 and 34 kDa components.

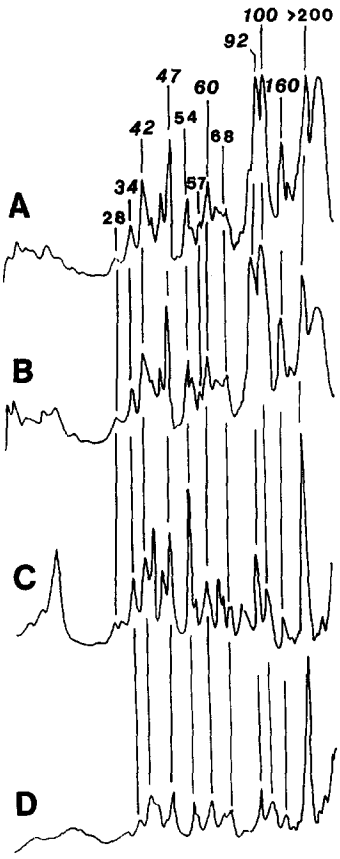


Fig. 17. Densitometry tracings of polyacrylamide gel electrophoretograms of solubilized and reconstituted synaptosomal membrane proteins in sodium dodecyl sulphate. (A, B) Membranes of synaptosomal lysate (A) before and (B) after washing with buffer D; (C) solubilized proteins; (D) liposomes. The numbers in (A) identify major components that copurify with the transporter (M_r > 200000, vesicle proteoglycan; 140000; 68000, acetylcholinesterase; 57000; 54000; 28000) or (in italics) do not (160000; 100000, 92000, ouabain-sensitive ATPase; 60000; 47000; 42000, actin; 32000). The minor M_r 140000 band copurifying with the transporter (Fig. 11) is not clearly resolved from the M_r 100000 component in the densitometric scan. The solubilization step and the transfer to liposomes effect a simplification of the pattern and cause the selective incorporation.

Discussion

Experiments with vesiculated presynaptic plasma membrane fragments

The isolation from synaptosomes of preparations of presynaptic plasma membranes which can be induced to form sealed vesicles and demonstrate choline uptake in the presence of an imposed ionic gradient is a first step towards the identification and purification of the specific transporter and in understanding how transport is coupled in vivo to sources of metabolic energy. Only one attempt, described as unsuccessful, has been reported [25] with mammalian cortical synaptosomal membrane preparations though limited success has been obtained with proteoliposomes derived from such membranes [25]. These showed both the high-affinity choline uptake characteristic of cholinergic terminals and the low affinity uptake present in all synaptosomes. However, work with synaptosomal membrane preparations from insect synaptosomes, which have a much higher contribution from cholinergic nerve terminals, has been more successful [23] and we have now shown that very active preparations can be derived from synaptosomes derived from the purely cholinergic *Torpedo* electromotor nerve terminals.

The apparent lability of the choline transporter justified a study of the optimum conditions for isolating and lysing synaptosomes with regard to the composition of the density gradient on which the synaptosomes were separated and the pH, temperature and composition of the lysing buffer. The slightly alkaline conditions (pH 8.5) established as optimum for lysis is known to remove proteins from membranes and promote the vesiculation of membrane fragments [47,48].

The kinetics of uptake showed four well-defined characteristics. Firstly, optimal uptake was ion-gradient dependent. Uptake was absolutely dependent on an inwardly directed Na^+ gradient; the rate of uptake quickly fell away from its initial value as the Na^+ gradient dissipated and was followed by a slower efflux of choline down its concentration gradient, producing an 'overshoot'. This overshoot is typical of uptake systems driven by an imposed Na^+ gradient [41]. Optimal uptake was dependent on K^+ as the loading ion and on an inwardly directed Cl^- gradient; Rb^+ and Br^-, respectively, could partially substitute for these two ions. Uptake was increased when the outwardly directed K^+ gradient was allowed to express itself electrochemically by the addition of the K^+-specific ionophore valinomycin. Secondly, the uptake of choline displayed simple saturation kinetics and the apparent dissociation constant of the putative choline-transporter complex (K_T) was 1.7 μM, very similar to that found for intact synaptosomes and characteristic of the high-affinity uptake system. Initial rates varied considerably between different preparations. Measured at Cl^- equilibrium, they varied between 25 and 60 pmol · (mg of protein)$^{-1}$ · min^{-1} with a mean of 44, values comparable to those reported [44] for electromotor synaptosomes; measured in the presence of an inwardly directed NaCl gradient (Figs. 5 and 8), they were higher and ranged between 75 and 160. This contrasts with a maximum of 75 pmol · (mg of protein)$^{-1}$ · min^{-1} for insect preparations under comparable conditions [23]. Thirdly, the uptake was very sensitive to hemicholinium, with a K_i of 25 nM. Finally, uptake was temperature dependent. That the optimum temperature temperature for uptake was no higher than 25°C is understandable in view of the fact that the membranes were derived from a poikilothermic animal.

We have also shown that synaptosomal membrane fragments containing the choline transporter can be purified on a Ficoll density gradient while retaining their capacity to bind choline specifically and to take it up. Such fragments are only able to take up choline as a result of their ability to form closed vesicles under the conditions of preparation used; this is shown by the sensitivity of the uptake to changes in osmotic pressure. It is possible to conceive of a vesicle subjected to osmotic dehydration in solutions of high osmolarity entirely losing its capacity to take up choline, and as a result of osmotic collapse, there would be no internal space into which choline could penetrate. However, under these circumstances, choline would still bind specifically to the transporter but would not be transported. Thus by measuring the rate of uptake at different osmotic pressures and extrapolating to infinite osmolarity, it is possible to estimate at least roughly the number of specific choline binding sites in the preparation. Extrapolation is simplified by assuming that uptake varies linearly with the reciprocal of the osmolarity of the suspension medium; although we have advanced no theoretical reason for this, it seems to be empirically true at least over the limited range of osmotic pressures studied. This method avoids differences in uptake among fractions caused by variations in the ability of the fractions to form sealed vesicles and may thus provide a better measure of the transporter content of the fraction. This is important when evaluating copurification of protein components with transporter activity. That fractions do vary in their capacity to form sealed membranes is shown by variations among the fractions in the slope of the line connecting uptake with the reciprocal of osmolarity. This is most marked as between fractions E and F; the latter had an uptake less than that of the former, but that this was partly due to its reduced capacity to form tightly sealed membrane fragments was shown by the fact that its uptake was less affected by changes in osmotic pressure than that of the neighbouring fraction E; thus when extrapolation was made to infinite osmotic pressure, the number of specific binding sites for choline per μg of protein proved to be higher in F than in E. It has not been rigorously excluded that cholinesterase and muscarinic binding sites as well as transporter

molecules contribute to these (to do so would require binding studies well beyond the scope of this paper), but this seems unlikely since choline has a very low affinity for cholinesterase (and, presumably, for muscarinic sites) at the concentration used and its binding was fully hemicholinium-sensitive.

Several proteins copurified with the choline binding sites, those of M_r ($\times 10^{-3}$) > 200, 140, 68, 57, 54 and 28 showing the best positive correlation. One component of the M_r 68 000 doublet was identified immunochemically as acetylcholinesterase; the other is probably adsorbed choline acetyltransferase. The M_r > 200 000 component was identified immunochemically as the synaptic vesicle-specific proteoglycan [39,40]; this leaves the components with M_r ($\times 10^{-3}$) 140, 57, 54 and 28 as possible candidates for the transporter, assuming this is a protein with an M_r of less than 200 000, as most identified transporters are. Of these, only the 54 kDa component was enriched in the proteoliposomes (as percent total protein).

Reconstitution experiments

In these, liposomes acquired all the properties of the transporter system observed in the native membranes, so far as these were tested: uptake of choline in the presence of an inwardly directed Na^+ gradient; dependence of the uptake on Na^+, partial dependence on Cl^-, overshoot; sensitivity to hemicholinium-3; electrogenicity and an optimum temperature of 25°C. That selective transfer of proteins to the liposomes occurred is suggested by the much higher initial rate of uptake of choline per mg of protein and the higher and quicker overshoot; that this is simply due to a lower Na^+ permeability of the lipid membrane seems to be ruled out by the fact that the ratio of the heights of the overshoots in the native and reconstituted systems was less than that of the initial rates. Furthermore the more rapid decline of the overshoot in the reconstituted system suggests that ionic gradients dissipate more rapidly across the proteoliposomal membrane than the native membranes.

It is instructive to compare the polypeptide composition of our proteoliposomes with that published by Israël et al. [29]. The most prominent proteins transferred in their preparation were described as having M_r ($\times 10^{-3}$) 90, 60 and 42; these may be the M_r 100 000 and 92 000, 60 000 and 42 000 components seen in Fig. 17. It should be pointed out that the 68 000 component, acetylcholinesterase, is transferred to our proteoliposomes as well as the transporter. Unless the esterase is completely inhibited (and Israël et al. [29] did not test for this) the acetylcholine entrapped in their proteoliposomes might well have been hydrolysed and choline, not acetylcholine, would have been released, possibly via the choline transporter working in reverse. Since the acetylcholine assay they used involves hydrolysis of the ester to choline and assay of choline, choline leaking out of the proteoliposomes would also have given a signal, which they would have misinterpreted as acetylcholine. Thus the relevance of their results to the problem of acetylcholine release at the nerve terminal under physiological conditions is doubtful, though they might be relevant to choline uptake.

We have also noted the presence of synaptic vesicles in electron micrographs of lysed synaptosomes and have demonstrated by the immunoblot technique the presence of the specific vesicle proteoglycan antigen in the synaptosomal plasma membrane fragments. Thus it would be difficult to exclude the possibility that some synaptic vesicle membrane constituents, including the vesicular acetylcholine transporter [49], are incorporated into proteoliposomes along with plasma membrane constituents. In the experiments of Israël et al. [29] this may also have happened; thus even if acetylcholine had survived hydrolysis by cholinesterase it might well have been released through the proteoliposome membrane via the vesicle acetylcholine carrier. By contrast, the presence of this carrier could not have affected our results since the vesicle acetylcholine uptake mechanism is known to have a very high K_T for choline (10 mM) [49].

Acknowledgements

We are grateful to Drs G.P. Richardson and J.H. Walker for gifts of antisera and to Dr. G.Q. Fox and Herrn D. Kötting for electron microscopy. I.D. was in receipt of an Auslandsstipendium of the Max-Planck-Gesellschaft.

References

1 Iversen, L.L. (1971) Br. J. Pharmacol. 41, 571–591
2 Bennett, J.P., Jr., Mulder, A.H. and Snyder, S.H. (1974) Life Sci. 15, 1045–1056
3 Whittaker, V.P. (1972) Biochem. J. 128, 73P–74P
4 Yamamura, H. and Snyder, S.H. (1972) Science 178, 626–628
5 Dowdall, J.M. and Simon, E.J. (1973) J. Neurochem. 21, 969–982
6 Iversen, L.L. and Neal, M.J. (1968) J. Neurochem. 15, 1141–149
7 Ross, S.B. and Renyi, A.L. (1967) Life Sci. 6, 1407–1415
8 Schuberth, J., Sundwall, A., Sörbo, B. and Lindell, J.O. (1966) J. Neurochem. 13, 347–352
9 Kuhar, M.J., Sethy, V.H., Roth, R.H. and Aghajanian, G.K. (1973) J. Neurochem. 20, 581–593
10 Guyenet, P., Lefresne, P., Rossier, J., Beaujouan, J.C. and Orlowski, J. (1973) Mol. Pharmacol. 9, 630–639
11 Haga, T. and Noda, H. (1973) Biochim. Biophys. Acta 291, 564–565
12 Pert, C. and Snyder, S. (1974) J. Pharm. Exp. Ther. 191, 102–108
13 Carroll, P.T. and Buterbaugh, G.G. (1975) J. Neurochem. 24, 917–924
14 Simon, J.R. and Kuhar, M.J. (1976) J. Neurochem. 27, 93–99
15 Colburn, R.W., Goodwin, F.K., Murphy, D.L., Bunney, W.E. and Davis, J.M. (1968) Biochem. Pharmacol. 17, 957–964
16 Weinstein, H., Varon, S., Muhleman, D.R. and Robert, E. (1965) Biochem. Pharmacol. 14, 273–388
17 Logan, W.J. and Snyder, S.H. (1972) Brain Res. 42, 413–431
18 Kanner, B.I. (1978) Biochemistry 17, 1207–1212
19 Kanner, B.I. and Sharon, I. (1978) Biochemistry 17, 3949–3953
20 Marvizón, J.G., Mayru, F., Jr., Aragón, M.C., Giménez, C. and Valdivieso, F. (1981) J. Neurochem. 37, 1401–406
21 Roskoski, R., Jr. (1981) J. Neurochem. 36, 544–550
22 Gordon, D., Zlotkin, E. and Kanner, B. (1982) Biochim. Biophys. Acta 688, 229–236
23 Breer, H. and Lueken, W. (1983) Neurochem. Int. 5, 713–720
24 King, R.G. and Marchbanks, R.M. (1980) Nature 287, 64–65
25 Meyer, E.M. and Cooper, J.R. (1982) Science 217, 843–845
26 Richardson, P.J. (1981) J. Neurochem. 37, 258–260
27 Feldberg, W. and Fessard, A. (1942) J. Physiol. (Lond.) 101, 200–216
28 Whittaker, V.P. (ed.) (1985) The *Torpedo* Electromotor System as a Neurobiological Model, Weinheim, Verlag Chemie, in the press
29 Israël, M., Lesbats, B. and Manaranche, R. and Morel, N. (1981) Biochim. Biophys. Acta 728, 438–448
30 Stadler, H. and Tashiro, T. (1979) Eur. J. Biochem. 101, 171–178
31 Morel, N., Manaranche, M., Israël, M. and Gulik-Krzywicki, T. (1982) J. Cell Biol. 93, 349–356
32 Israël, M., Manaranche, R., Mastour-Frachon, P. and Morel, P. (1976) Biochem. J. 160, 113–115
33 Bray, G.A. (1960) Anal. Biochem. 1, 279–285
34 Medzihradsky, F., Nandhasri, P.S., Idoyaga-Varge, V. and Sellinger, O.Z. (1971) J. Neurochem. 18, 1599–1603
35 Johnson, M.K. and Whittaker, V.P. (1963) Biochem. J. 88, 404–409
36 Racker, E. (1950) Biochim. Biophys. Acta 4, 211–214
37 Ellman, G.L., Courtney, K.D., Andres, V., Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95
38 Fonnum, F. (1975) J. Neurochem. 24, 407–409
39 Jones, R.T., Walker, J.H., Stadler, H. and Whittaker, V.P. (1982) Cell Tissue Res. 223, 117–126
40 Walker, J.H., Obrocki, J. and Zimmermann, C.W. (1983) J. Neurochem. 41, 209–216
41 Ducis, I. and Koepsell, H. (1983) Biochim. Biophys. Acta 730, 119–129
42 Ohta, M., Narahashi, T. and Keeler, R.F. (1973) J. Pharmacol. Exp. Ther. 181, 143–154
43 Dixon, D.M. (1953) Biochem. J. 55, 170–171
44 Richardson, P.J. and Whittaker, V.P. (1981) J. Neurochem. 36, 1536–1542
45 Whittaker, V.P. and Stadler, H. (1980) in Proteins of the Nervous System (Bradshaw, R.A. and Schneider, D.M., eds.), 2nd Edn. pp. 231–255, Raven Press, New York
46 Zechel, K. and Stadler, H. (1982) J. Neurochem. 39, 788–795
47 Fonnum, F. (1967) Biochem. J. 103, 262–270
48 Steck, T.L., Weinstein, R.S., Straus, J.H. and Wallach, D.F.H. (1970) Science 168, 255–257
49 Giompres, P. and Luqmani, Y.A. (1980) Neuroscience 5, 1041–1052