The incorporation of solubilized choline-transport activity into liposomes

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The choline-transport system has been solubilized from synaptic plasma membrane by using either sodium cholate or Triton X-100, and re-incorporated into unilamellar liposomes by using the technique of cholate dialysis. The criteria of choline-transport activity were saturability by excess choline, inhibition by hemicholinium-3, and trans-activation (i.e. stimulation of the uptake of [^3H]choline into liposomes by preloading them with non-radioactive choline). Liposomes prepared from detergent extracts of synaptic plasma membrane and added lipid showed uptake of [^3H]choline fulfilling these three criteria. Data on choline-transport activity of liposomes at various choline concentrations could be interpreted as implying that the transport system has two apparent K_m values (2–5 μM and 50–100 μM), or alternatively that the system is composed of two or more negatively co-operating subunits (or units). It was shown by t.l.c. that the transported radioactivity was choline and that it was not significantly acetylated. Replacing Na^+ by K^+ on the outside of these liposomes partially inhibited uptake, and the formation of a potential gradient (inside negative) with valinomycin increased the total but not the saturable components of uptake when liposomes were prepared in a K^+ medium, and transferred to an Na^+ medium.

The study of choline transport is of importance because choline transport is necessary for the synthesis of acetylcholine in nerve cells and indeed may be a rate-limiting or regulatory factor in this synthesis. Choline transport has been discovered in many places other than neurons [e.g., erythrocytes (Askari, 1966; Martin, 1968); skeletal-muscle fibres (Renkin, 1961)], so that it may also be of importance for the supply of choline for functions other than the synthesis of acetylcholine (e.g., for the synthesis of phosphatidylcholine). But the investigation of choline uptake in biological systems (such as intact cells, synaptosomes or brain slices) is impeded because of their complexity. One possible approach, however, is to try to solubilize the choline-transport system and then re-incorporate it into an artificial membrane (e.g. a unilamellar liposome or planar lipid bilayer) and measure choline transport across this artificial membrane. It may then be possible to experimentally control the environment on both sides of the membrane, thus allowing easier investigation of the molecular mechanism, control factors and the relation to acetylcholine synthesis. A further advantage of this approach is that choline transport in a functionally reconstituted system may be used as an assay for the choline carrier, thus enabling its purification, which

would resolve the question of the existence of two separate carriers of high and low affinity.

It was necessary to have some criteria that would enable one to decide if the reconstituted membrane system (in this case liposomes) demonstrated choline-transport activity. These criteria may then be said to be a definition of 'choline-transport activity'. The rate of choline uptake in most biological systems (e.g., synaptosomes and erythrocytes) demonstrates the following three properties. (1) It is inhibited by hemicholinium-3. (2) The inhibitable component of choline uptake demonstrates saturability (i.e. there is a maximum rate of inhibitable choline uptake, which is not increased by further increases of external choline concentration). Thus the initial rate of uptake of [^3H]choline chloride (1 μM) is inhibited by saturating concentrations (e.g., 10 mM) of non-radioactive choline. (3) The rate of choline uptake (or efflux) is increased by choline inside (or outside) the membrane-enclosed space; this is known as transactivation (Martin, 1968; Marchbanks *et al.*, 1981). These three properties were used as criteria of choline transport in the reconstituted membrane system. We report the solubilization from synaptic plasma membranes of a substance conferring these characteristics on the membrane of liposomes. A preliminary communi-

cation of some of these results has been made (King & Marchbanks, 1980).

Materials and methods

Materials

[Me-³H]Choline chloride (sp. radioactivity 60–85 Ci/mmol; ethanolic solution) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The ethanol was removed by a stream of N₂ before use. Hemicholinium-3 was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Dowex 50W (X8; H⁺ form, 50–100 mesh) was obtained from Sigma, Poole, Dorset, U.K.

Egg phosphatidylcholine (grade I), phosphatidylserine (grade I from bovine spinal cord) and phosphatidylethanolamine (grade I from egg yolk) were obtained from Lipid Products, Nutfield Nurseries, Nutfield, Surrey, U.K. Ox cortex lipid extract was prepared by the method of Brammer (1973), a modification of the original method of Folch *et al.* (1957). Immediately before the use of this lipid extract in experiments, chloroform was removed by a rotary evaporator, and residual chloroform removed by resuspending the lipid in diethyl ether, the ether being then removed in the same way. Asolectin, a preparation of soya-bean phospholipid, was purchased in granular form from Associated Concentrates, Woodside, NY, U.S.A., and was acetone-washed (to remove protein) by the method of Kagawa & Racker (1971) with 0.01% (w/v) *NN*-diphenyl-*p*-phenylenediamine as an anti-oxidant. Triton X-100, cholic acid and sodium dodecyl sulphate were obtained from Sigma. Millipore filters (GSPW 02500) were obtained from Millipore (U.K.) Ltd., London, England, and dialysis tubing (cellulose tubing, 16 mm inflated diameter, type 250 9U) from Sigma.

Synaptic plasma-membrane preparation

This was prepared by the method of Jones & Matus (1974). Guinea-pigs were killed by stunning and exsanguination, the cerebral cortices were quickly dissected, weighed and transferred to 9 vol. of ice-cold 0.32 M-sucrose containing 5 mM-Tris/HCl (pH 7.4). All subsequent procedures were performed at 4°C. A 10% (w/v) homogenate was prepared by using a Teflon/glass homogenizer (Aldridge *et al.*, 1960) with a clearance of 0.25 mm and a speed of 840 rev./min. Ten strokes of the pestle were routinely used. The homogenate was centrifuged at 1000 g (3000 rev./min in a Sorvall RC2B centrifuge, SS34 head) for 10 min. The supernatant was kept on ice, and the pellet was washed by re-homogenizing it in a small volume of 0.32 M-sucrose, and repeating the centrifugation. The supernatants were combined, and centrifuged at 12000 g (10000 rev./min; Sorvall RC2B apparatus).

for 20 min to yield a crude synaptosomal pellet containing mainly mitochondria, synaptosomes and myelin. The supernatant was discarded.

The pellet was hypo-osmotically ruptured by homogenizing (as above) in hypo-osmotic buffer (5 mM-Tris/HCl, pH 8.1; 4 ml for each original g of tissue) and left at 4°C for 60 min. For the production of the 'crude hypo-osmotically ruptured synaptosomal fraction' this was centrifuged at 95000 g for 20 min. For the preparation of the synaptic plasma membrane fraction an equal volume of 2.3 M-sucrose containing 5 mM-Tris/HCl (pH 7.4) was added and gently mixed. Flotation-sedimentation density-gradient centrifugation was then performed on this suspension by layering 10 ml of 0.92 M-sucrose containing 5 mM-Tris/HCl (pH 7.4) over 28 ml of the suspension, and spinning at 95000 g (27000 rev./min; Beckman L2-65 instrument, with an SW27 head) for 2 h. The particulate fraction floating at the interface of the 1.15 M-sucrose and 0.92 M-sucrose consists mainly of synaptic plasma membrane; mitochondria and heavier particles are found in the pellet, and myelin and lighter particles float on the 0.92 M-sucrose (Jones & Matus, 1974). This fraction (containing synaptic plasma membrane) was collected using a Pasteur pipette, diluted 1:7 with 100 mM-NaCl/10 mM-Tris/HCl (pH 7.4) and centrifuged at 95000 g (27000 rev./min; Beckman L265 instrument, with an SW27 head) for 20 min. The pellet was resuspended in the above buffer to approx. 2 mg of protein/ml, and is called the 'synaptic plasma-membrane' fraction.

Solubilization of membrane by using detergents

Sodium cholate solubilization. Membrane preparations were solubilized with sodium cholate by using a modification of the procedure of Bardin & Johnstone (1978), itself a modification of the method of Kagawa & Racker (1971).

To membrane preparations prepared as above (approx. 2 mg of protein/ml in 100 mM-NaCl/10 mM-Tris/HCl, pH 7.4) were added the following: 100 mM-dithiothreitol (to 1 mM), 100 mM-dipotassium EDTA (to 0.3 mM), trypsin inhibitor (type II-S from soya beans; Sigma; to 0.1 mg/ml), phenylmethanesulphonyl fluoride (to 100 µM), 20% (v/v) sodium cholate (to 2%). The suspension was stirred at 4°C for 30 min and then centrifuged at 100000 g (Beckman L2-65 instrument, with an SW27 rotor) for 1 h at 4°C. The supernatant is henceforth called the sodium cholate-solubilized fraction. (This is an empirical definition of membrane-component solubilization used by many workers; see Razin, 1972.)

Sodium cholate solubilizes membrane components including lipids (particularly phosphatidylcholine) and membrane proteins, forming mixed lipid/detergent micelles and lipid/detergent/protein micelles

(Helenius & Simons, 1975). The presence of dithiothreitol and proteinase inhibitor (e.g., trypsin inhibitor, phenylmethanesulphonyl fluoride) during and after the solubilization procedure has been found to protect the functional integrity of certain membrane proteins (Lang *et al.*, 1979). The synaptic plasma-membrane fraction contained originally 2.5 mg of protein and 1.5 mg of phospholipid in that volume derived from approx. 0.5 g wet wt. Of this, 60% of the protein and 40% of the phospholipid were solubilized by 2% sodium cholate. The amounts solubilized by 0.5% Triton X-100 were respectively 36% and 27%. The relationship between the initial protein concentration P (mg/ml) and the percentage solubilization by cholate was found to conform (at a fixed sodium cholate to protein ratio of 6.7:1 (w/w) and within protein limits of between 0.2–5 mg/ml) to the equation:

$$\text{Solubilization (\%)} \simeq 40 P^{0.36}$$

Triton X-100 solubilization. Membrane preparations were solubilized by the non-ionic detergent Triton X-100, by using a modification of the method of Kasahara & Hinkle (1977). To membrane preparations prepared as above (approx. 2 mg of protein/ml in 100 mM-Tris/HCl, pH 7.4) were added the following: 100 mM-dithiothreitol (to 1 mM), 100 mM-dipotassium EDTA (to 0.3 mM), trypsin inhibitor (to 0.1 mg/ml), phenylmethanesulphonyl fluoride (to 100 μ M), 10% (v/v) Triton X-100 (to 0.5%). The suspension was stirred at 4°C for 30 min, and centrifuged at 100 000 *g* (Beckman L265 instrument, with an SW27 rotor) for 1 h at 4°C. The supernatant is then called the Triton X-100-solubilized fraction.

Preparation of liposomes

Three methods were used to prepare liposomes; cholate dialysis, sonication and freeze-thaw/sonication.

Cholate dialysis. The procedure used was one based on that reported by Kagawa & Racker (1971), and modified by Bardin & Johnstone (1978). This method is designed to form unilamellar liposomes by the removal of detergent (during dialysis) from a mixture of solubilized protein, lipid and sodium cholate. All procedures were performed at 4°C. A mixture of purified lipids of similar composition to synaptic plasma membrane [40% phosphatidylcholine, 30% phosphatidylethanolamine, 20% cholesterol, 10% phosphatidylserine (w/w); Breckenbridge *et al.*, 1972] were suspended in 100 mM-NaCl/10 mM-Tris/HCl, pH 7.4, 0.3 mM-EDTA and 2% (w/v) sodium cholate to a concentration of approx. 25 mg/ml. The suspension was sonicated in a sealed tube under N_2 to clarity in a bath sonicator (Kerry Ultrasonics, Hitchin, Herts., U.K.), containing ice and water, for approx. 30 min.

This suspension was centrifuged at 100 000 *g* (Beckman L265 instrument, SW27 rotor) for 1 h to remove any insoluble material. The supernatant was then added to the cholate-solubilized membrane fraction (see above), to give a final protein concentration of approx. 1 mg/ml, and lipid concentration of 5–15 mg/ml.

This solution was mixed for 10 min, and then dialysed for 16–20 h against 300–1000 vol. of 100 mM-NaCl/10 mM-Tris/HCl (pH 7.2)/0.1 mM- $CaCl_2$ /0.1 mM- $MgCl_2$ / 10 μ M-phenylmethanesulphonyl fluoride/0–5 μ M-choline chloride. The choline chloride concentration used in the dialysis fluid was the same as the [3H]choline concentration during uptake (i.e. 1–5 μ M), except in preloading experiments, in which case there was no choline chloride during dialysis.

Sonication. Lipid preparations (see above) were suspended in 100 mM-NaCl / 10 mM-Tris / HCl (pH 7.4)/0.3 mM-EDTA to a concentration of 5–20 mg/ml at 4°C. If protein was to be included in the liposomes, then solubilized membrane protein was added to a concentration of approx. 1 mg/ml. In some experiments Triton X-100 was removed from the solubilized membrane protein after mixing with the lipid suspension [as above, but also containing 0.5% (v/v) Triton X-100]. The resulting mixture of lipid and protein was then sonicated in a sealed tube under N_2 to clarity in a bath sonicator (containing ice and water) for approx. 30 min.

Freeze-thaw/sonication. Sonicated liposomes were prepared as above, frozen in liquid N_2 , thawed at 25°C and then briefly sonicated in a bath sonicator (under N_2) for 10–30 s.

The centrifugation of liposomes before transport measurements. In some experiments where liposomes had been produced by cholate dialysis, 15–20 ml batches of liposome suspension were spun at 40 000 *g* (20 000 rev./min; Sorvall RC2B instrument, SS34 rotor) for 30 min at 4°C, and the pellets from each batch resuspended in 0.4–2 ml of supernatant (to a maximum lipid concentration such that 20 μ l of liposomes would not block the Millipore filters during filtration and that the process of filtration and washing could be done in less than 20 s, which was decided empirically for each batch of liposomes).

Measurement of uptake of [3H]choline by liposomes

Choline transport was measured by observing the uptake of [3H]choline chloride with or without the presence of a specific inhibitor of choline transport, hemicholinium-3 (MacIntosh, 1961) or saturating concentrations of non-radioactive choline chloride (10 mM). The liposomes were separated from the surrounding medium by Millipore filtration, followed by washing (Racker, 1972).

Liposomes were pre-incubated at 37°C for

15 min, and then 140 μ l was added to 70 μ l of dialysis medium (see under 'Cholate dialysis') at 37°C containing 1–2 μ Ci of [3 H]choline chloride (1–5 μ M), with or without the required amount of hemicholinium-3 or a saturating concentration of non-radioactive choline chloride. When the liposomes had been concentrated beforehand 20 μ l was added to an equal volume of incubation medium. The solutions were immediately mixed, incubated for the required time at 37°C and then transferred to 5 ml of ice-cold filtering solution (100 mM-NaCl/Tris buffer as in the dialysis medium, and 10 mM-choline chloride, the latter being present to reduce non-specific binding of radioactive choline to filters or liposomes). This was mixed, and immediately filtered through Millipore filters (type GSWP 02500) using an Amicon vacuum-filtration manifold. The filters were then washed with 10 ml of the ice-cold filtering solution. The order of filtering was randomized because pressure differences in the apparatus affect the blank value. Zero points were obtained by adding the liposomes suspension directly to the ice-cold filtering solution (which already contained [3 H]choline), and filtering immediately.

Preloading liposomes with non-radioactive choline. In experiments where liposomes were preloaded with non-radioactive choline chloride before the measurement of uptake of [3 H]choline chloride, the following procedure was followed. Liposomes were prepared as above, without the addition of choline chloride to the dialysis medium or sonication medium. They were then incubated for 2 h with the required concentration of non-radioactive choline chloride (0–15 μ M) at 37°C. Uptake of [3 H]choline chloride by these liposomes was then measured as above, by adding 1 vol. of liposome suspension (e.g., 20 μ l) to 2 vol. of dialysis medium at 37°C, containing 1–2 μ Ci of [3 H]choline chloride (0.1 μ M) and non-radioactive choline chloride, of the required molarity, such that the total choline concentration of the mixture (i.e. after the liposomes have been added) was 5 μ M. Incubation and filtering were then performed as above.

Other measurements

Radioactivity. The filters were dried in an oven at 80°C for 10 min, placed in 5 ml plastic vials and then 5 ml of scintillant [1.5% (w/v) 2,5-diphenyloxazole + 0.2% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)-benzene in toluene/Triton X-100/0.1 M-NH₃ in ethanol (2:1:0.2 by vol.)] was added. Radioactive standards were produced by placing 10 μ l of aqueous solution of known radioactivity on to filters, drying them, adding scintillant and counting as above. The liposome incubation medium containing [3 H]choline chloride was counted in the same way (i.e. by placing 10 μ l on filters, oven drying, adding scintillant and counting). The count-

ing efficiency for [3 H]choline using this method was between 15 and 20%.

Proteins in solution. The Lowry method was used with the addition of 0.5% (w/v) sodium dodecyl sulphate to overcome the interference of Triton X-100, and help micellize large quantities of lipid (Kasahara & Hinkle, 1977).

Proteins on Millipore filters. The method used was that of Schaffner & Weissmann (1973) and relies on the staining of protein on Millipore filters by Amidoschwarz 10B.

Phospholipid. Lipids were extracted from samples by the method of Bligh & Dyer (1959), and the phospholipid content then measured by a modification of the method of Bartlett (1959).

Results

Some preliminary considerations

Effects of disruptive treatment on [3 H]choline uptake by subcellular fractions. To gain some idea of the resistance of the choline-transport system to membrane disruption, the effect of various concentrations of Triton X-100 or sodium cholate on the uptake of [3 H]choline by synaptosomes (synaptosomes prepared by the method of Gray & Whittaker, 1962) was investigated. The uptake of [3 H]choline by synaptosomes separated after 4 min by rapid sedimentation was hemicholinium-3-sensitive and inhibited by 0.1% (v/v) Triton X-100, 0.2% (w/v) sodium cholate but not by 0.01% (v/v) Triton X-100 or 0.01% (w/v) sodium cholate.

The [3 H]choline uptake by synaptic plasma membrane, sonicated plasma membrane or synaptic plasma membrane co-sonicated with added lipid was measured. The [3 H]choline uptake by synaptic plasma membrane was sensitive to hemicholinium-3 but considerably less (about 2%) than the [3 H]choline uptake observed for synaptosomes. [3 H]Choline uptake by the sonicated synaptic plasma-membrane fraction was not inhibited by hemicholinium-3. However, if added lipid (7 mg/ml) and synaptic plasma membrane were co-sonicated, then the [3 H]choline uptake by the resulting particles was inhibited by hemicholinium-3. No inhibition by hemicholinium-3 was observed for lipid-only liposomes. The presence of added lipid during the sonication of synaptic plasma membrane appears to allow the retention of hemicholinium-3-sensitive choline uptake by the resulting particles. An explanation for these observations is that an increase in the amount of lipid present (with a fixed amount of membrane) may increase the enclosed space that is served by a membrane-transport function, thereby allowing the easier detection of such a function (Sorensen & Vidaver, 1978).

Attempts at the incorporation of choline-transport activity into liposomes (using sonication).

Certain membrane-transport systems have been solubilized by using Triton X-100, and then (after the removal of detergent) they have been incorporated into liposomes (by sonication) in a functional state. Synaptic plasma membrane was solubilized with either sodium cholate or Triton X-100, the detergents removed (by gel filtration or Biobeads SM2 respectively) from the soluble and insoluble fractions, and then liposomes formed by sonicating these fractions with presonicated purified lipid liposomes (Kasahara & Hinkle, 1977).

Liposomes formed from lipids alone, or from added lipids and Triton-soluble (or insoluble) or cholate-soluble (or insoluble) material (the detergents having been removed before the addition of lipid), did not show hemicholinium-3 sensitive uptake. However, we found that if the Triton X-100 was removed from the original mixture of Triton X-100 and synaptic plasma membrane (i.e. before centrifugation to separate Triton-soluble and Triton-insoluble material) and this material added to and then sonicated with purified lipid liposomes, the resulting suspension did demonstrate an uptake of [3 H]choline, which was inhibitable by hemicholinium-3.

Freeze-thaw/sonication (described by Kasahara & Hinkle, 1976, 1977) is said to result in the formation of larger liposomes than sonication alone. The freeze-thaw/sonication procedure for the production of liposomes using purified lipid and Triton X-100-solubilized synaptic plasma membrane (Triton removed before the addition of lipid) was carried out. No statistically significant hemicholinium-3-sensitive [3 H]choline uptake was observed in liposomes formed with lipid and Triton-soluble extract of synaptic plasma membrane.

The uptake of [3 H]choline by liposomes made by cholate dialysis

As discussed in the introduction, the three criteria of transport used were saturability, hemicholinium sensitivity and transactivation by preloading with non-radioactive choline. The starting material used for the preparation of liposomes was guinea-pig synaptic plasma membrane; this was solubilized using 2% cholate, and mixed with a suspension of purified lipids in 2% cholate. Unilamellar liposomes were then formed from this mixture by the cholate dialysis technique, and choline transport was measured by observing the uptake of [3 H]choline, the liposomes having been separated from the incubation medium by Millipore filtration followed by washing. Fig. 1 shows the time course of uptake. It can be seen that the uptake of [3 H]choline by these liposomes is reduced by the presence of saturating concentrations (10 mM) of non-radioactive choline chloride and hemicholinium-3 during the first 4 min of uptake. If the same experiment is

Fig. 1. Time course of uptake of [3 H]choline by liposomes containing synaptic plasma-membrane extract. Cholate-solubilized extract of synaptic plasma membrane was prepared as described in the Materials and methods section, and liposomes were formed from this by cholate dialysis at pH 7.4 using a mixture of commercially purified lipids of similar composition to synaptic plasma membrane (see the text) (3 mg of lipids/ml, 2 mg of protein/ml). Both uptake of [3 H]choline and protein trapped on the filters was measured as described in the Materials and methods section. ▲, [3 H]Choline concentration, 1 μ M, no additions; ●, plus 10 mM-choline chloride non-radioactive; ○, plus 100 μ M-hemicholinium-3. Vertical bars represent $\pm$ S.E.M. for four filtrations.

Fig. 2. The effect of various concentrations of hemicholinium-3 on [3 H]choline uptake by liposomes formed using solubilized membrane extract and added lipid. Liposomes formed from membrane extract that had been purified by DEAE-cellulose (R. G. King & R. M. Marchbanks, unpublished work) and asolectin (a mixture of phospholipids but little cholesterol from soya beans) by cholate dialysis (10 mg of phospholipid/ml and 0.3 mg of protein/ml) were concentrated by centrifugation. Uptake of [3 H]choline (5 μ M) was measured at different hemicholinium-3 concentrations, and proteins were measured on the filters. Vertical bars represent $\pm$ S.E.M. for four filtrations.

Fig. 3. The effect of preloading with non-radioactive choline on initial $[^3\text{H}]$ choline uptake by liposomes containing membrane extract

Liposomes were prepared as described in the legend to Fig. 1, and preloaded with non-radioactive choline chloride as described in the Materials and methods section. Uptake of $[^3\text{H}]$ choline ($5\mu\text{M}$) was measured after 30s and proteins were measured directly on the filters. Vertical bars represent $\pm$ S.E.M. for four filtrations.

conducted over a longer time course (30min or more) the effects of hemicholinium-3 or 10mM-choline are not statistically significant. Thus hemicholinium-3 and 10mM-choline chloride affect the initial rate of $[^3\text{H}]$ choline uptake by these liposomes (as shown by uptake during the first 4min), but do not affect the liposomal $[^3\text{H}]$ choline concentration when it is approaching an equilibrium value (2h or more). The concentration dependency of the inhibition of uptake by hemicholinium-3 is shown in Fig. 2. That the proteoliposomes also fulfil the third criterion of choline transport, i.e. transactivation by preloading with non-radioactive choline, is shown in Fig. 3.

Fig. 4 shows the effect of varying the choline concentration on the initial inhibitable rate of choline uptake by these liposomes. The results from Fig. 4 may be plotted reciprocally [i.e. (initial rate of inhibitable choline uptake) $^{-1}$ against (choline concentration) $^{-1}$] as shown in Fig. 4, inset (a), in which case the results from two separate experiments do not fall on a straight line. This phenomenon [i.e. bent plot of (initial rate of inhibitable choline uptake) $^{-1}$ against (choline concentration) $^{-1}$] has been observed in synaptosomes (Yamamura & Snyder, 1973) and has been interpreted to mean that there are two separate choline-transport systems, a high-affinity and a low-affinity system. If this interpretation is

used on the data in Fig. 4, inset (a), apparent K_m values of $2\text{--}5\mu\text{M}$ and $50\text{--}100\mu\text{M}$ are obtained.

Another possible interpretation of the non-linear double-reciprocal plot is that (at least in an enzyme-catalysed reaction) the combination and/or reaction of the substrate at one catalytic centre may not be independent of the presence of this reactant at another catalytic centre (e.g., if the enzyme is composed of two or more subunits). A plot of $\log [v/(V_{\text{max.}} - v)]$ against $\log$ (substrate concentration) (v = initial rate of reaction, $V_{\text{max.}}$ = maximum initial rate of reaction) is shown in Fig. 4, inset (b) ($V_{\text{max.}}$ estimated as 30pmol/mg of protein per min for both experiments), and gives maximum slopes of 1.5 (Exp. 1) and 1.45 (Exp. 2). This suggests that the choline-transport system has two or more subunits that interact in a negatively co-operative way.

Control experiments on choline uptake into liposomes

The uptake of $[^3\text{H}]$ choline by control liposomes made with lipid only. To ascertain whether the observed phenomena of choline transport described above were due to the presence of the extract of synaptic plasma membrane in the liposomes, experiments were performed on control liposomes containing lipid alone. $[^3\text{H}]$ Choline uptake by these control liposomes did not show inhibition by hemicholinium-3 or by saturating (10mM) concentrations of non-radioactive choline (Table 1). The values for $[^3\text{H}]$ choline uptake by pure lipid liposomes cannot be compared with those for liposomes containing membrane extract on a protein basis, but can be compared as a percentage of the 4h $[^3\text{H}]$ choline uptake, or on a phospholipid basis. From this Table it is seen that the 4min uptake of $[^3\text{H}]$ choline by liposomes made using cholate-solubilized membrane extract is inhibitable by hemicholinium-3 or non-radioactive 10mM-choline chloride (49% of the 4h value is inhibited to 37% and 32% respectively), whereas this inhibition is not observed in the case of the pure lipid liposomes. It can be seen that it is the presence of the membrane extract during the formation of the liposomes that confers the properties of the choline-transport system. Because the membrane extract is solubilized it is reasonable to suppose that the functional components are incorporated into liposomes formed principally from exogenous lipids, though it is possible that the extract forms a separate population of liposomes. Exposure to hypo-osmotic conditions did not release the radioactivity taken up because unilamellar liposomes, being small, are relatively resistant to hypo-osmotic rupture. Only liposomes of very large size (e.g. $1\mu\text{m}$) are sensitive (Reeves & Dowben, 1969; Johnson & Butters, 1973).

Fig. 4. Initial 10 mM-choline-sensitive rate of $[^3\text{H}]$ choline uptake at various $[^3\text{H}]$ choline concentrations by liposomes containing membrane extract

Liposomes were prepared as described in the legend to Fig. 1 and $[^3\text{H}]$ choline uptake was measured after 30 s. The inhibitable $[^3\text{H}]$ choline uptake was calculated from the difference between the control $[^3\text{H}]$ choline uptake and that in saturating (10 mM) concentrations of non-radioactive choline. Vertical bars represent $\pm$ s.e.m. for four filtrations. Inset (a) shows the data (●) of the main Figure and an additional experiment (○) as reciprocal plots. The breaks in the lines have been drawn by eye to illustrate the appearance of plots having two separate K_m values, as given in the text. Inset (b) shows the data (●) of the main Figure and an additional experiment (○) plotted as $\log [v/(V_{\max} - v)]$ against $\log$ (choline concentration).

Table 1. $[^3\text{H}]$ Choline uptake by pure lipid liposomes and liposomes made with lipid and membrane extract

Liposomes containing lipid plus membrane extract were made as described in Fig. 1. Pure lipid liposomes were made in the same way, using the same lipids, but without the addition of the cholate-solubilized extract of synaptic plasma membrane. The uptake of $[^3\text{H}]$ choline at 4 h expressed on the basis of phospholipid content was 1.5 pmol/mg of phospholipid (liposomes containing membrane extract) and 0.9 pmol/mg of phospholipid (lipid-only liposomes). The $[^3\text{H}]$ choline concentration was $1\text{ }\mu\text{M}$. Results are means $\pm$ s.e.m. for the numbers of observations shown in parentheses.

	Proportion of 4 h $[^3\text{H}]$ choline uptake (%)	
	4 min	60 min
Lipid alone	26 $\pm$ 5 (8)	79 $\pm$ 9 (8)
Lipid alone + 10 mM-choline chloride	30 $\pm$ 4 (8)	80 $\pm$ 12 (8)
Lipid alone + 100 μM -hemicholinium-3	25 $\pm$ 3 (8)	84 $\pm$ 10 (8)
Lipid + membrane extract	49 $\pm$ 5 (6)	78 $\pm$ 8 (6)
Lipid + membrane extract + 10 mM-choline chloride	32 $\pm$ 3 (6)	75 $\pm$ 10 (6)
Lipid + membrane extract + 100 μM -hemicholinium-3	37 $\pm$ 3 (6)	80 $\pm$ 9 (6)

Analysis of radioactivity taken up in liposomes

It was possible that the radioactivity measured on the millipore filters (on to which liposomes incubated with $[^3\text{H}]$ choline had been filtered) was not $[^3\text{H}]$ -

choline, but a metabolite. To exclude this possibility the radioactivity from the liposomes on Millipore filters was extracted and submitted to t.l.c. As shown in Table 2, all the detected radioactivity extracted from the filters was $[^3\text{H}]$ choline (as measured by

Table 2. *Extraction of radioactivity from millipore filters and t.l.c. of the extract to determine the [^3H]choline content*
Control Millipore filters were prepared by placing 1 μCi of [^3H]choline chloride (70 Ci/mmol) in 20 μl of dialysis medium on the filters and oven-drying them before extraction. Liposomes were prepared with synaptic plasma membrane extract as in Fig. 1 and incubated with [^3H]choline (5 μM) for 10 min and separated from the medium. The Millipore filters were then oven-dried and the radioactivity extracted by dissolving each filter in 2 ml of allyl cyanide; 6 ml of 0.1 M-HCl was added, mixed for 1 min, and spun at 2000 g in an MSE bench centrifuge for 10 min and 3 ml of the lower clear aqueous phase freeze-dried. This was dissolved in 25 μl of methanol containing 10 mM-choline chloride and 10 mM-acetylcholine chloride and submitted to t.l.c. as described by Marchbanks & Israel (1971). Values are means $\pm$ S.E.M. for three determinations.

	Radioactivity (%)		
	Extracted/total on filter	Recovered from choline band/ total placed on t.l.c. plate	Recovered from all other areas of t.l.c. plate/ total placed on t.l.c. plate
Control Millipore filters	88 $\pm$ 7	80 $\pm$ 8	<2
Millipore filters on to which liposomes had been filtered	91 $\pm$ 4	86 $\pm$ 4	<3

t.l.c.), and the percentage of radioactivity extracted was 91% of the total (approximately the same as the control extraction).

It has been suggested that there are two separate choline-transport systems, a high-affinity and a low-affinity system (Yamamura & Snyder, 1973), the former being directly coupled to choline acetyltransferase (Barker & Mittag, 1975). The lack of acetylation of transported choline suggests that if there is a separate high-affinity choline-transport system present and functional in the liposomes, it is not directly coupled to choline acetyltransferase, and even at low concentrations (e.g., 5 μM) choline can be transported without compulsory acetylation.

Further extraction of synaptic plasma membranes

The question of how much choline transport activity remains in the cholate-insoluble pellet after cholate solubilization of synaptic plasma membrane may be asked. Liposomes made from the re-extracted cholate-insoluble material exhibit choline-transport activity (as shown by hemicholinium-3 sensitivity and saturability by 10 mM-choline), but this activity (when expressed as inhibitable initial rate of [^3H]choline uptake on a protein basis) is only approx. 40% of that of liposomes prepared from the original cholate extract.

Solubilization of the choline-transport system using the non-ionic detergent, Triton X-100, was attempted. As noted previously several unsuccessful attempts were made (by using standard reconstitution methods) to produce liposomes from Triton X-100-solubilized membrane extracts that demonstrated choline-transport activity. One aspect

that these methods had in common was that the Triton X-100 was removed before exogenous lipid was added to form liposomes. It is known that in some cases the removal of detergent from solubilized membrane proteins causes their aggregation and loss of function (Razin, 1972; Wolosin, 1980). Therefore a method was used (a modification of that of Yu & Branton, 1976) whereby lipids in a solution of Triton X-100 were added to the Triton X-100-solubilized membrane components, the Triton X-100 was then removed, sodium cholate was added to 2% (w/v) and liposomes were formed by cholate dialysis. This method produced liposomes that demonstrated choline-transport activity [when concentrations of Triton X-100 of 0.5% (v/v) were used], i.e. [^3H]choline uptake by these liposomes was inhibited by 10 mM-choline and hemicholinium-3. However, liposomes formed by using the solubilized material from a 0.1% Triton X-100 extract did not show choline-transport activity (Table 3). One possible explanation of this latter observation may be that the choline-transport system is an integral part of the membrane, which is not solubilized by low concentrations of Triton X-100 (e.g., 0.1%).

The effects of various ions, ATP, and electrical gradients on liposomal [^3H]choline uptake

Choline transport in synaptosomes and erythrocytes has been found to be sensitive to the Na^+ and K^+ gradients across the membrane (Martin, 1968; Marchbanks, 1968). Whether or not this is due to a requirement for Na^+ co-transport, an inwardly directed negative electrical gradient, or some other mechanism is not clear.

Table 3. *Effect of different Triton X-100 concentrations on the solubilization of carrier activity*

Hypo-osmotically ruptured crude synaptosomal fraction from ox cortex was produced and solubilized with Triton X-100 at the concentrations shown as described in the Materials and methods section. To this solubilized material was added ox cortex lipid extract (see the Materials and methods section) suspended in 100 mM-NaCl/10 mM-Tris/HCl (pH 7.4)/0.3 mM-EDTA/2% (v/v) Triton X-100 (the lipid suspension had been centrifuged at 100 000 g for 1 h to remove any non-solubilized lipid; see the Materials and methods section). The solubilized lipid and protein (10 mg/ml and 1.5 mg/ml respectively) were mixed and Triton X-100 was removed by adsorption on Biobeads SM2 or Amberlite XAD2 (Holloway, 1973). Sodium cholate was added to 2% (w/v), the resulting solution stirred for 10 min and then dialysed as described in the Materials and methods section. The uptake of [3 H]choline (5 μ M, 30 s uptake period) by the liposomes thus formed, and protein measurements were as described in the Materials and methods section. Results are means $\pm$ S.E.M. for four observations.

	Initial rate of [3 H]choline uptake (pmol/mg of protein per min)		
	No inhibitors	+100 μ M-Hemicholinium-3	+10 mM-Choline
Liposomes formed using 0.1% (v/v) Triton X-100 extract	29.2 $\pm$ 1.2	33.2 $\pm$ 1.8	31.7 $\pm$ 2.0
Liposomes formed using 0.5% (v/v) Triton X-100 extract	48.7 $\pm$ 2.0	36.2 $\pm$ 2.3	30.0 $\pm$ 1.5
Liposomes formed using 1% (v/v) Triton X-100 extract	26.8 $\pm$ 2.1	20.2 $\pm$ 1.5	19.2 $\pm$ 1.7

Table 4. *Effects of Na⁺ and K⁺ on [3 H]choline uptake by liposomes prepared using DEAE cellulose purified solubilized membrane extract*

Triton X-100-solubilized neuronal membrane fraction was stirred with DEAE-cellulose (pH 7.2) (R. G. King & R. M. Marchbanks, unpublished work) and the supernatant was retained. To this was added Triton-solubilized ox lipid extract (Expt. 1) or Triton-solubilized asolectin (Expt. 2), the Triton was removed, and sodium cholate added to 2% (w/v) as described in the legend to Table 3. Liposomes were then formed from this solution by cholate dialysis, with the dialysis medium (pH 7.2) containing 100 mM-KCl instead of 100 mM-NaCl where indicated. Dialysate (1 ml) contained 0.3 mg of protein and 8 mg of phospholipid (Expt. 1) and 0.2 mg of protein and 9 mg of phospholipid (Expt. 2). Liposomes were concentrated by centrifugation (see the Materials and methods section); 30 s [3 H]choline uptake was measured as described in the Materials and methods section, except that 10 μ l of liposome suspension was added to 35 μ l of the NaCl or KCl dialysis medium (as specified; referred to as [3 H]choline-uptake medium) containing 1 μ Ci of [3 H]choline with or without hemicholinium-3 or non-radioactive 10 mM-choline chloride. [3 H]-Choline concentration during uptake was 5 μ M. Proteins were measured on filters as described in the Materials and methods section. Results are means ($\pm$ S.E.M., where indicated) from four filtrations.

	Dialysis in medium containing	[3 H]Choline-uptake medium containing	[3 H]Choline uptake (pmol/mg of protein per min)			Mean hemicholinium-3 inhibitable [3 H]choline uptake (pmol/mg of protein per min)
			No inhibitors	100 μ M-Hemicholinium-3	10 mM-Choline	
Expt. 1	NaCl	NaCl	15 $\pm$ 2	3 $\pm$ 1	1 $\pm$ 1	12
	NaCl	KCl	10 $\pm$ 2	3 $\pm$ 1	1 $\pm$ 1	7
	KCl	NaCl	61 $\pm$ 4	20 $\pm$ 2	15 $\pm$ 2	41
	KCl	KCl	48 $\pm$ 3	15 $\pm$ 1	10 $\pm$ 1	33
Expt. 2	NaCl	NaCl	50 $\pm$ 6	16 $\pm$ 7	12 $\pm$ 5	34
	NaCl	KCl	50 $\pm$ 10	21 $\pm$ 2	8 $\pm$ 4	29
	KCl	NaCl	71 $\pm$ 7	21 $\pm$ 6	6 $\pm$ 4	50
	KCl	KCl	60 $\pm$ 7	15 $\pm$ 8	3 $\pm$ 4	45

The effects of Na⁺ and K⁺ gradients across the liposomal membrane during [3 H]choline uptake were investigated. Liposomes were prepared from DEAE-cellulose-purified solubilized membrane extract (R. G. King & R. M. Marchbanks, unpublished work) and added lipid by cholate dialysis as in Table 4. The dialysis medium containing either 100 mM-NaCl (no K⁺) or 100 mM-KCl (no Na⁺). After concentration of the liposomes by centrifugation,

10 μ l of liposomal suspension was transferred to 35 μ l of [3 H]choline uptake medium [containing either 100 mM-NaCl (no K⁺) or 100 mM-KCl (no Na⁺)] for [3 H]choline uptake. Table 4 shows that in all conditions the liposomes demonstrated 30 s [3 H]choline uptake that was inhibitable by hemicholinium-3 or 10 mM-choline. The results from the two experiments described in Table 4 are quantitatively different, but they both show the same trends.

The quantitative differences may be due to the different lipids used for reconstitution (similar effects have also been observed by other workers, e.g. Bardin & Johnstone, 1978).

Those liposomes that were made in medium containing 100 mM-KCl (whether or not they were transferred to an NaCl or a KCl medium for [3 H]choline uptake) demonstrated greater choline transport than liposomes produced in medium containing 100 mM-NaCl. The presence of predominantly extraliposomal NaCl during [3 H]choline uptake resulted in greater transport activity than the presence of predominantly extraliposomal KCl. The maximum choline transporting activity was observed in liposomes produced in a KCl medium and transferred to an NaCl medium for [3 H]choline-uptake measurement. It does not therefore appear that an ionic gradient is necessary for the measurement of 'choline transport' in these liposomes, nor does it appear that NaCl is obligatory for choline-transport activity (liposomes produced in 100 mM-KCl demonstrated hemicholinium-3 inhibition of [3 H]choline uptake when no NaCl was present). However, the observation that in both experiments the mean 30 s hemicholinium-3-inhibitable [3 H]choline uptake/mg of protein appeared to be maximal in those liposomes produced in a KCl medium and transferred to an NaCl medium for [3 H]choline uptake suggests that the transport of choline from outside the liposomes to inside the liposomes may be stimulated by the predominant presence of NaCl on the same side and KCl on the opposite side. These results are also compatible with those found by Martin (1972), who studied the effects of cations on choline transport of erythrocytes (one of his findings was that influx of choline was stimulated by extracellular Na^+ , and efflux of preloaded choline was stimulated by extracellular K^+).

Table 5 shows the effects of transferring the liposomes produced in KCl medium to [3 H]choline uptake medium containing 100 mM-NaCl and either 3 mM- MgCl_2 or 5 mM- MgATP^{2-} . In both cases, the initial [3 H]choline uptake (pmol/mg of protein per min as assessed by 30 s uptake periods) was not statistically significantly different from the control, whether or not non-radioactive 10 mM-choline was present. This result thus suggests that 2.2 mM- MgCl_2 or 3 mM- MgATP^{2-} -extraliposomally during [3 H]choline uptake did not markedly affect the uptake of choline from outside the liposomes to inside the liposomes in this experiment.

Table 5 also shows the effect of pre-incubating the 'KCl-produced' liposomes with 8 μM -valinomycin, and then measuring [3 H]choline uptake after transferring these liposomes to NaCl-containing [3 H]choline-uptake medium. By this procedure it would be expected that valinomycin would produce a potential across the liposomal membrane (more negative on the inside of the liposomes than on the outside) by increasing the permeability of the membrane to K^+ (Lauger, 1978). It can be seen that pre-incubation of the liposomes with valinomycin appeared to cause an increased initial [3 H]choline uptake/mg of protein by the liposomes (4.7 ± 0.7 pmol/mg of protein per min, compared with 3.0 ± 0.6 pmol/mg of protein per min in liposomes not pre-incubated with valinomycin), but also the initial [3 H]choline uptake/mg of protein in the presence of 10 mM-choline chloride appeared to be increasing by pre-incubating the liposomes with valinomycin (3.1 ± 0.5 pmol/mg of protein per min, compared with 1.6 ± 0.0 pmol/mg of protein per min in liposomes not pre-incubated with valinomycin). These results therefore suggest that the creation of a potential gradient across the liposomal membrane (inside negative) by valinomycin as described above does not affect the inward trans-

Table 5. *Effects of Mg^{2+} , valinomycin and MgATP^{2-} on [3 H]choline uptake by liposomes*

Liposomes were prepared as described in the legend to Fig. 1, except that the dialysis medium (pH 7.4) contained 100 mM-KCl, instead of 100 mM-NaCl. Liposomes were pre-incubated with 8 μM -valinomycin where specified (37°C, 15 min), and then 30 s [3 H]choline uptake was measured as described in the Materials and methods section, except that 0.14 ml of the liposome suspension was added to 0.36 ml of NaCl dialysis medium (referred to as [3 H]choline-uptake medium) containing 2 μCi of [3 H]choline, additions as shown, with or without non-radioactive 10 mM-choline chloride. [3 H]Choline concentration during uptake was 5 μM . Proteins were measured on filters as described in the Materials and methods section. Results are means $\pm$ S.E.M. from six filtrations.

Additions to liposomes during pre-incubation	Additions to [3 H]choline-uptake medium	[3 H]Choline uptake (pmol/mg of protein per min)	
		Control	10 mM-Choline
None	None	3.0 ± 0.6	1.6 ± 0.2
8 μM -Valinomycin	None	4.7 ± 0.7	3.1 ± 0.5
None	3 mM- MgCl_2	2.7 ± 1.4	1.7 ± 0.4
None	5 mM- MgATP^{2-}	3.0 ± 0.6	1.5 ± 0.6

port of choline but does increase the total 30s [^3H]choline uptake/mg of protein by the liposomes. Choline is a positively charged ion, and therefore this latter result would not be entirely unexpected in that the creation of a potential gradient across the liposomal membrane (inside negative) would increase the total (diffusive and carrier-mediated components) [^3H]choline uptake.

Discussion

The results indicate that liposomes formed from a cholate-solubilized extract of guinea-pig cortical synaptic plasma membrane and added lipids (by the cholate-dialysis technique) demonstrate [^3H]choline uptake with the following properties. (1) The initial rate of uptake is inhibited by a saturating concentration (10 mM) of non-radioactive choline chloride. (2) The initial rate of uptake is inhibited by hemicholinium-3. From the results shown in Fig. 2 it may be estimated that, at a [^3H]choline concentration of $5\text{ }\mu\text{M}$, hemicholinium-3 has a half-maximal inhibitory effect at a concentration between $1\text{--}4\text{ }\mu\text{M}$. This is comparable with results found using synaptosomes, brain slices or erythrocytes (for review, see MacIntosh & Collier, 1976). (3) Preloading of the liposomes with non-radioactive choline chloride results in stimulation of the initial rate of [^3H]choline uptake. (4) Initial inhibitable rate of [^3H]choline uptake is half-maximal at a [^3H]choline concentration of $50\text{--}100\text{ }\mu\text{M}$.

These properties are also properties of the choline-transport system, where it has been examined in synaptosomes (Marchbanks, 1968; Marchbanks *et al.*, 1981) and in erythrocytes (Martin, 1968, 1972). If binding were responsible for the observed choline 'uptake', then preloading liposomes with non-radioactive choline would be expected to reduce this observed uptake by reducing the amount of [^3H]choline bound to the liposomes; however, the reverse is observed (i.e. preloading with choline increases the observed uptake). As control liposomes formed from the lipid alone did not demonstrate these properties in the above experiments, it is concluded that the most likely explanation is that a choline-transport system, or functional element of such a system, has been solubilized by cholate from synaptic plasma membrane, and re-incorporated in a functional or semi-functional state into liposomes. The radioactivity was transported as choline and we were unable to observe any acetylation, indicating that choline transport in this system is not coupled to choline acetyltransferase.

In synaptosomes prepared from cerebral cortex the initial rate of choline influx at a choline concentration of $1\text{ }\mu\text{M}$ is $10\text{--}20\text{ pmol/min per mg of protein}$ (Marchbanks, 1968). The rate in the above

protein-containing liposomes is $0.5\text{--}1.0\text{ pmol/min per mg of protein}$, so that only $5\text{--}10\%$ of the native activity is retained. Various reasons may be postulated for this observation. For example, (a) only a fraction of the total carrier has been extracted. That this is the case is shown by the fact that further carrier activity (some 40% of original) may be extracted from the cholate-insoluble pellet produced after the first extraction. (b) Functional activity may be lost during or after the extraction procedure. (c) A proportion of the carrier may be wrongly orientated in, or not even incorporated into, the liposomal membrane. (d) Only a small proportion of the liposomes may contain a functional carrier system [this has been shown to be the case in certain experiments on the erythrocyte glucose carrier (Goldin & Rhoden, 1978)]. (e) Incorporation of other solubilized membrane components into liposomes may cause non-specific effects (e.g. leakiness to cations) that obscure choline-transport activity. Liposomes made from Triton X-100-extracted ox brain membrane and added lipids (by removing the Triton X-100 after adding the lipids, adding sodium cholate, and then dialysing) also showed choline-transport activity.

The mean hemicholinium-3-sensitive [^3H]choline uptake by liposomes appears to be greatest in those liposomes where there is a predominant presence of extraliposomal NaCl and intraliposomal KCl (compared with liposomes where NaCl is not predominant extraliposomally or where KCl is not predominant intraliposomally) (Table 4). Thus Na^+ on one side of the membrane and K^+ on the other side favours the transport of choline from the Na^+ side to the K^+ side (e.g. binding of choline to a carrier molecule may be favoured by the presence of Na^+ , and then once this choline has crossed the membrane, its binding to the carrier molecule may be reduced by the presence of K^+).

Pre-incubating liposomes prepared in a predominantly KCl medium with $8\text{ }\mu\text{M}$ -valinomycin and then transferring them to a predominantly NaCl medium for [^3H]choline uptake would be expected to hyperpolarize the liposomes and therefore increase the influx of a positively charged ion such as choline. As described in Table 5 this treatment increased uptake both without inhibitors or in the presence of non-radioactive 10 mM-choline as compared with the same liposomes treated in the same way, but without pre-incubation with valinomycin, indicating that saturable transport is unaffected by the electrical gradient.

These results show that the choline-transport system has been solubilized from neuronal membrane preparations and re-incorporated into liposomes, where it retains many of the properties observed in synaptosomes or brain slices. Furthermore, the liposomal system has enabled the investi-

gation of certain aspects of mechanism not easily elucidated in intact biological systems, and may also be used as a future means of purification of the choline carrier.

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