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Choline transport across a carbon tetrachloride phase containing a chloroform-methanol extract of brain

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The presence of a chloroform-methanol extract of cat brain in a carbon tetrachloride phase separating two aqueous phases resulted in an increased passage of [^{3}H]choline across the organic phase which was inhibited by the choline transport inhibitor hemicholinium-3 and by high concentrations of non-radioactive choline. In the absence of cat brain extract, [^{3}H]choline passage across carbon tetrachloride was neither inhibited by hemicholinium-3, nor by non-radioactive choline.

The synthesis of the neurotransmitter, acetylcholine and the phospholipid, phosphatidylcholine depend upon an intracellular supply of choline, much of which is derived from the extracellular fluid via choline transport across the cell membrane [1–3]. Such a choline transport system is thought to be a rate limiting factor in the synthesis of acetylcholine [4], and wherever investigated has been found to be saturable and inhibited by hemicholinium-3 [2,3,5–7]. In order to further characterize the choline transport system, attempts have been made to isolate it from biological membranes, and then reincorporate it into liposomes [6–8]. One of the authors has used detergents for its solubilization, but unfortunately the presence of detergent has hindered purification [9,10]. In addition, when the solubilized extracts were reincorporated into liposomes, the variability of liposomal size hindered accurate assessment of the degree of purification [10]. Because of these problems, it was decided to investigate the possibility of solubilizing the choline transport system using organic solvents rather than detergents, and assaying transport activity by measuring the passage of choline across a bulk organic phase. Initial experiments showed that chloroform was unsuitable for use as the organic phase, since choline is partly soluble in chloroform [11,12]. However, choline is insoluble in carbon tetrachloride [11]. The present study was designed to investigate whether the incorporation of a chloroform-methanol extract of brain into a carbon tetrachloride phase resulted in passage of choline across the carbon tetrachloride which was saturable and inhibited by hemicholinium-3.

All common laboratory reagents and solvents were analytical grade and were obtained from Ajax Chemical Company, Sydney. Hemicholinium-3, L-α-phosphatidylcholine dipalmitoyl (synthetic, grade 1S) and L-α-phosphatidylethanolamine dipalmitoyl (Type V from *Escherichia coli*) were from Sigma Chemical Co. [*methyl*-^{3}H]Choline chloride (60 Ci/mmol) was from the Radiochemical Centre, Amersham.

Brains were removed from young adult cats (2.5–4 kg) immediately after they had been killed for use in other experiments within the department. A chloroform-methanol extract was then

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prepared by a modification of the method of Folch et al. [13]. Whole brains were homogenized in chloroform/methanol (1 : 1, v/v; 5 ml/g wet wt.) for 10 min using a Waring blender and centrifuged at $1000 \times g_{av}$ for 10 min. The supernatant was removed, and the pellet washed twice with chloroform/methanol (2 : 1, v/v). The supernatants were combined and the final mixture was made to the ratio of 4 : 3 : 2 (by vol.) of chloroform/0.9% (w/v) NaCl in water/methanol. After mixing and centrifugation ($1000 \times g$ for 10 min), the chloroform lower phase was removed and stored at 4°C. Prior to use, aliquots were evaporated to dryness in a rotary evaporator, and resuspended in carbon tetrachloride (CCl_4) at a concentration of 0.4 g original wet wt. of tissue/ml (approx. 1.6 mg protein per ml).

Measurement of [^{3}H]choline diffusion across a CCl_4 phase was performed by a modification of a method used by Hider and McCormack [14] as described previously by one of us [12]. Six ml of CCl_4 was placed in glass U tubes (internal diameter 1 cm; distance between inner edges of arms of the U, 3 cm; height of U, 10 cm). Two ml of 130 mM KCl, 8 mM $MgCl_2$, 0.01 mM $CaCl_2$ and 15 mM NaH_2PO_4/Na_2HPO_4 (pH 6.8) ('intracellular-type' buffer) was placed in one arm of the U, and 2 ml of 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 15 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) ('extracellular-type' buffer) containing 50 nM (3 μCi/ml) [^{3}H]choline chloride with or without unlabeled choline chloride or hemicholinium-3 was placed in the other arm of the U. The experiments were performed at room temperature (20°C) and the CCl_4 was continually stirred with a magnetic stirrer. 200-μl samples were taken every 24 h for one week from the aqueous phase which did not originally contain [^{3}H]choline. Immediately prior to taking samples this phase was mixed by ten repeated pipetting cycles of an automatic pipette. Immediately after taking samples, 200 μl of buffer was added to the phase from which the sample had been taken. The radioactivity was measured by liquid scintillation counting and the rate of [^{3}H]choline diffusion across the CCl_4 phase was calculated by linear regression [15] of the graph of total [^{3}H]choline which had crossed against time (24–168 h). The amount of [^{3}H]choline which traversed the CCl_4 during the course of an experiment (168 h) was never greater than 1% of the total. Extraction and thin-layer chromatography (TLC) [16] showed that 80% of the diffused radioactivity after 168 h was [^{3}H]choline. The presence of hemicholinium-3 or unlabelled choline did not significantly affect the amount of radioactivity which was recovered from the rest of the TLC plate (i.e., excluding the choline spot). In order to calculate the total [^{3}H]choline that had crossed the CCl_4 phase, TLC was performed and a correction factor applied to the total radioactivity crossed. Statistical analysis was performed by analysis of variance and Dunnett's *t*-test for multiple comparisons with a control. Protein was measured by the method of Lees and Paxman [18].

As shown in Fig. 1, the presence of the brain extract in the carbon tetrachloride phase resulted in an increased passage of [^{3}H]choline across this phase in comparison to control (no brain extract). When 1 mM hemicholinium-3 was added to the aqueous phase initially containing [^{3}H]choline, the passage of choline across the CCl_4 layer was reduced. Such a reduction was not observed in the absence of brain extract.

As shown in Fig. 2, the higher the concentration of hemicholinium-3, the greater the degree of inhibition of [^{3}H]choline passage across the CCl_4 phase containing brain extract. The mean rate of [^{3}H]choline passage in the absence of hemicholin-

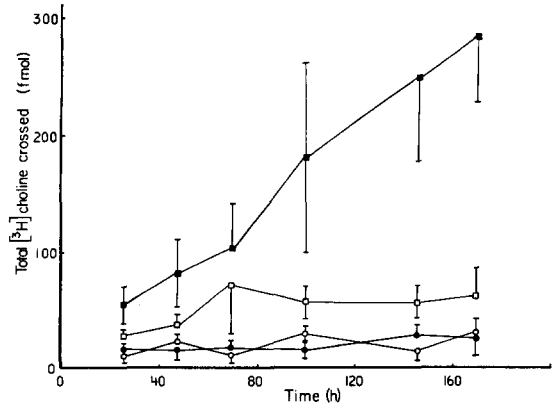

Fig. 1. [^{3}H]Choline passage across CCl_4 in the presence (open symbols) or absence (closed symbols) of 1 mM hemicholinium-3 in the aqueous phase which originally contained [^{3}H]choline. ■, □, brain extract present in CCl_4 phase; ●, ○, no brain extract present. Vertical bars show S.E. ($n = 4$).

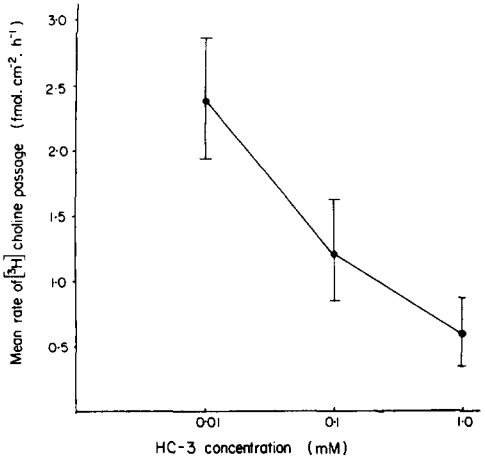

Fig. 2. Effect of hemicholinium-3 on [^{3}H]choline passage across CCl_4-containing brain extract. The rate of [^{3}H]choline passage in the absence of hemicholinium-3 (HC-3) was 2.60 ± 0.35 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} ($\pm$S.E., $n = 20$). Vertical bars show S.E. ($n = 8$).

ium-3 was 2.60 ± 0.35 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} ($\pm$S.E., $n = 20$). From the data in Fig. 2, the IC_{50} for hemicholinium-3 was 31 (13,77) μM (95% confidence limits, d.f. = 22), as calculated by linear regression.

Table I shows the effects of increasing concentrations of non-radioactive choline in the aqueous phase which initially contained [^{3}H]choline. From this table, it is seen that [^{3}H]choline passage across the CCl_4 phase (containing brain extract) was reduced by the presence of the non-radioactive choline. This reduction was significant at unlabelled choline concentrations of 1 μM and above ($p < 0.05$, $n = 8$). However, no significant reduction was observed in the absence of brain extract, even at the highest concentration of unlabelled choline used (10 mM).

TABLE I

EFFECTS OF NON-RADIOACTIVE CHOLINE ON [^{3}H]CHOLINE PASSAGE ACROSS CARBON TETRACHLORIDE

Rate of [^{3}H]choline passage across the CCl_4 phase was determined as described in the text, after non-radioactive choline had been added in the concentrations shown to the aqueous phase which originally contained [^{3}H]choline. Figures show means $\pm$S.E. (n).

	Non-radioactive choline concn. (M)	Rate of [^{3}H]choline passage (fmol $\cdot$ cm$^{-2} \cdot$ h^{-1})
CCl_4 plus brain extract	0	2.60 ± 0.35 (20)
	10^{-7}	2.41 ± 0.42 (8)
	10^{-6}	1.32 ± 0.37 (8) [a]
	10^{-5}	1.28 ± 0.28 (8) [a]
	10^{-4}	1.35 ± 0.43 (8) [a]
	10^{-2}	0.89 ± 0.24 (8) [a]
CCl_4 without brain extract	0	0.59 ± 0.17 (12) [a]
	10^{-2}	0.52 ± 0.16 (8) [a]

[a] Significantly different from CCl_4 plus brain extract without non-radioactive choline, $p < 0.05$.

Experiments were performed to ascertain whether hemicholinium-sensitive choline transport across the CCl_4 layer (in the presence of brain extract) could be demonstrated in the opposite direction. In this case, radioactive choline was initially placed in the 'intracellular-type' buffer. The rate of choline passage was 1.89 ± 0.61 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} in the absence of hemicholinium-3, and 1.40 ± 0.66 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} in the presence of 100 μM hemicholinium-3 ($n = 28$ in both cases, no significant difference between these two values). It was therefore concluded that transport of choline chould be demonstrated more readily in the normal direction (i.e. when radioactive choline was initially placed in the 'extracellular-type' buffer), and all further experiments were performed in this way.

An experiment was performed in order to ascertain whether heat denaturation of the brain extract caused a reduction of choline passage across the CCl_4 layer. In this case during the rotary evaporation stage prior to suspension in CCl_4, the extract was heated in a water bath at 80°C for 30 min. The rate of choline passage across the CCl_4 phase was 3.17 ± 0.70 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} in the absence of hemicholinium-3, and 1.38 ± 0.53 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} in the presence of 100 μM hemicholinium-3 ($n = 28$ in both cases). These figures were not significantly different from those obtained using non-heated extract. It was therefore concluded that if the observed hemicholinium-sensitive choline transport was due to a protein or lipoprotein, such a molecule was resistant to the effects of heating at 80°C for 30 min.

The extent of choline transport varied in proportion to the amount of protein in the CCl_4 phase. In the experiment shown in Fig. 2 there was 0.4 g original wet wt. of tissue per ml of CCl_4

(approx. 1.6 mg protein per ml). If the amount of extract and hence protein per ml of CCl_4 was increased, so did the transport. At a protein concentration of 1.8 mg/ml the rate of choline passage was 4.37 ± 1.40 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1}, and at a protein concentration of 3.2 mg/ml it was 8.42 ± 1.43 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} ($n = 28$ in both cases).

Experiments were performed to ascertain whether the addition of pure phospholipids to CCl_4 (in the absence of brain extract) resulted in hemicholinium-sensitive choline transport. Such transport was not demonstrated. In the presence of phosphatidylcholine (1 mg/ml) choline passage was 1.57 ± 0.24 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} (0.84 ± 0.31 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} with 100 μM hemicholinium-3); and in the presence of phosphatidylethanolamine (1 mg/ml) it was 1.78 ± 0.42 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} (1.01 ± 0.23 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} with 100 μM hemicholinium-3), $n = 4$ for all figures. In neither cases did hemicholinium-3 cause significant inhibition of choline passage, and all values were lower than those obtained in the presence of brain extract. When proteins and proteolipids were removed from the brain extract (whilst suspended in chloroform) by ether precipitation as described by Mokrasch [19], and the remaining lipid-containing fraction dried and reconstituted in CCl_4 (0.4 g original wet wt. tissue per ml), hemicholinium-sensitive choline transport was not demonstrated. In this experiment the rate of choline passage across the CCl_4 phase was 1.45 ± 0.33 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} in the absence of hemicholinium-3, and 1.21 ± 0.27 fmol $\cdot$ cm$^{-2} \cdot$ h^{-1} in the presence of 100 μM hemicholinium-3 ($n = 6$ in both cases; figures not significantly different from one another). These results suggest that the substance responsible for hemicholinium-sensitive choline transport may be a protein or proteolipid.

In those above experiments which involved the use of cat brain extracts suspended in chloroform, the chloroform was removed by evaporation prior to resuspension of the extract in CCl_4. It was still possible, however, that trace amounts of chloroform might be present in the dried cat brain extract, and that such trace amounts of chloroform might contributed to the observed results. Therefore experiments were performed to measure the passage of [^{3}H]choline across CCl_4 which had been previously mixed with known amounts of chloroform, but which did not contain brain extract. The volumes of chloroform used were 1, 2, 5, 10, 20, 50, 100 and 200 μl per 6 ml of CCl_4. The largest volumes of chloroform were considerably greater than the maximum amount of chloroform which could have been present in the dried cat brain extract. None of these volumes of chloroform resulted in [^{3}H]choline passage across CCl_4 which was significantly different from control (no chloroform) or inhibited by hemicholinium-3 (figures not shown). It was therefore concluded that the effects of the presence of brain extract in CCl_4 were probably not due to trace amounts of chloroform.

In summary the results of the present study show that the presence of a chloroform-methanol extract of cat brain in the CCl_4 phase caused an increase in [^{3}H]choline passage across this phase which was inhibited by the choline transport inhibitor hemicholinium-3 and by high concentrations of non-radioactive choline. Saturability and hemicholinium sensitivity are properties of the choline transport system in biological membranes [2,3,5], although the concentration of hemicholinium-3 required to inhibit choline uptake in vivo is considerably less than that required in the present study. It has been proposed that certain carriers in biological membranes may act as shuttles [17]. If this is the case for the choline carrier, it is not inconceivable that the results of our experiments could be due to the presence of such a shuttle within the CCl_4 phase. Indeed, it has been shown that the presence of certain complex molecules (bis(salicylamidato)copper complexes) in a bulk organic phase, can result in the transport or shuttling of amino acids across this phase [14], although most membrane carrer systems in mammals are thought to be of higher molecular weight than that of the substances used by these workers.

It is not possible to say whether our results are due to the presence of the same molecule or molecules in the CCl_4 phase which are also responsible for the transport of choline across biological membranes, although one of the authors has shown that choline uptake by liposomes containing chloroform-methanol brain extract demonstrates the same properties [10]. However, the methods we have used in the present study may enable the further purification from brain extracts of a sub-

stance or substances which can transport choline across an organic phase. If such a purified substance were also active in lipid bilayers, this would lend credence to the hypothesis that this substance may also play a role in the transport of choline across biological membranes. The purification and study of the properties of such a compound may lead to further understanding, on a molecular level, of the nature of the choline transport system. Furthermore, the methods used in the present study for solubilization and assay of choline transport across an organic phase may enable purification of the choline carrier without the problems associated with the presence of detergents or the variability in size of liposomes when the latter are used for choline transport assays.

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