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CHOLINE UPTAKE SYSTEMS OF RAT BRAIN SYNAPTOSOMES

TATSUYA HAGA* AND HARUHIKO NODA

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo (Japan)

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SUMMARY

The uptake of [^{3}H]choline into synaptosomes and the subsequent synthesis of [^{3}H]acetylcholine were examined as functions of choline, Na^+ and hemicholinium-3 concentrations in the incubation medium. The results indicate the presence of two carrier-mediated uptake systems for choline. One system requires Na^+ for the uptake and the other does not. About 60% of the choline taken up into synaptosomes by the Na^+-dependent system were converted to acetylcholine, whereas only a few percent of the choline taken up into synaptosomes by the Na^+-independent system were converted to acetylcholine. K_m, the choline concentration giving half-maximum transport, was 4–8 μM for the Na^+-dependent system and about 40 μM for the Na^+-independent system. K_i values for hemicholinium-3 were 0.05–0.1 μM for the Na^+-dependent system and 40–50 μM for the Na^+-independent system.

INTRODUCTION

It has been shown that nerve ending particles (synaptosomes) take up choline by a carrier-mediated process[1–3]. Potter[1] and Marchbanks[2] showed that the uptake is stimulated by the presence of Na^+, but Diamond and Kennedy[3] showed that Na^+ has an inhibitory effect on the uptake. There is also a disagreement among authors concerning the size of the choline fraction converted into acetylcholine. In a previous report[4] it was suggested that this disagreement was partly due to the difference in the choline concentrations used in the incubation media, and that the effect of choline concentration might be explained by assuming two mutually independent systems for the uptake of choline into synaptosomes. The present report is concerned with some evidence supporting this assumption.

METHODS

Reagents

[*Me*-^{14}C]Choline chloride (spec. act. 54 Ci/mole) and [*Me*-^{3}H]choline chloride (spec. act. 15 Ci/mmole) were obtained from the Radiochemical Center, Amersham, Bucks, England. Hemicholinium-3 was obtained from the Aldrich Chemical Co., Milwaukee, Wisc., U.S.A. Tetrodotoxin was supplied by the Sunkyo Co., Japan.

* Present address: Institute of Brain Research, Faculty of Medicine, University of Tokyo, Tokyo, Japan.

Preparation of a crude synaptosomal fraction

Female rat cerebrum was homogenized in 0.32 M sucrose in a Teflon homogenizer (clearance about 0.02 cm) at about 2500 rev./min to give a 10 % homogenate. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was then centrifuged at 11000 × *g* for 20 min and the pellet was suspended in 0.32 M sucrose (10 ml per g original tissue). This suspension was centrifuged at 11000 × *g* for 30 min and the pellet was resuspended in 0.32 M sucrose. 1 ml of the suspension, derived from about 0.8 g wet wt of original tissue, contained about 27 mg of protein when assayed by the method of Lowry *et al.*[5]. This crude synaptosomal fraction was used immediately after preparation. The uptake of choline and the synthesis of acetylcholine by this fraction were confirmed as being due to the synaptosomes by means of uptake experiments using fractions obtained by discontinuous Ficoll density gradient centrifugation[4].

Incubation conditions

The standard solution contained 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.8 mM $CaCl_2$, 50 mM Tris–HCl buffer (pH 7.5), 1 mM Tris–phosphate buffer (pH 7.5) and 10 mM glucose. In some experiments Na^+ was replaced by sucrose at double the concentration. O_2 was bubbled through the solution for 1 min prior to use. Each standard tube contained synaptosomes derived from about 0.16 g wet wt of original tissue in 1 ml of the standard solution containing unlabeled choline (0–74 μM). Each tube was preincubated at 25 °C for 20 min with shaking before the addition of 10 or 20 μl of [^{3}H]- or [^{14}C]choline, and was then incubated usually for 12 min. Unlabeled choline was added to control tubes to make a final concentration of 50 mM.

Extraction and assay

After incubation, 8 ml of ice-cold standard solution containing 6.25 mM unlabeled choline and 0.1 mM neostigmine were added to each incubation tube, and each suspension was centrifuged at 15000 × *g* for 20 min. Each pellet was extracted with 0.3 ml of acidic ethanol solution (95 % ethanol, 0.2 % acetic acid, 2 mM acetylcholine, 3 mM choline, 0.1 mM neostigmine and water) at 3 °C for 20 min. About 15 % of the [^{3}H]acetylcholine was broken down either by extraction at room temperature or by extraction in the absence of neostigmine. After centrifugation at 15000 × *g* for 20 min, a fraction of the supernatant (50 μl) was added to glass fiber filter paper (Toyo Roshi GB-100) and the radioactivity counted. Another fraction (50 μl) was used for paper chromatographic analysis. Counts of this fraction amounted to 16 % of total counts in the initial pellets. This value was estimated by the procedure described below. After centrifugation of incubated suspensions, the pellets were extracted 4 times with 0.3 ml of acidic ethanol solution, and the final pellets were dissolved in 2 % sodium dodecyl sulfonate. The proportional counts for each fraction obtained by this procedure were 79.0, 16.1, 3.7, 0.8 and 0.5 %, respectively, from the first extraction to the final dissolution.

The separation of choline and acetylcholine was carried out by paper chromatography using a solvent system of acetone and 0.2 M ammonium acetate adjusted to pH 4.8 with acetic acid (4:1, by vol.)[3]. The chromatogram was developed for about 13 h on Toyo Roshi No. 50 paper, 2 cm × 40 cm. Choline and acetylcholine spots were detected by exposing the paper to iodine vapor, and were then cut out.

The glass fiber filter paper or the paper chromatogram strip was dipped into 10 ml of toluene containing PPO (4 g/l) and dimethyl-POPOP (0.2 g/l) after the iodine color had vanished, and counts were measured with a Beckman LS-200B liquid scintillation counter. The efficiency of counting for ^{3}H, assessed by an internal standard, was 26 % when the glass fiber filter paper was used, and 5.7 % when the paper chromatogram strip was used. The cross contamination of choline and acetylcholine with paper chromatography was about 0.5 %. In some instances a gas flow counter was used for measurements of ^{14}C after elution of the choline or acetylcholine spots from the paper chromatogram with water, followed by evaporation of the eluates.

For samples containing both ^{14}C and ^{3}H, any channel contamination of one species by the other was corrected by using linear simultaneous equations, whose parameters were determined by measurements of authentic samples.

RESULTS

Uptake of choline into synaptosomes and their synthesis of acetylcholine

Differences between the amounts of [^{14}C]choline or [^{14}C]acetylcholine in the pellets of standard tubes and those in the pellets of the control tubes which contained 50 mM of unlabeled choline were assumed to be due to uptake into the synaptosomes. This assumption was confirmed by procedures such as washing the pellets, applying osmotic shock, and incubating at 0 °C, as described in the previous report[4]. Another supporting fact is described below.

When synaptosomal suspensions were diluted with different volumes of the ice-cold standard solution after incubation, the amounts of [^{14}C]choline or [^{14}C]-acetylcholine recovered in the pellets (y) was a function of the volumes of the suspensions obtained after the dilution (x), and the relation was expressed in the form of $y = a + b/x$, where a and b were constants (Fig. 1). For values of y for [^{14}C]choline in the pellets of the control tubes, the value of a was almost 0. This means that under these conditions [^{14}C]choline either is bound to synaptosomes in forms exchangeable with the unlabeled choline of the medium at low temperature and/or is in solution in the medium. For values of y for [^{14}C]choline in the pellets of the standard tubes, neither a nor b were 0, and the value of b did not differ from that for the control tubes. This means that the differences between the amounts of [^{14}C]choline in the pellets of the standard tubes and those in the pellets of the control tubes represent the amount of [^{14}C]choline in synaptosomes which is not exchanged with unlabeled choline in the medium at low temperature. The exchange of [^{14}C]choline in the synaptosomes with the unlabeled choline added at the end of the incubation did not occur since the termination of [^{14}C]choline uptake by cooling the tubes or by the addition of hemicholinium-3 without addition of unlabeled choline did not affect the amounts of [^{14}C]choline in pellets. The amounts of [^{14}C]acetylcholine in the pellets of the standard tubes did not diminish by the dilution: this suggested that [^{14}C]acetylcholine was not in the medium but was inside the synaptosomes. [^{14}C]-Acetylcholine was not found in the pellets of the control tubes.

Time course

The rates of [^{3}H]choline uptake and [^{3}H]acetylcholine synthesis at Na^{+} concentrations of 0, 50 and 140 mM are shown for choline concentrations of 3.7 and

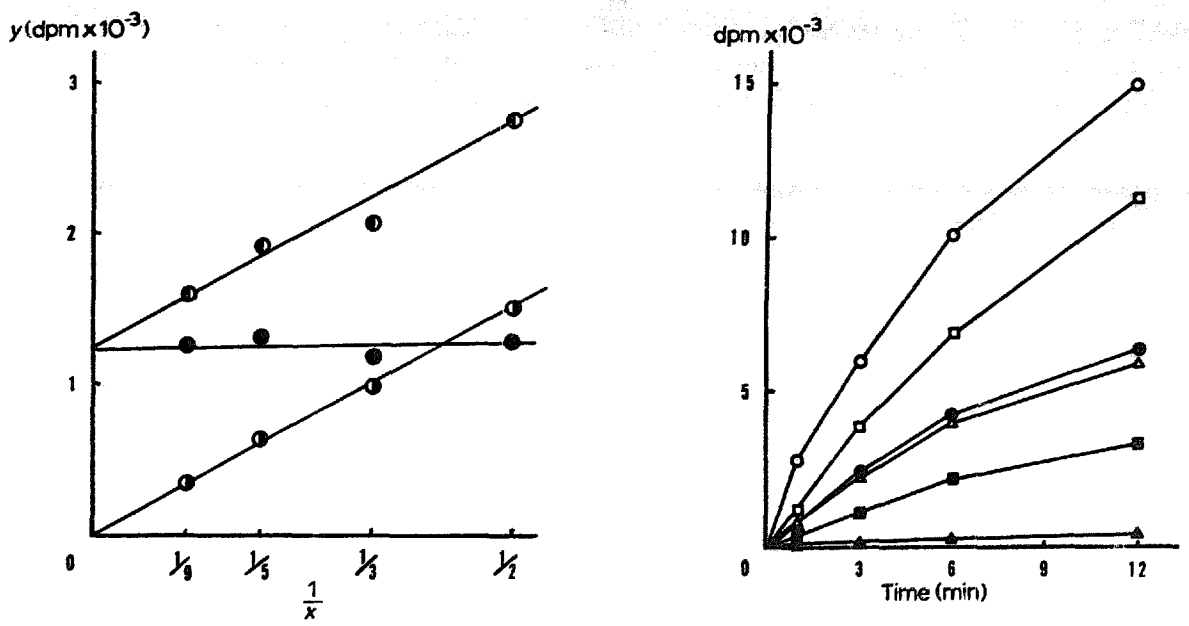


Fig. 1. Effects of dilution on the amounts of [^{14}C]choline or [^{14}C]acetylcholine recovered in the pellets. Each incubation tube contained 4.4 mg of protein and 3.7 μM [^{14}C]choline (spec. act., 54 Ci/mole). Each incubate (1 ml) was diluted with various volumes of ice-cold standard solution containing unlabeled choline and neostigmine at concentrations which became 25 mM and 0.1 mM, respectively, after the dilution. x represent volumes attained after the dilution. See Methods for condition of incubation and assay. ◐—◐, choline from standard tube; ◑—◑, choline from control tubes; ●—●, acetylcholine from standard tube.

Fig. 2. The uptake of [^{3}H]choline and the synthesis of [^{3}H]acetylcholine with various incubation times and a choline concentration of 3.7 μM. Pre-incubation was carried out at 25 °C for 20 min in the presence of 3.7 μM unlabeled choline. Each tube contained 4.68 mg protein and 1.2 μCi [^{3}H]choline, and was incubated for the period indicated in the abscissa. A portion of extract (50 μl) was directly assayed and its count (○, □, △) taken as measure of [^{3}H]choline uptake. Another portion (50 μl) was analyzed by paper chromatography and the acetylcholine fraction count is shown by ●, ■, ▲. The concentration of Na^+ in the incubation medium was 0 mM for △ and ▲, 50 mM for □ and ■, and 140 mM for ○ and ●. The values indicated have been corrected for the blank values obtained by the same procedure on control tubes which contained 50 mM unlabeled choline.

37 μM in Figs 2 and 3, respectively. The uptak of [^{3}H]choline is expressed by direct counting assay of the extract, but not by that of the paper chromatogram choline fraction.

The plot of [^{3}H]choline uptake against incubation time is not linear, but is similar for various concentrations of choline or Na^+, and it is possible to make them overlap with each other within experimental error by adjusting the scale of ordinate. This fact permits the assumption that the ratio of the amounts of [^{3}H]choline at an incubation time of 12 min under two different conditions is identical to that of the initial uptake rates. The fraction of [^{3}H]choline converted into [^{3}H]acetylcholine hardly changed during the incubation process, although there was slight increase under some conditions. This implies that the rate of conversion of choline in synaptosomes into acetylcholine is not the limiting step for the accumulation of [^{3}H]acetylcholine in synaptosomes.

Effect of Na^+ on the conversion of choline into acetylcholine

Na^+ stimulated the uptake of choline and the synthesis of acetylcholine. Even in the absence of Na^+, a considerable amount of choline was taken up, but acetyl-

choline was hardly synthesized (Figs 2 and 3). In order to test the effect of Na^+ on the conversion of choline in synaptosomes into acetylcholine, synaptosomes were first incubated in the absence of Na^+ with [^{14}C]choline, washed twice and re-incubated in the absence or presence of Na^+. As shown in Table I, Na^+ did not stimulate the conversion of choline in synaptosomes into acetylcholine. This fact suggests two up-

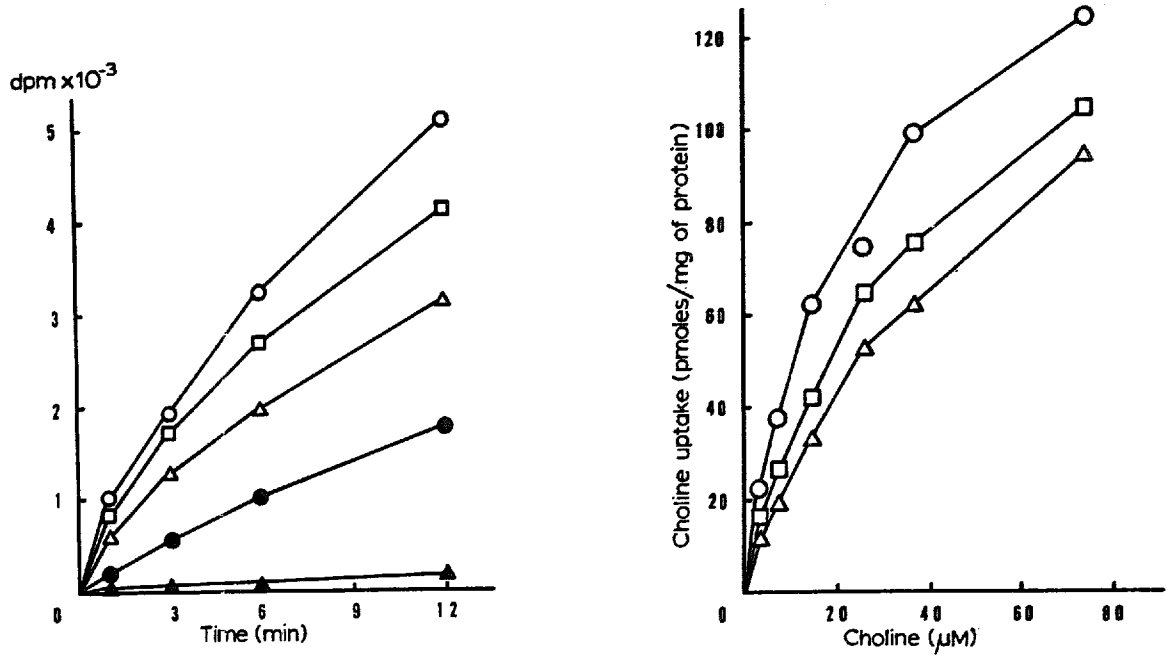


Fig. 3. The uptake of [^{3}H]choline and the synthesis of [^{3}H]acetylcholine with various incubation times and a choline concentration of 37 μM. Conditions and symbols were the same as in Fig. 2 except that choline concentration was 37 μM during pre-incubation and incubation.

Fig. 4. The uptake of [^{3}H]choline with various concentrations of choline at an incubation time of 12 min. Each incubation tube contained 3 μCi [^{3}H]choline and 5.58 mg protein. Conditions, other than choline concentration and incubation time, were the same as in Fig. 2. The absolute amounts were calculated by correcting the counting and extraction efficiencies as described in Methods. The concentration of Na^+ was 0 (△), 40 (□) or 80 mM (○).

TABLE I

EFFECT OF Na^+ ON THE CONVERSION OF CHOLINE INTO ACETYLCHOLINE

Synaptosomes were incubated at 25 °C for 15 min in the presence of 3.7 μM [^{14}C]choline or unlabeled choline in the absence of Na^+. They were then washed twice with 0.32 M sucrose, and reincubated with 3.7 μM of unlabeled choline or [^{14}C]choline in the presence or absence of Na^+. Na^+ was replaced by sucrose at twice the concentration. See Methods for assay details.

Incubation I	*Incubation II*	*Acetylcholine (cpm)*	*Choline (cpm)*
[^{14}C]Choline	Unlabeled choline		
	0 min	116	961
	15 min, without Na^+	92	657
	15 min, with Na^+	96	539
Unlabeled choline	[^{14}C]Choline		
	0 min	88	345
	15 min, without Na^+	162	1590
	15 min, with Na^+	1074	1871

take systems in synaptosomes: one is related to the uptake of choline in the absence of Na^+, and the other is an Na^+-dependent uptake system having a high capacity to synthesize acetylcholine.

Effect of choline concentration

The uptake of [^{3}H]choline into synaptosomes tended to reach a saturation point as the choline concentration increased (Fig. 4), and can be expressed by a Michaelis–Menten type equation. K_m, the concentration for the half-maximum uptake of choline, was about 40 μM for the incubation in the absence of Na^+ (Fig. 6). The amount of acetylcholine found in the pellet also reached a saturation point as the choline concentration increased, regardless of Na^+ concentration (Fig. 5). K_m, the concentration of choline in medium for the half-maximum amount of acetylcholine, was about 4–8 μM for the incubation in the presence of 40 or 80 mM Na^+ (Fig. 6). This value may be interpreted as the concentration for the half-maximum uptake of choline for the Na^+-dependent uptake system. When the same plot was applied to the increase by Na^+ (40 or 80 mM) of the amounts of [^{3}H]choline in synaptosomes, an apparent K_m of 4–8 μM was obtained.

Effect of Na^+ concentration

The uptake of [^{3}H]choline and the synthesis of [^{3}H]acetylcholine were found to reach a saturation point as the Na^+ concentration increased, regardless of choline concentration (Fig. 7). The concentration of Na^+ for half saturation was 40–60 mM. When a Hill plot was made after the correction of all the values for a Na^+ concentration of 0 mM, the value of n was 2–3, depending on the value at saturation, which

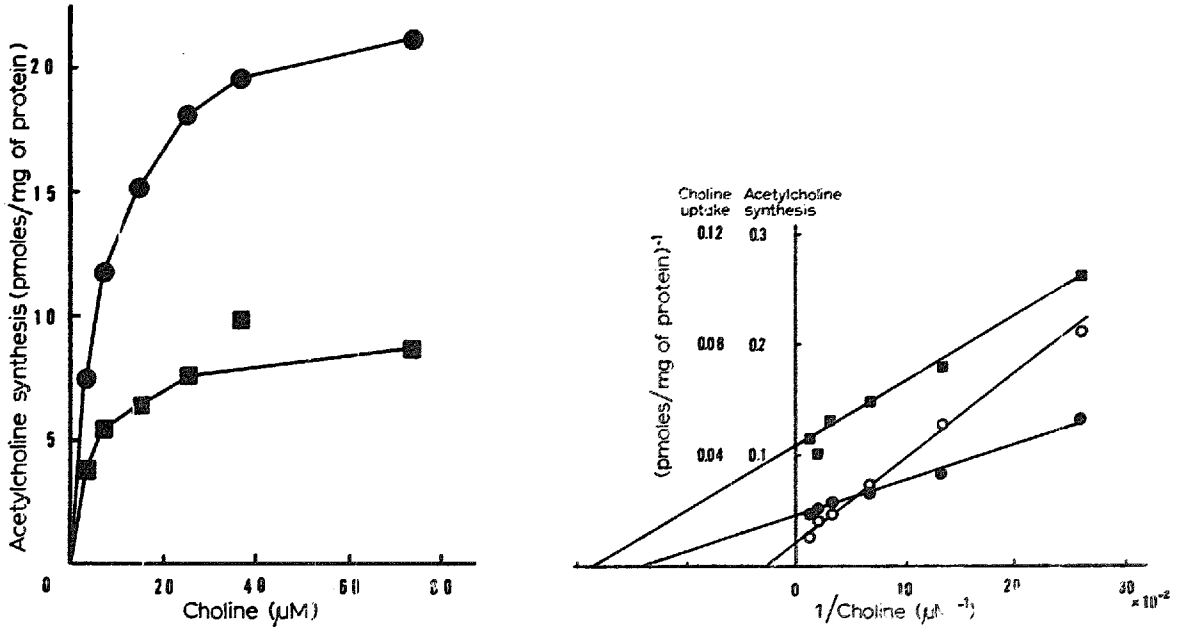


Fig. 5. The synthesis of [^{3}H]acetylcholine with various concentrations of choline at an incubation time of 12 min. Conditions for experiments were the same as in Fig. 4. The concentration of Na was 40 (■) or 80 mM (●).

Fig. 6. Double reciplocal plots for the uptake of [^{3}H]choline at an Na^+ concentration of 0 mM (○), and for the synthesis of [^{3}H]acetylcholine at an Na^+ concentration of 40 mM (■) and 80 mM (●). The original data were the same as in Figs 4 and 5.

was not estimated with sufficient accuracy. The small decrease of choline uptake caused by an increase of the Na^+ concentration from 0 to 10 mM were incompatible with the Hill plot.

Effect of hemicholinium-3

The concentration of hemicholinium-3 for 50% inhibition of the choline uptake was about 50 μM when synaptosomes were incubated in the absence of Na^+ (Fig. 8). When synaptosomes were incubated in the presence of Na^+, 5 μM of hemicholinium-3 almost completely inhibited acetylcholine synthesis, but only partially inhibited choline uptake, and a higher concentration of hemicholinium-3 was necessary for the complete inhibition of choline uptake (Fig. 9). The apparent K_i for acetylcholine synthesis was 0.1 μM, and the apparent K_i values for the choline uptake were 0.1 and 50 μM. These results were compatible with the concept of two uptake systems. The K_i for the Na^+-independent system was calculated at 46 μM, and that for Na^+-dependent system as 0.05–0.07 μM. Hemicholinium-3 was assumed to be a competitive inhibitor in this calculation, and therefore the K_i value was equal to $I_{50}/[1+(\text{chol})/K_m]$, where I_{50} was the concentration of hemicholinium-3 for 50% inhibition.

The amount of [^{3}H]choline taken up in the absence of Na^+ was about 40% of that taken up in the presence of Na^+ at a choline concentration of 3.7 μM. Only about 25% of the [^{3}H]choline, however, was taken up in the presence of 5 μM hemicholinium-3 (Fig. 8). In this case the Na^+-independent uptake system was hardly

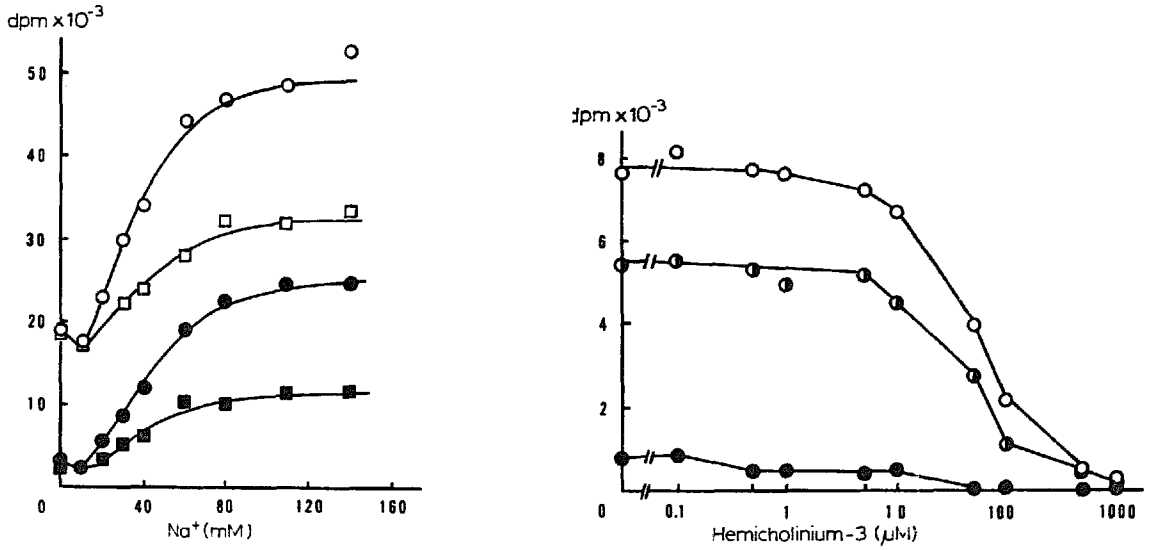


Fig. 7. The uptake of [^{3}H]choline and the synthesis of [^{3}H]acetylcholine with various concentration of Na^+ at an incubation time of 12 min. Each incubation tube contained 3 μCi [^{3}H]choline (0.2 μM) and 5.88 mg protein. Pre-incubation was carried out in the presence of unlabeled choline at 0 μM (○, ●) or 18.5 μM (□, ■) and Na^+ at the concentrations shown in abscissa. Osmolarity was adjusted with sucrose. Other conditions were the same as in Fig. 2. The two lower curves (●, ■) represent counts of the acetylcholine fraction.

Fig. 8. The effect of various concentrations of hemicholinium-3 on the uptake of [^{3}H]choline and on the synthesis of [^{3}H]acetylcholine during incubation in the absence of Na^+. Pre-incubation was carried out in the presence of 3.7 μM unlabeled choline and hemicholinium-3 at the concentrations shown in abscissa. Each tube contained 1.2 μCi [^{3}H]choline and 4.68 mg protein. Incubation time was 12 min. Other conditions were the same as in Fig. 2. ○—○, counts in the extract; ◑—◑, counts in the choline fraction; ●—●, counts in the acetylcholine fraction.

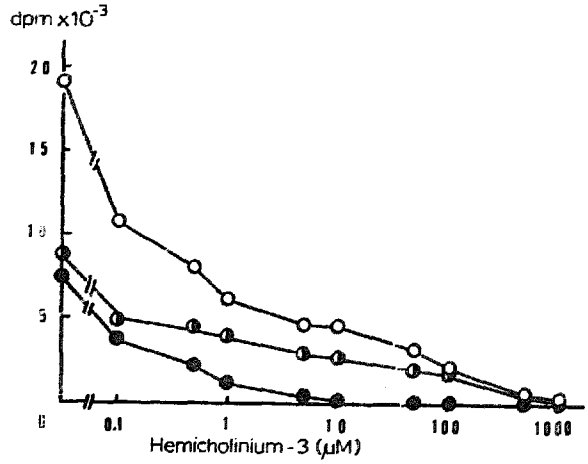
inhibited, in contrast to the almost complete inhibition of the Na^+-dependent uptake system (Figs 8 and 9). The above difference between 40 and 25 % may be due to a Na^+ inhibition of the uptake system which is assumed to be independent of Na^+.

Sucrose density gradient centrifugation

The following experiments were carried out to test the possibility of separating the two uptake systems. A crude synaptosomal fraction containing 18.8 mg of protein was incubated at 25 °C for 20 min with the standard solution containing either [^{14}C]choline (2 μCi, 7.4 μM) or [^{3}H]choline (30 μCi, 7.4 μM) *plus* 5 μM of hemicholinium-3. After incubation, the pellets were combined, washed with 0.32 M sucrose and suspended in 0.32 M sucrose. The suspension (3 ml) was mounted on the linear gradient of sucrose of 0.8–1.5 M (24 ml). After centrifugation at 25000 rev./min for 2 h, fractions of 30 drops (0.71 ml) were collected and centrifuged after the addition of ice-cold 0.32 M sucrose containing 0.1 mM neostigmine (8 ml). The pellets were extracted and assayed as described in Methods. In this system the ^{14}C in the acetylcholine fraction and the ^{3}H in the extract represented the uptake of the Na^+-dependent system and that of the Na^+-independent system, respectively. As shown in Fig. 10, both radioactivities were found in fractions considered to be rich in synaptosomes[6], but they were not separated from each other. When synaptosomes were incubated in the solution containing sucrose instead of Na^+, radioactivities were also found in those fractions considered to be rich in synaptosomes.

Effects of various reagents

The effects of various reagents are shown in Table II. The omission of Ca^{2+} from the incubation medium, or the addition of EDTA or EGTA (ethyleneglycol-bis-



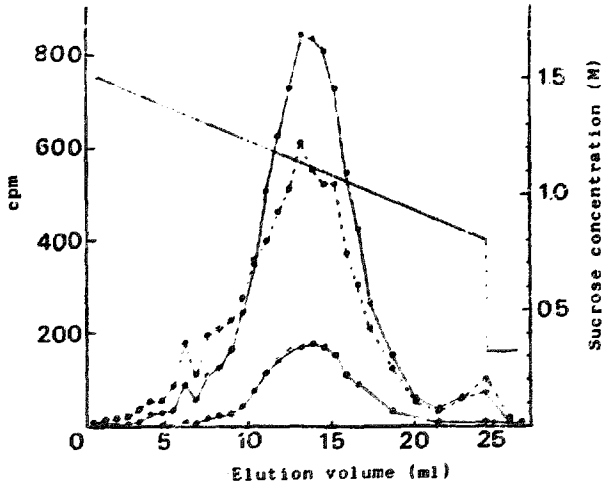


Fig. 9. The effect of various concentrations of hemicholinium-3 on the uptake of [^{3}H]choline and on the synthesis of [^{3}H]acetylcholine during incubation in the presence of Na^+. Conditions and symbols were the same as in Fig. 8 except that 140 mM Na^+ was included in the incubation medium.

Fig. 10. Continuous sucrose density gradient centrifugation. See text for experimental details. Counts in the extract or the acetylcholine fraction derived from a tube incubated in the absence of hemicholinium-3 but in the presence of ^{14}C were represented by ●—●. The lower curve represents counts in the acetylcholine fraction. ●---●, counts in the extract derived from a tube incubated in the presence of 5 μM hemicholinium-3 and ^{3}H. The straight line represents sucrose concentration. Counting efficiency of ^{3}H was 13%, that of ^{14}C was 52 % for the extract and 32 % for the acetylcholine fraction.

(β-aminoethylether)-*N*,*N*′-tetraacetic acid), hardly affected the uptake of choline and the synthesis of acetylcholine.

Sodium cyanide and 2,4-dinitrophenol decreased the amount of [^{14}C]acetylcholine recovered in the synaptosomal pellets but did not affect the amount of [^{14}C]-choline recovered in them. This suggests that the uptake of choline is not directly coupled with energy supplied by metabolic processes. The amount of both [^{14}C]choline and [^{14}C]acetylcholine was decreased by ouabain. Ouabain, however, also has effects on the release of acetylcholine[4] and it was not certain whether this affected the uptake.

Putative transmitter substances, such as serotonin, glutamate, γ-aminobutyric acid and epinephrine, did not affect the uptake of choline nor the synthesis of acetylcholine. Tetrodotoxin, used as an inhibitor of Na^+ transport, at a concentration of 10 μg/ml also had no effect.

TABLE II

EFFECTS OF SOME REAGENTS

Synaptosomes were incubated with 3.7 μM [^{14}C]choline at 25 °C for 10 min.

	Extract	*Acetylcholine* (% *of control*)	*Choline*
Ca^{2+} omitted	97	85	98
EGTA* (1 mM)	110	115	110
EDTA* (1 mM)	99	98	92
NaCN (4 mM)	70	23	98
2,4-Dinitrophenol (0.2 mM)	80	45	96
Ouabain (0.2 mM)	69	39	74
γ-Aminobutyric acid (1 mM)	100	105	
Serotonin (1 mM)	104	94	
Epinephrine (1 mM)	95	103	
Glutamate (1 mM)	98	102	

* Concentrations of $CaCl_2$ and $MgCl_2$ in the standard solution changed to 0 and 2 mM, respectively.

DISCUSSION

Na^+ was observed to have a stimulating effect on the amount of choline taken up into synaptosomes and, to a greater extent, on the amounts of acetylcholine which they synthesized; therefore the fraction of choline converted to acetylcholine increased as the Na^+ concentration increased. Na^+, however, had no stimulating effect on the conversion of choline in synaptosomes into acetylcholine. In order to explain the above results we could assume that Na^+ had a stimulating effect on the conversion of choline into acetylcholine only when choline was taken up in the presence of Na^+. This, however, is not likely since choline acetyltransferase is considered to occur in the cytoplasm and not bound to membranes[7,8], and the enzyme has been reported to be activated by both NaCl and KCl[9]. KCl had no stimulating effect on choline uptake and acetylcholine synthesis. Another explanation is to assume two uptake systems: one is an Na^+-independent uptake system with a low capacity for synthesizing acetylcholine and the other is an Na^+-dependent uptake system with a high

capacity for synthesizing acetylcholine. The fraction of choline converted into acetylcholine was about 60 %, independent of the Na^+ concentration for the Na^+-dependent system. With this assumption, we need not postulate any effect of Na^+ on the synthesis or storage of acetylcholine apart from that on the uptake of choline. The effect on the synthesis and/or storage was discussed by Birks[10], Bhatnagar and McIntosh[11] and suggested by Marchbanks[12].

The effects of choline concentration on the uptake of choline and the synthesis of acetylcholine can be analyzed on the basis of the two uptake systems mentioned above. The effect of the choline concentration in the medium on the synthesis of acetylcholine is not due to an effect on the conversion of choline in synaptosomes into acetylcholine. This follows because both the increase in the amount of choline in synaptosomes caused by the presence of Na^+, and the amount of acetylcholine in synaptosomes show the same dependence on choline concentration in the medium. The K_m values for choline were 4–8 μM for the Na^+-dependent system, and about 40 μM for the Na^+-independent system. This difference in K_m values explains the smaller effect of Na^+ and the smaller proportion of acetylcholine synthesis for greater concentration of choline, as suggested in the previous report[4], and also explains the apparent difference of effect of Na^+ found by various authors[1–3]. The inhibitory effect of Na^+ on the uptake of choline was not observed even at choline concentration of 74 μM under the present condition, which is in contrast with both the report of Diamond and Kennedy[3] and the results reported in the previous report[4], where synaptosomes were incubated at 37 °C. It is probable, however, that the uptake system assumed to be independent of Na^+ suffered an inhibition by Na^+, and that the inhibition was concealed by the stimulating effect of Na^+ on the other system. This possibility was suggested by experiments in the presence of hemicholinium-3 as described in the preceding section, together with the small decrease of choline uptake when the Na^+ concentration is changed from 0 to 10 mM (Fig. 7).

The assumption of two uptake systems was supported by the fact that by increasing the concentration of hemicholinium-3 the uptake of choline was inhibited in one step in the absence of Na^+ but in two steps in the presence of Na^+. The inhibitory effect of hemicholinium-3 on the uptake of choline was greater by a factor of 500 for the Na^+-dependent system, which has a high capacity to synthesize acetylcholine, than for the Na^+-independent system. The K_i value for the Na^+-independent uptake system is comparable to that reported by Diamond and Kennedy[3]. The K_i value for the Na^+-dependent uptake system was rather low as compared to results reported for the inhibitory effect of hemicholinium-3 on acetylcholine synthesis[13–15]: Bhatnagar *et al.*[15] reported that with minced mouse brain 63 % of the acetylcholine synthesis was inhibited by 1 μM of hemicholinium-3. The amount of [^{3}H]choline and [^{3}H]acetylcholine assumed to be taken up and synthesized by the Na^+-dependent system decreased roughly in parallel with each other as the hemicholinium-3 concentration increased (Fig. 9). This result suggests that hemicholinium-3 affects the uptake of choline but does not affect the synthesis or storage of acetylcholine in this system: the effect on acetylcholine synthesis and/or storage was suggested by experiments using cervical ganglion[16] and subarachnoidal administration of this reagent[17].

The kinetic results for the Na^+-dependent system are explained by assuming that a complex of a carrier with a choline molecule and two or three Na molecules

could carry out transport across membranes[13]. Three simple cases can be considered for the formation of the complexes: the carrier binds Na^+ and then binds choline (this means that Na^+ stimulates the binding of choline to the carrier) (case I), the carrier binds choline and then binds Na^+ (case II), or the carrier binds choline and Na^+ independently (case III). If it is assumed that the transport of the complex through membrane is slow as compared to the rate of binding of choline or Na^+ to the carrier, we can obtain kinetic equations for the three simple cases. The amount of choline taken up and the amount of acetylcholine synthesized are expected to be independent of Na^+ concentration at a saturated choline concentration for case I, and independent of choline concentration at saturated concentration of Na^+ for case II. For case I, the apparent K_m value (the concentration for the half-maximum uptake of choline) would be expected to decrease as the Na^+ concentration increased. Results obtained were contrary to these expectations for both cases (Figs 4–7). There are also some discrepancies between the results obtained experimentally and those expected from the formula for case III, but it was much less than that for cases I and II. Thus it is likely that Na^+ affects the transport of a complex of choline and carrier across membranes, but does not affect the binding of choline to the carrier. This is in contrast to the biogenic amine transport system in synaptosomes, where Na^+ has been suggested to increase the affinity of a substrate for the carrier[19]. This treatment, however, is an oversimplified one and a conclusive assessment must await detailed studies which cover an examination of a rate-limiting step. Recently, Thomas and Christensen[20] proposed a random order mechanism for a co-transport system of Na^+ and neutral amino acids for pigeon erythrocytes, though they claimed that the transport of a carrier complex was not a rate-limiting step.

It is possible that there is only one uptake system *in situ* and that, due to the preparation procedures, some synaptosomes lost both their sensitivity to Na^+ and their capacity to synthesize acetylcholine, thus showing apparent Na^+-independent uptake. On the other hand, it is tempting to speculate that synaptosomes in general have an Na^+-independent uptake system, and that synaptosomes from cholinergic synapses have an Na^+-dependent uptake system. In this respect, it is to be noted that the K_m value for the Na^+-dependent system is 5–10 times lower than that for the Na^+-independent system. Recently, Johnston and Iversen[21] showed two uptake systems for glycine in both slices and homogenates, and it was suggested that the uptake system having lower the K_m value existed in synapses where glycine is the transmitter.

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