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Inhibition by Hemicholinium-3 of [^{14}C]Acetylcholine Synthesis and [^{3}H]Choline High-Affinity Uptake in Rat Striatal Synaptosomes

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SUMMARY

GUYENET, PATRICE, LEFRESNE, PIERRE, ROSSIER, JEAN, BEAUJOUAN, JEAN CLAUDE, AND GLOWINSKI, JACQUES: Inhibition by hemicholinium-3 of [^{14}C]acetylcholine synthesis and [^{3}H]choline high-affinity uptake in rat striatal synaptosomes. *Mol. Pharmacol.* **9**, 630–639 (1973).

Hemicholinium (HC-3), a neuromuscular blocking agent which inhibits the synthesis and the release of acetylcholine (ACh) was investigated for its effects both on [^{14}C]ACh synthesis from [2-^{14}C]pyruvate or [6-^{14}C]glucose and on [^{3}H]choline uptake in purified rat striatal synaptosomes. The synthesis of the transmitter was reduced to 15% of control by 1 μM HC-3 in the presence of eserine (170 μM). The drug produced half-maximal inhibition at 0.06 μM; this effect was totally reversed by 30 μM choline. Choline concentrations as low as 10 μM stimulated significantly the synthesis of [^{14}C]ACh in a diluted suspension of synaptosomes. The existence of a high-affinity uptake system for choline, recently observed by Yamamura and Snyder, was confirmed in our preparation: its K_m was found to be 3.5 μM. Eserine (170 μM) produced only 10% inhibition of the uptake process in the presence of 1 μM choline. HC-3 was found to be a purely competitive inhibitor of the choline high-affinity uptake (K_i = 25 nM). A very good correlation was found between the inhibitory effects of HC-3 on [^{14}C]ACh synthesis and on [^{3}H]choline high-affinity uptake. The results suggest that the high-affinity system for the transport of choline is present in cholinergic synaptosomes and plays an important role in sustaining and perhaps regulating the synthesis of the transmitter.

INTRODUCTION

Hemicholinium-3, an agent producing respiratory paralysis and ganglion blockade (1, 2), inhibits the synthesis of acetylcholine *in vitro* in central (3–7) as well as in peripheral (2, 4, 8, 9) nervous tissues. As shown initially by Schueler (1), the toxic action of the drug can be prevented by the administration of choline; in fact, this precursor of ACh[1] prevents the inhibition of transmitter synthesis induced by HC-3 (2, 4, 9). A carrier-mediated transport system for the uptake of choline was demonstrated in brain slices (10) and in various synaptosome-rich fractions (11–15) obtained from whole brain or cortical areas ($K_m \cong 60$ μM). Recently Yamamura and Snyder (16) described a second choline

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[1] The abbreviations used are: ACh, acetylcholine; HC-3, hemicholinium-3.

uptake system in a synaptosomal fraction of rat striatum, characterized by its high affinity (K_m = 1.1 μM). Moreover, at these low concentrations, choline taken up in synaptosomes is almost completely converted into ACh (16). The hypothesis of competition between HC-3 and choline at the neuronal membrane level was first proposed by MacIntosh *et al.* (4). HC-3 was indeed found to inhibit low-affinity choline transport in brain slices (10) and in various synaptosomal preparations (7, 12–15).

The present study was undertaken to examine simultaneously the effects of HC-3 on ACh synthesis and on the choline high-affinity uptake system in a purified synaptosomal preparation obtained from rat striatum, a region of the brain especially rich in cholinergic structures (17, 18). HC-3 in very low concentrations (10–100 nM) was found to inhibit both processes. These results support the view that choline transport plays an important role in the biosynthesis of ACh.

MATERIALS AND METHODS

Chemicals

[2-^{14}C]Pyruvic acid (10–20 mCi/mmole) was purchased from Commissariat à l'Energie Atomique, France. [*acetyl*-^{3}H]ACh chloride (250 mCi/mmole), [*methyl*-^{3}H]choline chloride (17 Ci/mmole), and [6-^{14}C]glucose (45 mCi/mmole) were provided by the Radiochemical Centre, Amersham, England. Eserine salicylate was obtained from Sigma Chemical Company, and hemicholinium-3 from Aldrich-Europe (Beerse, Belgium).

Preparation of Striatal Synaptosomes

Male Charles River rats weighing 200–300 g were killed by a blow on the neck followed by decapitation. Their brains were immediately removed and dissected in the cold at 4° with glass manipulators under a dissecting microscope (19). Each animal yielded 100 mg (fresh weight) of striatal tissue, and a total of 15–30 rats was used for each experiment. Synaptosomes (B fraction) were then prepared according to the method of Gray and Whittaker (20) as modified by Israel and Frachon-Mastour (21); band B of the discontinuous sucrose gradient was diluted with an equal volume of water and centrifuged at 25,000 × *g* for 30 min. The synaptosomal pellet was resuspended in various volumes of a standard physiological medium (NaCl, 136 mM; KCl, 5.6 mM; $NaHCO_3$, 16.2 mM; NaH_2PO_4, 1.2 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.2 mM). Choline acetyltransferase (EC 2.3.1.6) activity was estimated on an aliquot by the method of Fonnum (22); this value was used as an index of the number of intact cholinergic synaptosomes present in each incubation tube. The activity was found to be 0.20–0.35 μmole of ACh per hour per sample (0.3–0.5 mg of protein).

Synthesis Experiments

Method. The synaptosomal pellet obtained from 3 g of striatal tissue (initial wet weight) was resuspended in 9–15 ml of the cold standard physiological medium, and eserine (170 μM) and [2-^{14}C]pyruvate or [6-^{14}C]glucose were then added. Aliquots of this synaptosomal suspension (0.25–0.50 ml) were introduced into small glass tubes (12 ml), and choline and HC-3 were added (0.1 ml). Each tube contained 3–7 μCi of [2-^{14}C]pyruvate or [6-^{14}C]glucose. The incubation was carried out at 37° under a constant stream of 95% O_2–5% CO_2 in a Dubnoff metabolic shaker and was stopped by adding 8 ml of ice-cold 10 mM sodium phosphate buffer, pH 7.2. This solution, as well as an additional 8 ml of the buffer used to rinse the incubation tube, was rapidly transferred to a 50-ml Sorvall SS 34 tube containing 6 ml of a solution of tetraphenylboron in ethyl butyl ketone (3.3 mg/ml) and 25 μl of [*acetyl*-^{3}H]ACh (5 nCi) as an internal recovery standard. The tubes were shaken for 5 min and centrifuged for 5 min at 25,000 × *g* in a Sorvall RC2-B centrifuge. Five milliliters of the organic phase were added in conical glass tubes containing 0.5 ml of 1 N HCl. After shaking and centrifugation, 0.4 ml of the acidic phase was transferred into counting vials. All results were corrected using the blank values obtained in the absence of incubation at 37°.

Specificity of technique. To make sure

that [^{14}C]ACh was totally extracted by the tetraphenylboron–ethyl butyl ketone solution, this procedure was compared with the classical trichloracetic acid extraction method. At the end of the incubation a 0.25-ml aliquot of synaptosomal suspension was treated as previously described. A second 0.25-ml aliquot was added to an equal volume of trichloracetic acid (10%, w/v) containing [*acetyl*-^{3}H]ACh (5 nCi) as an internal recovery standard. After standing for 1 hr at 0°, the trichloracetic acid was extracted with water-saturated ether until pH 4 was reached. The aqueous solution was then added to 34 ml of 10 mM sodium phosphate buffer (pH 7.2), and ACh was further isolated as described above. The ^{14}C radioactivities recovered in the acid phase using these two processes were then compared.

To identify further the nature of the ^{14}C radioactivity recovered in the HCl phase, a 0.2-ml aliquot of this solution was processed for thin-layer chromatography (23). The radioactivity at the level of the ACh spot was measured as described previously (23) and compared with that found in the rest (0.3 ml) of the HCl phase.

Measurement of specific radioactivity of newly synthesized ACh. The specific radioactivity of the transmitter newly synthesized from [2-^{14}C]pyruvate was measured using the method already described for striatal slices (23).

Uptake Experiments

The synaptosomal pellet obtained from 1.5 g of striatal tissues (initial wet weight) was resuspended in about 40 ml of the cold standard physiological medium, which in some cases contained 170 μM eserine. Portions of this solution (0.9 ml) were placed in 15-ml Sorvall SM 24 polyethylene tubes, which were then incubated at 0° (blank) or at 37° for 2 min under 95% O_2–5% CO_2 in a Dubnoff metabolic shaker. [*methyl*-^{3}H] Choline (0.5–2 μCi; 0.1 μM–1 mM final concentration), in a volume of 0.1 ml, was then added to each tube, and the incubation was continued for 1 min at 0° (blank) or 37°. The incubation was stopped by adding 8 ml of the ice-cold incubation medium, and the tubes were centrifuged at 25,000 × *g* for 5 min. The total radioactivity used in each assay was estimated in the supernatant fraction, which was then discarded. After a gentle wash with 2.5 ml of the ice-cold incubation medium, the pellet was solubilized with 1 ml of Triton X-100 (1% in water) and kept overnight at room temperature. Finally 0.9-ml aliquots of the Triton solution were transferred to counting vials.

Radioactivity estimation. The radioactivity was estimated with a Packard Tri-Carb liquid scintillation spectrometer. Packard Instagel was used as the medium (10 ml/vial). All analyses pertaining to each experiment were performed simultaneously, and the values obtained were corrected for respective recoveries. In some experiments the statistical significance of the observed differences was calculated by Student's *t*-test (24).

RESULTS

[^{14}C]*ACh Synthesis from* [*2*-^{14}C]*Pyruvate or* [*6*-^{14}C]*Glucose in Rat Striatal Synaptosomes*

[^{14}C]ACh synthesized at 37° from [2-^{14}C]-pyruvate could be completely extracted from striatal synaptosomes by simple shaking in the presence of an ethyl butyl ketone solution of tetraphenylboron (Table 1). Indeed, the [^{14}C]ACh recovery was indentical with that obtained by the classical trichloracetic acid extraction method. The blank value obtained by incubation at 0° is shown in this table and was routinely subtracted in the results described below.

The ^{14}C radioactivity measured in the HCl phase after dissociation of the tetraphenylboron complex was considered to be due to labeled ACh. When synaptosomes were incubated in the presence of eserine (200 μM) and either [2-^{14}C]pyruvate or [6-^{14}C]glucose, no significant difference was observed between the ^{14}C radioactivity measured in HCl and that found in the ACh spot on thin-layer chromatography plates (Table 2).

In the presence of eserine (200 μM) and [2-^{14}C]pyruvate total ACh synthesis, measured by the increase in ACh in tissue and

TABLE 1

Extraction of [^{14}C]ACh synthesized from [2-^{14}C]-pyruvate in striatal synaptosomes

Striatal synaptosomes were incubated at 37° for various periods in the presence of choline (2.6 mM) and [2-^{14}C]pyruvate (5.4 mM, 8.8 μCi/ml). The suspension was then extracted either with trichloracetic acid (5%) or directly by shaking in the presence of a solution of tetraphenylboron in ethyl butyl ketone (see MATERIALS AND METHODS). ^{14}C radioactivity recovered in HCl was then measured in both cases. The values are means ± standard errors of four determinations.

Incubation time	Trichloracetic acid extraction		No trichloracetic acid extraction	
	Total radioactivity	Minus blank	Total radioactivity	Minus blank
min	*dpm*	*dpm*	*dpm*	*dpm*
0	94 ± 32	0	398 ± 50	0
10	1657 ± 103	1563	1920 ± 35	1520
20	2300 ± 140	2200	3060 ± 140	2670
30	4000 ± 130	3910	3830 ± 150	3430
40	4767 ± 166	4670	4690 ± 70	4230
50	5517 ± 301	5420	5370 ± 120	4970
60	5580 ± 420	5480	5960 ± 270	5560

in the incubation medium, was identical using either the leech dorsal muscle or the radiometric technique (Table 3). In the latter case the amount synthesized (nanomoles) was calculated by assuming that the specific radioactivity of the newly synthesized ester was equal to that of the added precursor.

Effect of HC-3 on [^{14}C]ACh Synthesis

Striatal synaptosomes were incubated for 15 min in the presence of 5.4 mM [2-^{14}C] pyruvate and 170 μM eserine. Final concentrations of HC-3 ranged from 10 nM to 3 mM. HC-3 inhibited total [^{14}C]ACh synthesis (tissue plus medium) even at 10 nM, with half-maximal inhibition occurring at 60 nM. The blocking effect of the drug on [^{14}C]ACh synthesis never exceeded 85% even when its concentration was raised to 3 mM (Fig. 1). Similar results were obtained when synaptosomes were incubated for 30 min; however, HC-3 then appeared slightly more effective.

Since the specificity of the isolation procedure used for the estimation of [^{14}C]ACh had been tested in the absence of any drug, the following experiment was performed to confirm the identity of the ^{14}C radioactivity finally extracted in the presence of HC-3. Striatal synaptosomes were incubated as described previously, but the samples were analyzed chromatographically. Synthesis of the ester was estimated by counting the radioactivity present in the ACh spot on thin-layer chromatographic plates (see MATERIALS AND METHODS). The values for [^{14}C]ACh synthesis obtained with this technique (Table 4) were very similar to those described previously (Fig. 1); in particular, ^{14}C radioactivity, corresponding to approximately 20% of control, was still detectable in the presence of high HC-3 concentrations (10 μM).

TABLE 2

Specificity of isolation procedure for [^{14}C]ACh synthesis from [2-^{14}C]pyruvate or [6-^{14}C]glucose

Striatal synaptosomes were incubated for various periods at 37° in the presence of choline (0.1 mM), eserine (0.2 mM), and [2-^{14}C]pyruvate (4.6 mM, 12.1 μCi/ml) or [6-^{14}C]glucose (1.5 mM, 3.2 μCi/ml). The radioactivity in the HCl phase was counted on a sample, and the remaining incubation mixture was analyzed for ^{14}C radioactivity on thin-layer chromatography plates (see MATERIALS AND METHODS). The values were corrected for recovery of [^{3}H]ACh used as an internal standard.

Precursor	Incubation time	*n*	^{14}C	
			HCl step	ACh spot on thin-layer plate
	min		*dpm*	*dpm*
[2-^{14}C]Pyruvate	10	2	4,560	4,550
	20	2	8,500	9,020
	30	2	12,000	12,000
	40	2	14,700	14,700
	50	2	17,100	17,000
	60	2	18,500	18,600
[6-^{14}C]Glucose	15	6	981 ± 26	1,002 ± 54

Effect of Choline on [^{14}C]Ach Synthesis in the Absence or Presence of HC-3

In one experiment [^{14}C]ACh synthesis from [6-^{14}C]glucose was estimated in striatal

TABLE 3

Biological and radiometric estimation of ACh synthesis in striatal synaptosomes

Striatal synaptosomes were incubated for various periods at 37° in the presence of [2-^{14}C]-pyruvate (5.7 mM, 5.8 μCi/ml), eserine (0.2 mM), and choline (1 mM). Then the suspension (tissue plus medium) was extracted with trichloracetic acid (5%). After removal of the trichloracetic acid by washing with ether, a portion (pH 4) was set aside for biological measurement of total ACh. The rest was treated as described under MATERIALS AND METHODS for determination of [^{14}C]ACh synthesis.

Incubation time	ACh		
	Biological		Radiometric (synthesis)[a]
	Total	Synthesis	
min	*nmoles*		*nmoles*
0	0.48	0	0
10	1.15	0.67	0.69
20	1.57	1.09	1.26
60	3.44	3.00	2.98

[a] Obtained by dividing disintegrations per minute of ^{14}C by the specific radioactivity of the precursor.

synaptosomes incubated for 3 or 8 min in the presence or absence of added choline (final concentration, 1 μM–1 mM). To minimize the possible influence of choline released from synaptosomes, the synaptosomal pellet was diluted 3-fold more than previously and incubations were carried out in a volume of 0.6 ml instead of 0.35 ml.

As indicated in Fig. 2, the presence of choline stimulated [^{14}C]ACh synthesis. The stimulatory effect was proportionally larger for the 3-min incubation than for the 8-min incubation. The maximum effect was obtained in both cases with concentrations of choline as low as 10 μM.

In another experiment striatal synaptosomes were incubated for 15 or 30 min with [2-^{14}C]pyruvate and eserine, as described above, in the presence of the concentration of HC-3 (60 nM) which induced half-maximal inhibition of [^{14}C]ACh synthesis. Choline concentrations ranging from 1 to 250 μM were used. Choline at 10 μM slightly reversed the inhibitory effect of HC-3 on [^{14}C]ACh synthesis, which re-

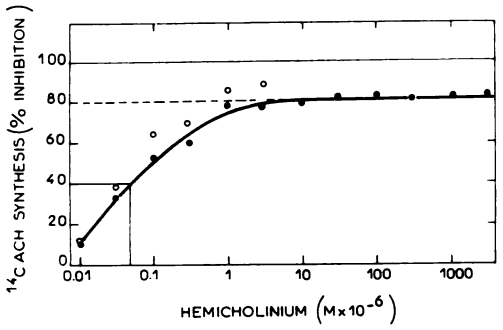

FIG. 1. *Effect of HC-3 on [^{14}C]ACh synthesis in rat striatal synaptosomes*

Striatal synaptosomes were incubated at 37° in the presence of [2-^{14}C]pyruvate (5.4 mM, 6.6 μCi/assay) for 15 or 30 min in a physiological medium containing eserine (170 μM). No choline was added in this experiment. HC-3 (final concentration, 10 nM–1 mM) was introduced in a volume of 0.1 ml. Total [^{14}C]ACh synthesis (tissue plus incubation medium) was determined as described under MATERIALS AND METHODS. The maximal ACh synthesis was 0.87 nmole/15 min and 1.78 nmoles/30 min. Each point represents the mean of two assays: ●, 15-min incubations; ○, 30-min incubations.

TABLE 4

Further identification of [^{14}C]ACh synthesized from [2-^{14}C]pyruvate in the presence of HC-3 in rat striatal synaptosomes

Rat striatal synaptosomes (0.35 ml) were incubated at 37° for 10 min in the presence of [2-^{14}C]pyruvate (5.3 mM, 5.5 μCi/assay) and eserine (170 μM). Final concentrations of HC-3 ranged from 0.01 to 10 μM. ^{14}C radioactivity was measured in the ACh spot obtained on thin-layer chromatography plates from a 0.2-ml aliquot of the HCl phase as described under MATERIALS AND METHODS. The results were corrected for recovery of [*acetyl*-^{3}H]choline introduced as an internal standard at the end of the incubation and are the means ± standard errors of five assays.

HC-3	^{14}C in ACh spot	
	Incorporation	Inhibition
M	*dpm*	%
0	3600 ± 120	0
10^{-8}	2900 ± 120	19 ± 3
10^{-7}	2000 ± 180	44 ± 5
10^{-6}	1200 ± 170	67 ± 5
10^{-5}	800 ± 60	78 ± 2

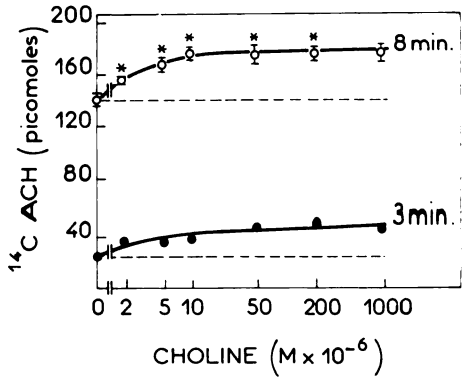

FIG. 2. *Effect of choline on [^{14}C]ACh synthesis in rat striatal synaptosomes*

Striatal synaptosomes were incubated at 37° with [6-^{14}C]glucose (2 mM, 3.2 μCi) for 3 or 8 min in the presence of eserine (170 μM) and various concentrations of choline. Total [^{14}C]ACh synthesis (tissue plus incubation medium) was estimated as described under MATERIALS AND METHODS. Each point is the mean ± standard error of four assays.

* $p < 0.02$.

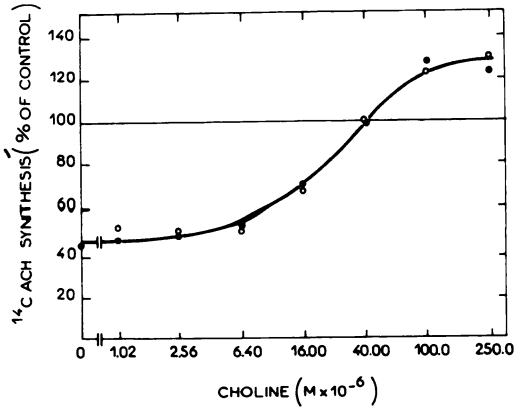

FIG. 3. *Reversal by choline of inhibitory effect of HC-3 on [^{14}C]ACh synthesis in rat striatal synaptosomes*

Striatal synaptosomes were incubated at 37° with [2-^{14}C]pyruvate (5.3 mM, 3.7 μCi) for 15 or 30 min in the presence of eserine (170 μM), HC-3 (60 nM), and various final concentrations of choline. Total [^{14}C]ACh was estimated as described under MATERIALS AND METHODS. The results are expressed as a percentage of the maximal synthesis (0.60 nmole/15 min, 1.12 nmoles/30 min) obtained in the absence of HC-3 and choline, and are the means of two assays. ●, 15-min incubations; ○, 30-min incubations.

turned to control levels in the presence of 40 μM choline. Higher concentrations of choline stimulated transmitter synthesis (Fig. 3).

Effect of HC-3 on Choline High-Affinity Uptake

In our experiments half-maximal inhibition of [^{14}C]ACh synthesis was obtained with 60 nM HC-3, a concentration too low to inhibit significantly the low-affinity choline uptake described by previous authors. This prompted us to confirm the presence of a high-affinity transport system for choline and to study the effect of HC-3 on this uptake process.

The time course of choline uptake was found to be linear for 1 min at each choline concentration tested (Fig. 4). Therefore this incubation time was retained in further experiments.

Striatal synaptosomes were then incubated at 37° or 0° for 1 min in the presence of various concentrations of [^{3}H]choline (1–10 μM) but in the absence of eserine. In this range of concentrations the net uptake of choline (difference between uptake at 37° and 0°) was a saturable process, with

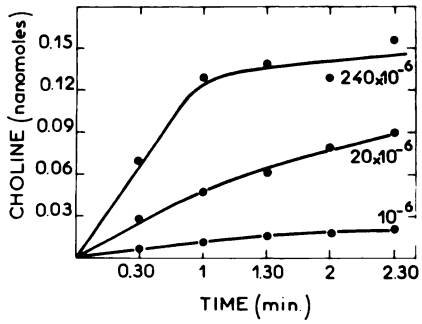

FIG. 4. *Time course of [^{3}H]choline uptake by rat striatal synaptosomes*

Striatal synaptosomes (0.35-ml samples) were incubated as described under MATERIALS AND METHODS for various periods at 37° in the presence of eserine (170 μM) and three different concentrations of [^{3}H]choline (1, 20, and 240 μM; 5 μCi). Results are the means of two assays and are expressed in nanomoles (disintegrations per minute divided by choline specific radioactivity).

a K_m of 4 μM (Fig. 5). The V_{max} of choline uptake was equal to 3 nmoles of choline per minute per choline acetylase unit (arbitrarily defined as the total activity found

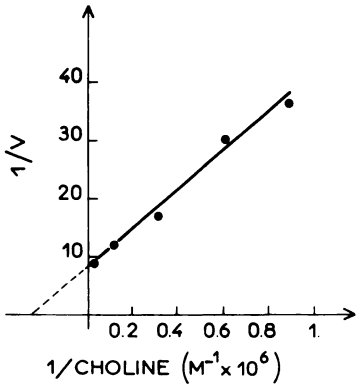

FIG. 5. *Choline high-affinity uptake in rat striatal synaptosomes*

Striatal synaptosomal suspensions (1 ml) were incubated for 1 min at 37° or 0° in the presence of various concentrations of labeled choline (2.2 μCi) as indicated under MATERIALS AND METHODS. The figure represents a reciprocal plot of the net uptake (v) with respect to the concentration of [^{3}H]choline. The velocity is expressed in nanomoles of choline per minute (disintegrations per minute divided by choline specific radioactivity) per sample. Each point is the mean of four assays.

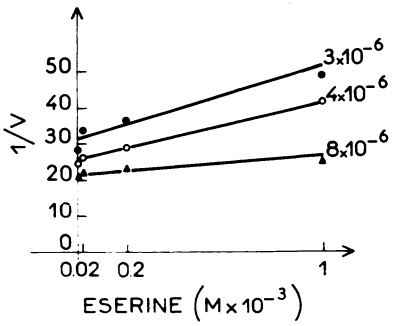

FIG. 6. *Effect of eserine on choline high-affinity uptake in rat striatal synaptosomes*

Striatal synaptosomes (1-ml samples) were incubated at 37° or 0° for 1 min with labeled choline (3, 4, or 8 μM; 2 μCi) in the presence of various concentrations of eserine. The figure represents a plot of the reciprocal net uptake of choline (uptake at 37° minus uptake at 0°) ($1/v$) with respect to eserine concentration. Each point is the mean of two assays.

in 1 g of fresh striatal tissue, 18 μmoles of ACh per hour). (As indicated under MATERIALS AND METHODS, the constant amount of choline acetylase activity present in each sample was taken as an index of the number of intact cholinergic synaptosomes.) In the presence of eserine the K_m of high-affinity uptake was found to be slightly lower (2.5 μM).

In order to test more precisely the influence of eserine on the high-affinity uptake of choline, striatal synaptosomes were incubated for 1 min with various concentrations of eserine (up to 1 mM) in the presence of three different concentrations of labeled choline. As indicated in Fig. 6, 170 μM eserine, the concentration generally used in the synthesis experiments, had very little effect on the net uptake of choline.

Finally, the effect of HC-3 on choline high-affinity uptake was examined. Striatal synaptosomes were incubated for 1 min at 37° or 0° in the presence of various concentrations of HC-3 (1, 5, or 8 μM; 2 μCi). HC-3 inhibited the net uptake of choline in a purely competitive manner (K_i = 25 nM) (Fig. 7).

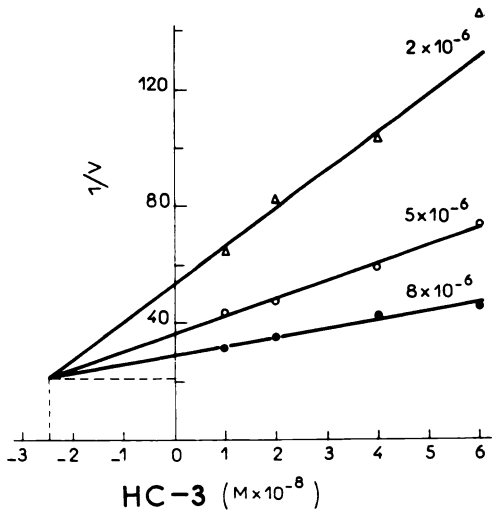

FIG. 7. *Effect of HC-3 on choline high-affinity uptake*

Striatal synaptosomes were incubated at 37° for 1 min in the presence of choline (2, 5, or 8 μM; 2 μCi/assay) and various concentrations of HC-3. Control samples were incubated at 0° for 1 min in the presence of the same amounts of labeled choline in order to subtract the uptake corresponding to passive diffusion. v represents net uptake (37° minus 0°) calculated as nanomoles by dividing the disintegrations per minute by choline specific radioactivity. The figure is a plot of $1/v$ with respect to the concentration of HC-3 for the various choline concentrations tested.

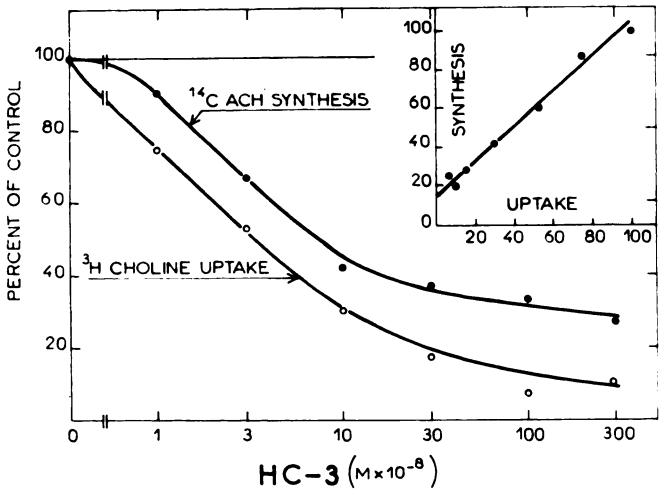

FIG. 8. *Simultaneous estimation of inhibitory effects of HC-3 on [^{14}C]ACh synthesis and [^{3}H]choline high-affinity uptake*

Rat striatal synaptosomes (0.32 ml) were incubated for various periods in the standard physiological medium containing pyruvate (5 mM), in the presence of eserine (170 μM) and various concentrations of HC-3. Labeled pyruvate (6.5 μCi) was added to half the samples, which were incubated for 15 min at 37° to estimate [^{14}C]ACh synthesis. The other samples were first incubated for 7 min at 37°. [^{3}H]Choline (1 μM, 0.52 μCi) was immediately added to one group of samples, and the incubation was continued for 1 min to estimate [^{3}H] choline uptake at 37°. The other group of samples was allowed to stand at 0° for 2 min after the preliminary incubation period. [^{3}H]Choline was then added, and the incubation was continued for 1 min to estimate the uptake at 0°. Each point is the mean of two determinations, and results are expressed as a percentage of maximal synthesis (0.88 nmole/15 min) or maximal net uptake (uptake at 37° minus uptake at 0°: 190 pmoles/min) obtained in the absence of HC-3. The inset represents a plot of [^{14}C]ACh synthesis vs. [^{3}H]choline high-affinity uptake.

Comparison of Inhibitory Effects of HC-3 on [^{14}C]ACh Synthesis and Choline High-Affinity Uptake

The similarity between the concentration of HC-3 giving half-maximal inhibition of [^{14}C]ACh synthesis and the K_i of HC-3 for choline high-affinity uptake led us to compare the inhibition of both processes in the same experiment.

Striatal synaptosomes were incubated in the presence of eserine (170 μM) and choline (1 μM). Labeled pyruvate was added to half the samples, which were incubated at 37° for 15 min to estimate the synthesis of [^{14}C]ACh. [^{3}H]Choline uptake was studied in the remaining samples, which were first incubated for 7 min at 37°. Labeled choline (a tracer amount, 2 μCi) was then added to one batch, and the incubation was stopped 1 min later (uptake at 37°); a second batch was transferred to a cold bath for 2 min for temperature equilibration, the same amount of labeled choline was then added, and the incubation was continued for 1 min at 0° (uptake at 0°). A linear correlation between inhibition of [^{14}C]ACh synthesis and inhibition of the net uptake of [^{3}H]choline was obtained (Fig. 8).

DISCUSSION

The present results, obtained with a purified synaptosomal preparation from rat striatum, confirm the existence of a high-affinity uptake system for choline. The K_m for this uptake process is between 2.5 and 4 μM. It is thus slightly higher than the K_m value (1.1 μM) obtained by Yamamura and Snyder (16). However, these authors used an irreversible cholinesterase inhibitor in their experiments, and this may explain the small difference observed. On the other hand, in contrast to the report by Yamamura and Snyder, we failed to detect a second saturable transport process for choline under our experimental

conditions. A possible explanation for this discrepancy is that our results represent the net uptake of choline obtained by subtracting from the uptake at 37° that observed at 0°, which corresponds to the amount of choline penetrating the intracellular spaces by simple diffusion. Using different experimental conditions, other authors also found a unique, temperature-dependent, saturable uptake process for choline in brain synaptosomes. However, it was characterized by a much higher K_m (50 μM) (12–15).

The present results confirm that HC-3 is a competitive inhibitor of choline uptake in brain synaptosomes (12–14). Nevertheless the kinetic parameters described in this study (K_i = 25 nM) differ markedly from previous observations (K_i = 20 μM) made under experimental conditions producing a low K_m for choline uptake. As shown in Fig. 3, a ratio of approximately 1000:1 between choline and HC-3 is necessary to totally reverse the inhibitory effect of the drug on ACh synthesis. Interestingly, this same ratio was found to be necessary to reverse the toxicity of the drug on the superior cervical ganglion (2). Both results strongly suggest the existence of a large difference between the affinities of choline and HC-3 for the uptake system. Indeed, a K_m/K_i ratio of 150 can be calculated from the present data.

Eserine appeared to be a very weak inhibitor of high-affinity choline uptake, and preliminary studies revealed that ACh is a poor competitive inhibitor of choline transport (K_i = 50 μM).

The concentrations of HC-3 used by previous authors to demonstrate inhibition of ACh synthesis were always higher than 10 μM, with one exception: Bhatnagar and MacIntosh (5) reported that half-maximal inhibition of ACh synthesis was achieved at 4 μM HC-3 in minced mouse brain. The present results demonstrate that in striatal synaptosomes much lower concentrations of HC-3 can inhibit ACh synthesis (half-maximal inhibition at 60 nM). This discrepancy may be attributable to differences in the experimental material; brain slices, generally obtained from the cortex, were used by other workers. These preparations release large amounts of choline into the incubation medium (5, 23), which may lower the activity of the drug by a competition mechanism occurring at the level of the uptake system.

The inhibition of ACh synthesis by HC-3 never exceeded 85% in our experiments. The remaining 15% of radioactivity very likely corresponds to [^{14}C]ACh, as it migrates together with this ester on cellulose thin-layer chromatography plates. However, the possibility cannot be completely ruled out that part of this radioactivity represents some labeled derivative of HC-3: indeed, it has been reported that HC-3 penetrates into the neuron (25) and that it can be acetylated by choline acetylase with about 20% of the efficiency obtained for choline (3, 15, 26, 27). However, the latter hypothesis is not supported by the observation that the remaining radioactivity was not increased by raising the doses of the drug up to 10 μM.

Added choline is required to sustain ACh synthesis in the electrically stimulated superior cervical ganglion (2) or rat diaphragm (9). In brain tissue (unstimulated slices or minces) an excess of choline has been shown not to influence greatly the synthesis of ACh (28, 6, 23). However, in rat striatal synaptosomes, as shown in this study, choline is able to stimulate ACh synthesis. This effect, which is better seen in incubations of short duration with a diluted synaptosomal suspension, is very likely masked in brain slices by extraneuronal choline released very quickly from tissues. Moreover, the maximal effect of choline on ACh synthesis is obtained at a concentration of 10 μM, which is just above the K_m of choline high-affinity uptake. In addition, reported plasma choline (29, 30) concentrations are similar (3–6 μM) to the K_m described in the present study. The linear correlation between the level of [^{14}C]ACh synthesis and that of [^{3}H]choline uptake, observed at all HC-3 concentrations, demonstrates the presence of the choline high-affinity uptake mechanism on cholinergic terminals. This further underlines the major role of newly taken up choline in sustaining the synthesis of the transmitter and suggests a close link between choline uptake and ACh

synthesis. In conclusion, the various results obtained suggest that the high-affinity uptake of choline may contribute to the physiological regulation of ACh synthesis.

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REFERENCES

1. F. W. Schueler, *J. Pharmacol. Exp. Ther.* **115**, 127–143 (1955).
2. R. I. Birks and F. C. MacIntosh, *Can. J. Biochem. Physiol.* **39**, 787–827 (1961).
3. J. F. Gardiner, *Biochem. J.* **81**, 297–303 (1961).
4. F. C. MacIntosh, R. I. Birks, and P. B. Sastry, *Nature* **178**, 1181–1182 (1956).
5. S. P. Bhatnagar and F. C. MacIntosh, *Can. J. Physiol. Pharmacol.* **45**, 249–268 (1967).
6. E. T. Browning and M. P. Schulman, *J. Neurochem.* **15**, 1391–1405 (1968).
7. R. M. Marchbanks, *Biochem. Pharmacol.* **18**, 1763–1766 (1969).
8. B. Collier, *J. Physiol.* (*London*) **205**, 341–352 (1969).
9. L. T. Potter, *J. Physiol.* (*London*) **206**, 145–166 (1970).
10. J. Schuberth, A. Sundwall, B. Sörbo, and J. O. Lindell, *J. Neurochem.* **13**, 347–352 (1966).
11. J. Schuberth and A. Sundwall, *J. Neurochem.* **14**, 807–812 (1967).
12. L. T. Potter, *in* "The Interaction of Drugs and Subcellular Components on Animal Cells" (P. N. Campbell, ed.), pp. 293–304. Churchill, London, 1968.
13. I. Diamond and E. P. Kennedy, *J. Biol. Chem.* **244**, 3258–3263 (1969).
14. I. Diamond and D. Milfay, *J. Neurochem.* **19**, 1899–1909 (1972).
15. R. M. Marchbanks, *Biochem. J.* **110**, 533–541 (1968).
16. H. I. Yamamura and S. Snyder, *Science* **178**, 626–628 (1972).
17. F. C. MacIntosh, *J. Physiol.* (*London*) **99**, 436–442 (1941).
18. C. O. Hebb and A. Silver, *J. Physiol.* (*London*) **134**, 718–728 (1956).
19. J. Glowinski and L. L. Iversen, *J. Neurochem.* **13**, 655–669 (1966).
20. E. G. Gray and V. P. Whittaker, *J. Anat.* **96**, 79–88 (1962).
21. M. Israel and P. Frachon-Mastour, *Arch. Anat. Microsc. Morphol. Exp.* **59**, 383–391 (1970).
22. F. Fonnum, *Biochem. J.* **115**, 465–472 (1969).
23. P. Lefresne, P. Guyenet, and J. Glowinski, *J. Neurochem.* **20**, 1083–1097 (1973).
24. D. Schwartz, "Methods Statistiques," pp. 152–162, Ed. Medicales Flammarion, 20, rue de Vaugirard, Paris 6, 1963.
25. G. Rodríguez de Lores Arnaiz, L. M. Zieher, and E. de Robertis, *J. Neurochem.* **17**, 221–229 (1970).
26. B. A. Hemsworth, K. I. Darmer, and H. B. Bosmann, *Neuropharmacology* **10**, 109–120 (1971).
27. B. A. Hemsworth, *Eur. J. Pharmacol.* **15**, 91–100 (1971).
28. P. J. G. Mann, M. Tennenbaum, and J. H. Quastel, *Biochem. J.* **32**, 243–261 (1938).
29. J. Bligh, *J. Physiol.* (*London*) **117**, 234–240 (1952).
30. J. E. Gardiner and W. D. M. Paton, *J. Physiol.* (*London*) **227**, 71–86 (1972).