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KINETICS OF [^{3}H]CHOLINE UPTAKE IN ABDOMINAL GANGLIA OF *LIMULUS POLYPHEMUS*

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Abstract—The uptake of [^{3}H]choline by isolated ganglia of the ventral nerve cord of *Limulus polyphemus* was studied. The uptake process was linear for 90 min and was concentration dependent. Kinetic analysis suggested the existence of a high affinity uptake process (K_t 14 μM and V_{max} 0.19 pmole/mg/min) and a low affinity uptake process (K_t 180 μM and V_{max} 0.89 pmole/mg/min). The high affinity uptake system showed a greater dependence on sodium ions and was more sensitive to inhibition by hemicholinium-3. Neither uptake system was greatly influenced by the absence of calcium or potassium. It is suggested that this system may be important in supplying choline for the biosynthesis of acetylcholine in cholinergic neurons.

Nervous tissue is unable to synthesize choline [1, 2], which is an immediate precursor of acetylcholine (ACh). Thus, the supply of choline for ACh synthesis is functionally important. It has been demonstrated that the transport of choline within the nervous system is mediated by two systems: one is a high affinity uptake process with a K_t ranging from 1 to 10 μM and the other a low affinity uptake process with K_t ranging from 10 to 100 μM [3–7]. The low affinity uptake is Na^+-independent, is present in non-neural tissue and is thought to contribute very little to the synthesis of ACh in cholinergic neurons [4, 8, 9]. The Na^+-dependent high affinity system contributes substantially to ACh sythesis and has been demonstrated in synaptosomes [4, 5, 10] and cholinergic neurons found in the brain and periphery [11, 12]. Furthermore, it has been suggested that the Na^+-dependent high affinity uptake system is specifically localized within cholinergic terminals [4, 11, 13–16].

ACh has been implicated as a central nervous system (CNS) neurotransmitter in horseshoe crabs (*Limulus polyphemus*) [17, 18]. Choline acetyltransferase activity has been demonstrated in the brain as well as the abdominal ganglia of *Limulus* [19, 20]. Additionally, this laboratory has reported preliminary studies on choline uptake and its conversion to ACh by *Limulus* abdominal ganglia [21]. The present study was undertaken to determine the kinetics and nature of choline uptake by *Limulus* abdominal ganglia.

MATERIALS AND METHODS

Tissue preparation. Limulus polyphemus were maintained in moist seaweed at an ambient temperature of 4–6°. Animals of either sex and averaging 10–18 cm across the carapace were used in all experiments. Individual abdominal ganglia were dissected out and placed in cold Chao's solution [22] containing 440 mM NaCl, 9 mM KCl and 37 mM $CaCl_2$ buffered at pH 7.2 (room temperature) with 10 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonate (HEPES). Each ganglion was split into halves, blotted, weighed and each half was placed in 0.2 to 0.5 ml of HEPES Chao's solution. A comparison of the kinetics of uptake in intact and split ganglia revealed no significant differences. One half of each ganglion was used for experiments performed at room temperature and the other half in experiments at 0–4°. In the extracellular space and homoexchange experiments, whole ganglia rather than split ganglia were used.

Uptake studies. Abdominal ganglia were incubated at room temperature in 210 μl of Chao's solution containing 0.1 to 100 μM [^{3}H]choline (0.5 to 550 × 10^{-9} Ci) for a prescribed period (10 min–2 hr). Parallel experiments were conducted at 0–4° and the results were subtracted from all values to correct for passive diffusion. After incubation each ganglion was washed in four 5-min changes of Chao's solution, blotted and placed in scintillation vials. The four 5-min changes were experimentally determined to reduce the efflux of radioactivity from the ganglia to a low baseline level (data not presented). The initial high level of efflux was presumed to reflect the release of loosely bound or trapped radioactivity. Tissue solubilizer (0.8 ml) was added to each vial, which was then capped and left overnight at 45–50°. Ten ml scintillant [4 g 2,5-diphenyloxazole (PPO), 0.05 g 1,4-bis-[2,(5-phenyloxazolyl)]benzene (POPOP) in 1 liter toluene] was added to each vial, and the radioactive content of the samples determined in a Beckman LS 3133P liquid scintillation system. Counting efficiency varied from 19 to 25 per cent. Units of uptake are expressed per mg of tissue.

Water compartment determinations. Individual ganglia were weighed to the nearest 0.1 mg, placed in small vials and desiccated under vacuum (24–48 hr) to constant weight. Total water content was subsequently calculated. The extracellular water space (ECS) was determined by 10 min incubations of the chain of abdominal ganglia in 500 μl of Chao's solution which contained 0.5 μCi each of [^{3}H]inulin (0.55 μM) and [^{14}C]mannitol (8.3 μM). Both mannitol and inulin were determined experimentally to equilibrate within 10 min. After incubation each chain was rinsed, blotted and the individual ganglia were dissected from the chain and weighed to the nearest 0.1 mg. Each ganglion was then digested and counted as previously described. An aliquot of each incubation medium was counted in

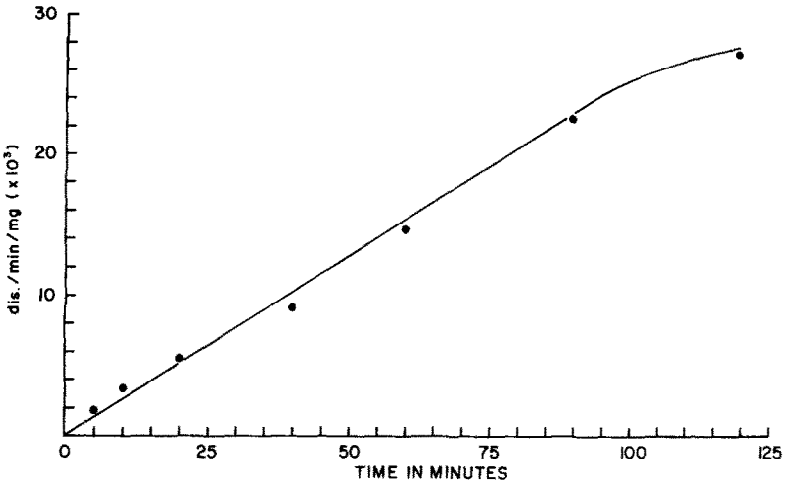

Fig. 1. Time course for the uptake of [^{3}H]choline by the abdominal ganglia of *Limulus*. The media contained 1 μM [^{3}H]choline. The uptake at 25° was corrected by subtracting the uptake at 0°. Values are the mean of at least four determinations.

10 ml Aquasol. Counting efficiency varied from 41 to 52 per cent.

The per cent ECS was calculated as follows:

$$\frac{\text{dis./min}_{\text{gang}}\,\mu\text{l}_{\text{med}}}{\text{dis./min}_{\text{med}}}\;\frac{1}{\mu\,1^{*}_{\text{gang}}}100 = \%\ \text{ECS}$$

Homoexchange studies. Individual ganglia were incubated for 30 min in 200 μl of Chao's solution containing 0.5 μM [^{3}H]choline (0.5 μCi). After incubation each ganglion was placed in a small chamber and perfused first for 10 min with Chao's solution and then for 10 min with either 1, 10 or 100 μM unlabeled choline in Chao's solution. The average perfusion rate was 250 μl/min and fractions were collected every 2 min. Aliquots of the fractions were counted in 10 ml Aquasol. Ganglia were digested and counted as previously outlined.

Sources of materials. Horseshoe crabs were obtained from the Gulf Specimen Co., Panacea, FL, and the Marine Biological Laboratories, Woods Hole, MA. Chemicals were obtained from the following sources: calcium chloride, choline chloride, decamethonium bromide, 2,4-dinitrophenol, PPO, POPOP, HEPES, ouabain, potassium chloride, and sodium chloride from Sigma Chemical Co., St. Louis, MO; [^{3}H]inulin (900 mCi/m-mole), [^{14}C]mannitol (60 mCi/m-mole), methyl[^{3}H]choline chloride (8.4 and 10.1 Ci/m-mole), and NCS tissue solubilizer from Amersham Corp.; Aquasol from New England Nuclear Corp.; hemicholinium-3 from Aldrich Chemical Co.; and sucrose from Fisher Scientific Co., Fairlawn, NJ.

RESULTS

Water compartment determinations. In order to distinguish between the extracellular and intracellular water compartments within the ganglia, both inulin and mannitol distribution spaces were measured. Individual ganglia were found to have a mean fresh weight of 10.1 mg ($\pm$ 3 mg, S.D.). After vacuum desiccation to constant weight, the total water space was calculated to be 85 per cent ($\pm$ 2 per cent S.D.). Of the total water volume, inulin distributed in 40.8 per cent ($\pm$ 11 per cent, S.D.) and mannitol in 44.9 per cent ($\pm$ 2 per cent, S.D.).

Uptake of [^{3}H]choline. When isolated abdominal ganglia of *Limulus* were incubated at room temperature in Chao's solution containing [^{3}H]choline, accumulation of the label was observed to be linear for 90 min and continued at a slower rate for up to 2 hr (Fig. 1). The mannitol space was used to correct for label in the extracellular space and, as a result, the tissue to medium (T/M) ratio represents the intracellular/extracellular distribution of the radioactivity. A T/M ratio of approximately 20: 1 was achieved at 90 min (data not shown). When parallel experiments were conducted at 0–4°, a marked reduction (81 per cent) in uptake of the label was observed, and a T/M ratio less than 1 was seen at 45 min. The uptake of [^{3}H]choline was subsequently studied at varying concentrations ranging from 1 to 100 μM. A Lineweaver–Burk plot [23] of the data revealed two distinct components: one with high affinity properties and the other with low affinity properties (Fig. 2). The K_t and V_{max} for the high affinity component were 14 μM and 0.19 pmole/mg/min respectively. The values for the same parameters of the low affinity component were 180 μM and 0.89 pmole/mg/min.

Effect of selected cations on uptake of [^{3}H]choline. To determine the effects of the cations in Chao's solution on both high and low affinity uptake, isolated ganglia were incubated in low (1 μM) and high (100 μM) choline concentrations with each cation in Chao's solution in turn replaced by equimolar sucrose (Table 1). Replacement of Na^+ reduced the high affinity uptake by 81 per cent and the low affinity uptake by 60 per cent. Replacement of either Ca^{2+} or K^+ by sucrose resulted in only nominal inhibition.

The role of Na^+ in the high affinity [^{3}H]choline uptake system was further investigated by varying the concentration of sodium in the incubating medium. When the Na^+ concentration was varied from 0 to 400 mM (by replacement with equimolar sucrose), increasing concentrations of Na^+ resulted in increasing

*One mg tissue is assumed to occupy a volume of 1 μl.

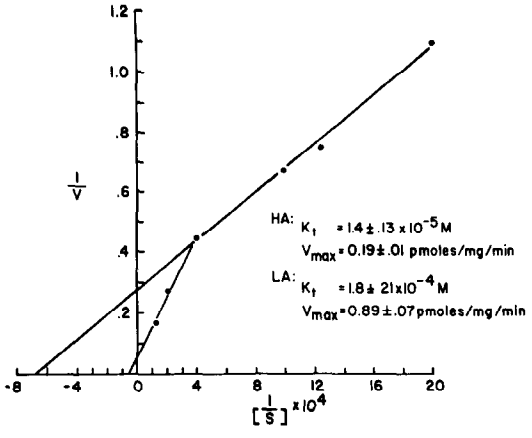

Fig. 2. Double reciprocal plot of choline uptake by the abdominal ganglia as a function of choline concentration. Each point represents the mean of at least five determinations. Lines were determined by regression analysis.

uptake up to 400 mM (Fig. 3). When the Na^+ concentration was increased more than 400 mM, a decrease in uptake was observed. Such concentrations of Na^+ resulted in hypertonic incubation media. Consequently, the possible role of hypertonicity in the decline in uptake was investigated. Hypertonicity produced by adding equimolar sucrose to Chao's solution resulted in a pattern of inhibition similar to that seen with Na^+, suggesting that the decline in uptake was due to hypertonicity rather than to an ionspecific effect.

Effect of inhibitors on uptake of [^{3}H]*choline.* The effects of a variety of substances known to inhibit the uptake of choline were examined (Table 2). Ouabain and 2,4-dinitrophenol had minimal effects on both high and low affinity uptake. The maximum inhibition obtained with ouabain was 44 per cent and with 2,4-dinitrophenol was 35 per cent. Decamethonium at low concentration (10 μM) had a marginal inhibitory effect, but at high concentration (500 μM) produced significant inhibition of both uptake systems. Hemicholi-

Table 1. Influence of ions on [^{3}H]choline uptake by the abdominal ganglia of *Limulus* *

Incubation condition	Choline uptake (as % of control at choline concn indicated)	
	1 μM	100 μM
Chao's solution (control)	100	100
Sodium replaced by sucrose (440 μM)	18.78$^+$ ± 3.0	40.33 ± 10.2$^+$
Potassium replaced by sucrose (9 mM)	76.61 ± 18.8	89.72 ± 11.6
Calcium replaced by sucrose (37 mM)	60.92 ± 14.7	71.83 ± 9.0

* Values are the mean ± S.D. for four determinations. Tissues were preincubated in the altered medium for 15 min before addition of [^{3}H]choline (1 or 100 μM) and subsequent incubation for 10 min. Values in parentheses are the ion concentrations in Chao's medium. Control uptake values were 0.2 ± 0.04 pmole/mg.

$^+$ Statistically significant compared to Chao's medium controls: ($P < 0.01$, Student's t-test).

Table 2. Effects of inhibitors on the uptake of [^{3}H]choline by the abdominal ganglia of *Limulus* *

Substance	Inhibitor concn (μM)	Choline uptake (as % of control)	
		Choline concn	
		(1 μM)	(100 μM)
Chao's solution (control)		100	100
Ouabain	1000	55.9 ± 9.8$^+$	63.6 ± 8.5
	100	74.3 ± 12.9	69.9 ± 11.8
	10	86.6 ± 5.7	77.8 ± 8.8
2,4-Dinitrophenol	1000	65.1 ± 7.4	69.3 ± 10.0
	100	82.8 ± 10.9	74.9 ± 9.0
	10	82.0 ± 7.3	90.2 ± 6.8
Decamethonium	500	35.3 ± 14.2$^+$	44.4 ± 7.7$^+$
	50	56.4 ± 9.72$^+$	56.2 ± 10.5
	10	81.0 ± 11.9	85.0 ± 10.4
Hemicholinium	500	7.4 ± 2.1$^+$	24.4 ± 9.5$^+$
	100	7.3 ± 1.8$^+$	31.7 ± 7.7
	10	25.3 ± 8.2	46.7 ± 7.4

* Values are the mean ± S.D. for four determinations, and conditions of experiment were as described in Table 1.

$^+$ Significantly different from controls: ($P < 0.01$, Student's t-test).

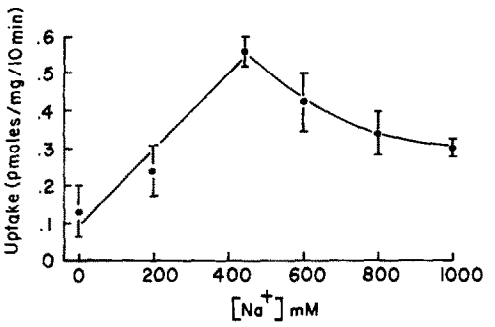

Fig. 3. Effect of sodium ion concentrations on high affinity choline uptake. Choline concentration in the media was 1 μM, and sodium chloride was varied from 0 to 1000 mM. Osmolarity was maintained constant by addition of sucrose, except for 600, 800 and 1000 mM sodium, where the media were hypertonic. Each point is the mean for five observations: vertical bars show $\pm$ S.D.

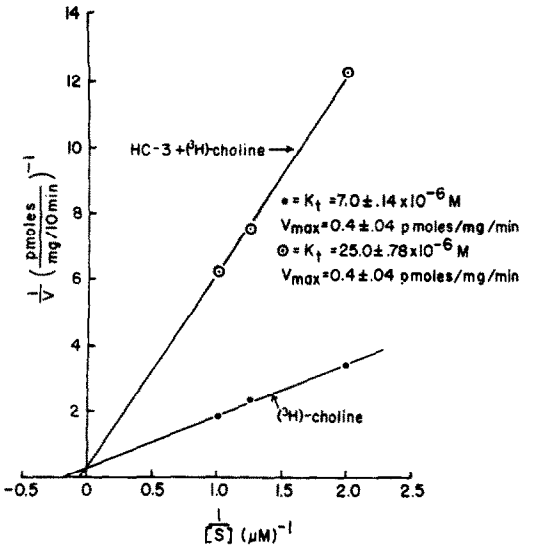

Fig. 4. Double reciprocal analysis of the high affinity [^{3}H]choline uptake in the presence of hemicholinium-3 (10 μM) at choline concentrations varying from 0.1 to 1 μM. The rate of choline accumulation (V) is expressed as pmoles/mg/10 min. Key: [^{3}H]choline alone (●); and [^{3}H]choline plus 10 μM hemicholinium-3 (○). Each point represents the mean for four experiments.

nium-3 (HC-3) was the most potent inhibitor of choline uptake.

To determine the nature of the inhibition by HC-3, the effect of varying concentrations of this compound on choline uptake was studied at low (1 μM) and high (100 μM) choline concentrations. The results, presented in Table 2, show that 10 μM HC-3 produced 75 per cent inhibition of high affinity choline uptake; 100 μM HC-3 produced maximal inhibition (93 per cent). Ten μM HC-3 produced 53 per cent inhibition when the choline concentration was increased to 100 μM. Maximum inhibition by HC-3 (500 μM) was 76 per cent. The effect of 10 μM HC-3 on uptake at varying choline concentrations (0.1 to 1.0 μM) was examined. A Lineweaver–Burk plot of the data is shown in Fig. 4 and suggests that HC-3 acts as a competitive inhibitor of choline uptake in *Limulus* abdominal ganglia.

Effect of homoexchange. It has been reported that low concentrations of GABA can trigger the release of GABA from the endogenous pool of rat synaptosomes [24]. In such cases, this homoexchange of neurotransmitter exhibited characteristics of high affinity uptake, including Na^+ dependence, concentration dependence and high tissue:medium ratios of radioactivity. The possibility that the entry of [^{3}H]choline into the ganglia resulted from homoexchange was investigated. When individual ganglia were incubated for 30 min in 0.5 μM [^{3}H]choline and subsequently washed with Chao's solution, a basal amount of radioactivity appeared in additional washes. Perfusion of washed labeled ganglia with varying concentrations of unlabeled choline (1–100 μM) for 10 min produced no concentration-dependent efflux of the radioactivity (Table 3). These results indicate that homoexchange did not occur at significant rates.

Table 3. Effects of different concentrations of unlabeled choline on [^{3}H]choline release from *Limulus* abdominal ganglia*

Fraction No.	Choline concn (μM)			
	0.0	1.0	10.0	100.0
4	1.16 $\pm$ 0.7	0.96 $\pm$ 0.5	1.41 $\pm$ 1.0	0.93 $\pm$ 0.6
5	1.00	1.00	1.00	1.00
6	0.84 $\pm$ 0.3	0.73 $\pm$ 0.3	0.49 $\pm$ 0.3	0.58 $\pm$ 0.3
7	1.00 $\pm$ 0.8	0.85 $\pm$ 0.4	0.61 $\pm$ 0.3	0.71 $\pm$ 0.5
8	0.64 $\pm$ 0.3	0.87 $\pm$ 0.6	0.74 $\pm$ 0.5	0.71 $\pm$ 0.4
9	0.48 $\pm$ 0.2	0.65 $\pm$ 0.4	0.57 $\pm$ 0.2	0.76 $\pm$ 0.6
10	0.45 $\pm$ 0.2	0.66 $\pm$ 0.4	0.54 $\pm$ 0.2	0.60 $\pm$ 0.3

* Abdominal ganglia were incubated for 30 min in 0.5 μM [^{3}H]choline. Fractions 1–5 (only 4 and 5 presented) were washes in Chao's while fractions 6–10 were washes with the indicated concentrations of unlabeled choline. Values are means $\pm$ S.D. of the release index [i.e. the radioactivity appearing in the experimental fraction compared to that in the reference fraction (#5) which immediately preceded the exposure period]. Reference fraction contained 1 per cent ($\pm$ 0.8 per cent) of total radioactivity in ganglia.

DISCUSSION

The results presented here demonstrate that the abdominal ganglia of *Limulus* avidly take up choline from the extracellular environment. The uptake process exhibits characteristics of a carrier-mediated transport system similar to those reported for nervous tissue from various mollusks, other invertebrates and several vertebrates [2, 3, 5, 6, 9, 25, 26]. At room temperature, [^{3}H]choline was rapidly accumulated from media containing low concentrations of choline. The uptake process has both a high affinity and low affinity component. The high affinity uptake is markedly Na^+ dependent (81 per cent inhibited in Na^+ free media) but relatively unaffected by the absence of Ca^{2+} or K^+. These observations are in good agreement with those for active choline uptake mechanisms described for *Aplysia* [2], the squid [3], the lobster [26] and the snail [6]. The high affinity uptake in *Limulus* is strongly inhibited (81 per cent) by cold temperatures and almost completely inhibited by 100 μM HC-3 (Table 2). The inhibition by HC-3 appears to be competitive in nature and is more pronounced on the high affinity component (Fig. 4). HC-3 is known to compete with choline for carrier transport sites [8, 27]. The kinetics of choline uptake in *Limulus* ganglia are not unlike carrier-mediated uptake processes for neurotransmitters or their precursors reported for other invertebrate and vertebrate systems.

There is increasing evidence that Cl^- may play a significant role in high affinity transport systems. It has been reported that high affinity uptake of choline by rat synaptosomes was reduced when incubated in Cl^- free media [7]. More recently, similar results were reported for approximately a dozen neurotransmitters, neurotransmitter precursors and amino acids [28]. These results suggest caution in the interpretation of high affinity uptake data in which sucrose was used to replace NaCl. In the present study whole ganglia were incubated in media containing reduced Cl^-. The significance of partial withdrawal of Cl^- in such cases is unclear. Thus the involvement of Cl^- in high affinity uptake of choline in *Limulus* abdominal ganglia merits further study.

The high affinity uptake process presented here exhibited a K_t of 14 μM and V_{max} of 0.19 pmole/mg/min. The K_t is in agreement with that reported for *Aplysia* [2] but somewhat higher than that reported for the snail [6]. The V_{max} is comparable to that reported for both the snail and *Aplysia*. The K_t and V_{max} for the low affinity uptake system in *Limulus* are 180 μM and 0.89 pmole/mg/min respectively. This K_t is similar to that reported for the snail [6] but significantly lower than that reported for *Aplysia* [2] and the leech [29]. The rate of choline uptake is clearly concentration dependent and exhibits two linear regions over the concentration range investigated (0.1 to 100 μM). Studies conducted on vertebrate species indicate that high affinity uptake occurs in cholinergic neurons and is characteristic of these neurons [11, 13, 30]. In addition, choline taken up by high affinity systems is thought to be used primarily in ACh synthesis by cholinergic neurons. Whether this is also true in the abdominal ganglia of *Limulus* is not certain.

Ouabain, 2,4-dinitrophenol and decamethonium only partially inhibit high affinity uptake (Table 2) at inhibitor concentrations as high as 1 mM. A similar effect of ouabain has been observed in the uptake of 5-hydroxytryptamine (5-HT) by snail ganglia [31]. In that same study, significant inhibition (15–25 per cent) of 5-HT uptake at 0° was produced by ouabain. It was also shown that near maximum inhibition of the Na^+ pump and the Na^+–K^+ ATPase was produced by 0.1 μM ouabain. In a few separate experiments, 1–30 mM ouabain produced up to 49 per cent inhibition of choline uptake by *Limulus* ganglia incubated at 0–4°, indicating active choline uptake in that temperature range. In addition, ganglia incubated at 0–4° in 2 μM [^{3}H]choline for 2 hr showed accumulation of the label (T/M ratio = 2:1). While subtraction of uptake values obtained at 0–4° is an accepted method for correcting for passive diffusion, particularly in mammals, it should be noted that the active uptake of dopamine and 5-HT by snail ganglia [31] and choline by *Limulus* ganglia occurs at 0°.

It has been reported that low concentrations of transmitter substances can simulate high affinity uptake by a homoexchange mechanism. Levi and Raiteri [24] demonstrated a concentration- and Na^+-dependent homoexchange of labeled GABA and glycine in rat synaptosomes which exhibited characteristics of high affinity uptake. The basal efflux rate of labeled GABA was increased 2-fold when the synaptosomes were perfused with 1 μM unlabeled GABA, and 8-fold with 10 μM unlabeled GABA. In the present study, ganglia incubated with [^{3}H]choline and perfused with varying concentrations (1–100 μM) of unlabeled choline resulted in no detected increase in efflux of the label. Thus homoexchange does not appear to be operative in choline uptake in the abdominal ganglia of *Limulus*.

From the results presented here, it is concluded that both high and low affinity choline uptake systems are present in the abdominal ganglia of *L. polyphemus*. The high affinity system shows strong Na^+ dependence and is completely inhibited by low concentrations of HC-3 but only partially inhibited by metabolic inhibitors. The presence of such a choline transport system adds support to the contention that the abdominal ganglia of *Limulus* contain a population of cholinergic neurons.

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