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Choline: High-Affinity Uptake by Rat Brain Synaptosomes

Abstract. *Synaptosomes from rat brain accumulate choline by two kinetically distinct processes, a high-affinity uptake system [Michaelis constant* (K_m) $= 1 \times 10^{-6}$M], *and a low-affinity system* ($K_m = 9 \times 10^{-5}$M). *The high-affinity uptake system requires sodium, and is associated with considerable formation of acetylcholine. The low-affinity uptake system is less dependent on sodium, and does not appear to be associated with a marked degree of acetylcholine formation. The high-affinity choline uptake appears to represent selective choline accumulation by cholinergic neurons.*

As it lacks the capacity to synthesize choline, the nervous system depends on choline uptake for cholinergic function (*1*). Choline transport systems in the kidney (*2*), erythrocytes (*3*), brain slices (*4*), and brain synaptosomes (*5*) have a relatively low affinity for choline, and have little associated acetylcholine formation, so that it is not clear whether the choline is accumulated selectively into cholinergic neurons. Recent reports that brain synaptosomes form a significant amount of acetylcholine when low concentrations of choline are present suggest that there may be more than one uptake system for choline (*6*).

We have demonstrated two kinetically distinct transport systems for choline into synaptosomes in the rat brain. A high-affinity uptake system, highly dependent upon the presence of sodium, is associated with considerable acetylcholine formation, while the low-affinity choline uptake system is much less dependent on sodium, and results in little acetylcholine formation (*7*).

The corpus striatum of male Sprague-Dawley rats (body weight, 150 to 200 g) was dissected (*8*), and homogenized in 30 volumes of ice-cold 0.32*M* sucrose containing $10^{-7}M$ Soman (pinacolyl-methylphosphorofluoridate) in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. At the concentration employed, Soman completely inhibited acetylcholinesterase without influencing choline uptake. The homogenate mixture was centrifuged at 1,000*g* for 10 minutes, the pellet discarded, and the supernatant fluid centrifuged at 17,000*g* for 60 minutes. The resultant pellet was reconstituted to the original volume with 0.32*M* sucrose, and 0.1 ml of the tissue suspension was added to 1.9 ml of a Krebs-phosphate solution, *p*H 7.4, containing 11.1 m*M* glucose and varying concentrations of [^{3}H]choline (17 curie/mmole; Amersham/Searle). Under these incubation conditions, even if all the endogenous choline had leaked out of the particles and choline levels increased during incubation (*9*), the resultant concentration of choline in the medium would be only $1 \times 10^{-7}M$, and hence contribute much less than the exogenous [^{3}H]choline to the final concentration of choline in the medium. The mixture was incubated at 30°C for 4 minutes, after which 50 μl each of choline (0.4*M*) and neostigmine (0.04*M*) were added, and the mixture was centrifuged at 27,000*g* for 15 minutes. The large amount of nonradioactive choline terminated the reaction by diluting the [^{3}H]choline. Neostigmine was added to inactivate any cholinesterase that may have regenerated. After the pellets were washed with ice-cold 0.9 percent NaCl containing 1.0 m*M* neostigmine, they were centrifuged at 48,000*g* for 10 minutes and the accumulated radioactivity was extracted into Triton X-100 : toluene scintillation fluid, and assayed by liquid scintillation spectrometry.

The relative amounts of labeled choline, acetylcholine, betaine, and phosphorylcholine were determined by high-voltage paper electrophoresis (*10*). The amount of radioactivity increased linearly with time for at least 4 minutes at all concentrations of [^{3}H]choline employed, and with varying concentrations of brain tissue. When pellets obtained from these incubations were layered on continuous sucrose density gradients (0.32 to 1.5*M*) and centrifuged for 90 minutes (*11*), the radioactivity was localized to an area of the gradient rich in synaptosomes, and separable from the bulk of activity of monoamine oxidase, a mitochondrial marker. Hypotonic shock completely liberated the radioactivity found in particulates. These observations indicate that radioactivity accumulated after incubation with [^{3}H]choline was localized predominantly in the synaptosomal fraction. The homogenates can be prepared rapidly, and therefore presumably undergo less deterioration than do purified synaptosomes. As essentially all radioactivity accumulated by homogenates is localized in the synaptosomes, all kinetic studies, unless otherwise indicated, were performed with homogenates. The kinetics of uptake were determined by double-reciprocal plots (*12*), and by least-squares fitting of a substrate velocity curve to the data by computer programs; this provided the Michaelis constants (K_m) and their standard errors (*13*) (Fig. 1).

Crude synaptosomal preparations of the corpus striatum were incubated with concentrations of [^{3}H]choline varying from $5 \times 10^{-7}M$ to $2 \times 10^{-2}M$. Double-reciprocal plots of the accumulated radioactivity always resulted in curves (Fig. 2) that could be resolved into two components graphically, as well as by computer analysis, with a calculated K_m of $1.2 \times 10^{-6}M$ and about $9.4 \times 10^{-5}M$, for the high- and low-affinity uptake systems, respectively. We found a similar dual affinity uptake system for [^{3}H]choline in synaptosomal preparations of the rat cerebral cortex. After separating the crude synaptosomal preparation into synaptosomal and mitochondrial fractions (*14*), we found the high-affinity choline transport system localized in the synaptosomal fraction.

Electrophoretic analysis of the radioactivity accumulated in pellets of homogenates showed that at a [^{3}H]choline concentration of $5 \times 10^{-7}M$, 70 percent of accumulated radioactivity was present as acetylcholine. This proportion decreased progressively to 61 percent, 47 percent, 23 percent, and 16 percent at [^{3}H]choline concentrations of $1 \times 10^{-6}M$, $1 \times 10^{-5}M$, $5 \times 10^{-5}M$, and $1 \times 10^{-4}M$, respectively. Negligible amounts of [^{3}H]phosphorylcholine were present until concentrations of [^{3}H]choline in the medium reached $5 \times 10^{-5}M$ and $1 \times 10^{-4}M$. At these concentrations, phosphorylcholine accounted for 8 percent and 10 percent, respectively, of the accumulated radioactivity. No [^{3}H]betaine was detected

until the concentration of [^{3}H]choline in the medium was $10^{-4}M$. At this concentration, [^{3}H]betaine accounted for 10 percent of the accumulated radioactivity.

It seems unlikely that the high-affinity choline uptake simply reflects saturation of choline acetyltransferase, because the K_m for choline acetyltransferase preparations (*15*) is considerably higher than the K_m for the high-affinity choline uptake. More probably, the high-affinity transport represents choline accumulation by subcellular components, presumably cholinergic synaptosomes, which synthesize acetylcholine; and the low-affinity system involves constituents that do not synthesize acetylcholine. Hence, with higher concentrations of [^{3}H]choline, more of it would enter the compartments not associated with acetylcholine formation.

The effects of ouabain and of varying ionic conditions on the two choline transport systems were examined by incubating preparations with low ($5 \times 10^{-7}M$) and high ($10^{-4}M$) concentrations of [^{3}H]choline (Table 1). Replacement of sodium by lithium or sucrose in the incubation medium reduced the uptake of radioactivity at low concentrations of choline by more than 95 percent, but lowered the uptake at the high concentration of choline by only 45 to 60 percent. Ouabain ($10^{-4}M$), an inhibitor of adenosine triphosphate (ATP) pyrophosphohydrolase (E.C. 3.6.1.8), reduced the uptake of low concentrations of choline by 45 percent, but lowered the uptake of high choline concentrations by only 13 percent. Thus, the high-affinity choline uptake appears to depend on sodium and on the ATP pyrophosphohydrolase system, while the low-affinity choline uptake is much less sensitive to these ionic manipulations.

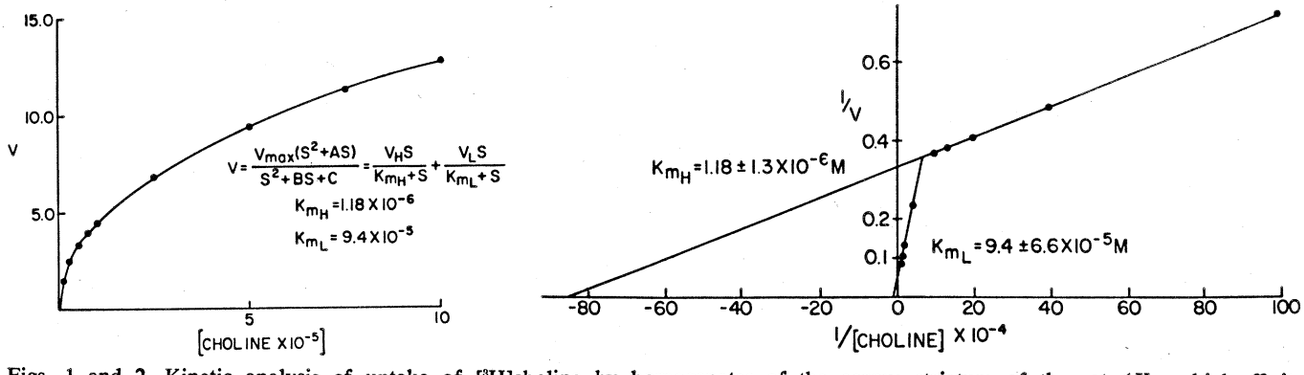
Table 1. Influence of sodium omission on choline uptake by striatal synaptosomes. Standard incubation medium contained Krebs-PO$_4$ buffer, Soman (0.1 μM), and either 0.5 μM or 100 μM [^{3}H]choline at 30°C. Results are the mean values of triplicate determinations which varied by less than 10 percent.

Incubation conditions	[^{3}H]Choline accumulation (percent of control) at	
	$5 \times 10^{-7}M$	$1 \times 10^{-4}M$
Sodium replaced by lithium*	2.9	39.1
Sodium replaced by sucrose†	5.3	55.8
Calcium replaced by sucrose†	88.5	95.8
Ouabain‡	54.4	87.3

* 140 mM. † 320 mM. ‡ 0.1 mM.

Does the high-affinity choline transport described here represent uptake into cholinergic nerve terminals? Our finding that the high-affinity choline uptake was associated with a marked degree of acetylcholine formation supports this possibility. Moreover, Kuhar *et al.* (*16*) found that lesions in the medial septal nucleus, and in the nucleus of the diagonal band, of the rat sharply reduce the uptake of low concentrations of [^{3}H]choline into synaptosomal preparations from the hippocampus. In contrast, the uptake of higher concentrations of [^{3}H]choline ($10^{-4}M$) is unaffected by such lesions. The medial septal nucleus and nucleus of the diagonal band contain a presumably cholinergic tract that projects to the hippocampus (*17*). The experiments of Kuhar *et al.* strongly suggest that the high-affinity choline transport, which mediates the accumulation of low concentrations of this compound, represents accumulation into cholinergic neurons, while the low-affinity uptake is mediated by some other tissue component. Recent experiments in our laboratory (*18*) have shown high- and low-affinity uptake systems for choline in the guinea pig ileum with almost identical characteristics to those described here for the uptake systems in brain synaptosomes. Thus, in the guinea pig ileum, the high-affinity choline uptake is associated with a marked degree of acetylcholine formation and is dependent on sodium, while the low-affinity uptake is much less dependent on sodium and is not associated with acetylcholine formation. Removal of the myenteric plexus from the guinea pig ileum results in a decrease of choline transport by the high-affinity choline uptake system, while the low-affinity system is unaffected. Studies with the denervated rat diaphragm also suggested that some choline uptake was localized in neuronal structures.

In summary, evidence indicates that the transport of choline by the high-affinity transport system into rat brain synaptosomes reflects accumulation by cholinergic nerve terminals. Because its K_m is about one-tenth that of choline acetylase, the high-affinity choline transport may be rate limiting in the regulation of acetylcholine formation in vivo, provided that the total capacity in vivo (V_{max}) for acetylating choline is not markedly lower than the capacity of the high-affinity choline transport.



Figs. 1 and 2. Kinetic analysis of uptake of [^{3}H]choline by homogenates of the corpus striatum of the rat (K_{m_H}, high-affinity system; K_{m_L}, low-affinity system). Fig. 1 (left). Computed least-squares fit of the velocity (v) of uptake of radioactivity expressed as nanomoles of [^{3}H]choline accumulated in 4 minutes per gram of brain tissue at different concentrations of [^{3}H]choline. The concentration of [^{3}H]choline is expressed in molar quantities. The plot was made on the assumption of a two-component transport system; analysis of the data on the basis of a one-component system gave a poor fit. Data are presented for concentrations ranging from $10^{-6}M$ to $10^{-4}M$; each point is the mean of four determinations. Symbols: A, constant; S, concentration of substrate. Fig. 2 (right). Double-reciprocal plot of velocity (v) of [^{3}H]choline uptake at different choline concentrations, using the same uptake data as in Fig. 1. Concentration of [^{3}H]choline and the velocity of uptake are as defined in the legend to Fig. 1.

Drugs which selectively inhibit this high-affinity choline uptake may be valuable tools in the study of cholinergic nerve transmission. Moreover, this high-affinity choline uptake system may furnish a heuristic approach to the labeling of cholinergic neurons in the brain and the peripheral nervous system.

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Adenosine 3′,5′-Monophosphate: Regional Differences in Chick Embryos at the Head Process Stage

Abstract. *The concentrations of adenosine 3′,5′-monophosphate and adenosine triphosphate and the activity of phosphodiesterase were determined in different regions of chick embryos at the head process stage. Adenosine 3′,5′-monophosphate, adenosine triphosphate, and phosphodiesterase were estimated to be higher in the mesoderm-forming portions of the hypoblast than in portions that form neural structures from Hensen's node or the epiblast.*

We have examined a few of the practical problems involved in the search for morphogenetic substances in embryonic development. By means of the recently proposed models for developing systems (*1*), such a search probably would require measuring relative differences in concentrations of compounds (morphogens) in the molecular weight range of 300 to 500. Concentration gradients of these morphogens would be set up within a time range of 3 to 6 hours over distances of 50 to 100 cells.

What differences in quantities of a presumed morphogen would constitute a functional gradient, and how can concentrations of morphogens be measured if turnover and metabolism of isotope compounds has taken place in only 3 to 6 hours? A variety of experiments could be designed for each type of presumed morphogen to answer the numerous technical questions involved.

Another approach to the problem of morphogen identification would be to study some of the effects of these substances. If adenosine 3′,5′-monophosphate (cyclic AMP) can be considered a second messenger for morphogenesis, as it is in numerous other biological messenger systems (*2*), then the finding of regional differences in cyclic AMP concentrations in chick embryos may indicate the possible action of a presumed morphogen. Thus, the investigation of regional differences in cyclic AMP and adenosine triphosphate (ATP) concentrations and phosphodiesterase activity provides a reasonable basis from which to begin the more laborious search for specific morphogens.

As a test system we have studied the conversion of [^{14}C]adenine into ATP and cyclic AMP in selected regions of explanted chick embryos. Since the tissues that develop from these regions have been determined (*3, 4*), differences in the concentration of cyclic AMP among these regions may indicate differences in morphogenetic action.

The embryos were removed from White Leghorn eggs (Truslow Farms) incubated 16 to 24 hours. The vitelline membranes to which the embryos remained attached were secured to a glass ring and suspended over a pool of albumen by means of the technique of New (*5*). Two groups of embryos were labeled by placing [8-^{14}C]adenine (0.5 μc in 0.1 ml of physiologic saline solution) on the hypoblast surface; these embryos were then incubated again for 2.5 to 4.5 hours, until they had reached the head process state (*6*), and were washed six times with 1 to 2 ml of saline at room temperature (*4*). Explanted embryos in a third group were again incubated to the head process stage and used to determine phosphodiesterase activity.

Selected portions of each embryo (Fig. 1) were dissected free with a tungsten needle (*4*). These portions consisted of a few thousand cells; many were 4 to 5 cells thick and 50 to 100 cells wide. The fragments were pooled in test tubes cooled with liquid nitrogen to which a known amount of 10 percent trichloroacetic acid was added to precipitate the protein.

The following tissues were represented in the embryonic fragments:

1) *Endoderm.* Almost all of the endoderm in fragment A was destined for the ventral portion of the foregut, for example, liver diverticula and a variable portion of the yolk sac (*3, 4*). Fragments B to H also contained some endoderm.

2) *Lateral plate mesoderm.* The splanchnic mesodern in fragments A to C was destined for heart and lung, and the somatic mesoderm was destined for limb and body wall. Kidney-forming, liver, limb, and area vasculosa mesoderm were found primarily in fragments D and E (*3, 4*).

3) *Paraxial mesoderm.* Fragment F eventually would form head mesoderm, anterior somites, and nephrotome. Fragment G would form more posterior portions of paraxial mesoderm, that is, the posterior somites (*4*).

4) *Hensen's node.* Fragment H consisted of cells that would form paraxial mesoderm, notochord, dorsal gut endoderm, and ventral portions of the neural tube (*4*).