MOLECULAR PHARMACOLOGY, 14, 868–878

Ionic and Nucleotide Cofactor Requirements for Uptake of [^{3}H]-Norepinephrine by Rat Brain Synaptic Vesicle Preparations

THEODORE A. SLOTKIN[1], FREDERIC J. SEIDLER, WILLIAM L. WHITMORE, MARIA SALVAGGIO, AND CHRISTOPHER LAU

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

(Received May 12, 1978)
(Accepted May 15, 1978)

SUMMARY

SLOTKIN, THEODORE A., SEIDLER, FREDERIC J., WHITMORE, WILLIAM L., SALVAGGIO, MARIA & LAU, CHRISTOPHER (1978) Ionic and nucleotide cofactor requirements for uptake of [^{3}H]norepinephrine by rat brain synaptic vesicle preparations. *Mol. Pharmacol.*, **14**, 868–878

Norepinephrine uptake into a crude preparation of rat brain synaptic vesicles showed a marked dependence on Mg^{2+} concentration. Mn^{2+} or Co^{2+} could substitute for Mg^{2+}, but displayed lower affinities. Zn^{2+}, Ni^{2+} and Ca^{2+} stimulated uptake only slightly and other divalent cations were ineffective. ATP, GTP and UTP produced stimulation of norepinephrine uptake, but only ATP was fully effective. ADP and AMP inhibited the ATP-induced stimulation. The irreversible inhibitor of ATPases, N-ethylmaleimide (NEM), blocked norepinephrine uptake; the effect was enhanced by pre-incubation of the vesicle preparation with NEM prior to addition of the cofactors and the enhancement was partially prevented by addition of ATP-Mg^{2+} during the pre-incubation. Replacement of K^+ by Na^+ in the medium did not alter norepinephrine uptake, but Li^+ inhibited uptake by competing with Mg^{2+}. The use of hypertonic medium inhibited uptake, while hypotonic medium markedly enhanced only the nonspecific uptake component (not ATP or Mg^{2+}-dependent, not reserpine-sensitive). The similarities of these data to those obtained with adrenomedullary or peripheral nerve vesicles validate the historical use of the peripheral preparations as models of central nervous system vesicles, and the simple and sensitive technique utilized in this study affords direct detailed evaluation of the effects of *in vivo* drug administration on neurotransmitter storage in the brains of small animals.

INTRODUCTION

Synaptic storage vesicles play a critical role in the function of neurons. They are responsible for maintenance of transmitter stores, protection of biogenic amines from destruction by intraneuronal monoamine oxidase, and for exocytotic release of neurotransmitter into the synaptic cleft upon stimulation of the neuron (1–4). In the noradrenergic system, vesicles also participate in transmitter synthesis, as the conversion of dopamine to norepinephrine occurs within the vesicle (5). A wide variety of antihypertensive and psychoactive drugs elicit their pharmacologic effects through direct or indirect actions on storage vesicles, storage vesicle amine uptake systems or on vesicular release of neurotransmitters. These include reserpine, tetrabenazine, adrenergic false transmitters and Li^+ (6–10). While the neuropharmacology of these

Supported by USPHS HD-09713.

[1] Recipient of Research Scientist Development Award DA-00006 from the National Institute on Drug Abuse.

0026-895X/78/0145-0868$02.00/0

agents has been studied extensively, the data pertaining to actions at the vesicular level have been arrived at predominantly by indirect means, e.g., patterns of formation of transmitter metabolites, subcellular fractionation of neural tissues, or degree of reserpine sensitivity of uptake into perfused whole organs, brain slices or synaptosomes (2, 11, 12). Direct evaluation in the central nervous system of pharmacologic effects on vesicular amine uptake or on vesicle properties has proven difficult for a number of reasons: yields of intact isolated vesicles are generally low, purification of vesicles from the rest of the microsomal fraction is difficult, and interpreting the effects on specific vesicle populations is confounded by the heterogeneity of the transmitters present. For these reasons, nearly all the studies on isolated vesicles have utilized peripheral nervous tissue (adrenal medulla, splenic nerve) obtained from large species (cow, pig, sheep) as the source of transmitter vesicles, and it has been assumed that vesicles from the central nervous system behave similarly (2, 3, 13).

More recently, studies by Philippu and coworkers (13–17) have detailed properties of crude synaptic vesicle preparations obtained from pig brain. Their methods used large amounts of tissue and were not suitable for studies in small animals, but with slight modifications (18, 19), evaluation of vesicular uptake is possible in as little as 130 mg of rat brain. The development of this technique has enabled detailed studies on the properties of rat brain vesicles and of pharmacologic effects of administration of reserpine (18–22). In general, the properties seem to resemble those of peripheral synaptic vesicles: total dependence on ATP and Mg^{2+}, inhibition *in vitro* and *in vivo* by reserpine, and saturation kinetics with an affinity constant for norepinephrine or dopamine of about $1–4 \times 10^6$ liters/mole (18, 19). Vesicles isolated from rat brain also display the same lack of substrate specificity seen in rat or cow adrenomedullary vesicles and in pig brain vesicles (15, 17–19, 23–27); while uptakes of norepinephrine and dopamine occur primarily in catecholaminergic vesicles, the vesicle uptake systems fail to distinguish between the two amines and also can take up indoleamines (18, 19, 23). Indeed, despite the heterogeneity of the endogenous transmitters and the probable presence of vesicles from terminals, axons and cell bodies, rat brain vesicular uptake *in vitro* proceeds as though there were only a single population of organelles (18, 19, 23). The current study was undertaken to examine in detail the nucleotide and ionic requirements for uptake of [^{3}H]norepinephrine by rat brain vesicles to enable comparison with results obtained in peripheral neuronal or adrenomedullary vesicles. These experiments also constitute in part a test of the historical use of the peripheral systems as models for storage vesicles of the central nervous system.

METHODS

Preparation of synaptic vesicles. Subcellular fractions containing brain synaptic vesicles were prepared by the method of Philippu and Beyer (15) as modified by Seidler *et al.* (18). Male Sprague-Dawley rats (Zivic-Miller), weighing 200–300 g were decapitated and brains removed, pooled and homogenized in 4 volumes of 300 mM sucrose containing 25 mM Tris (pH 7.4) and 10 μM iproniazid (irreversible monoamine oxidase inhibitor), using 5 up-down strokes in a Duall ground-glass homogenizer. The homogenate was centrifuged at $1000 \times g$ for 15 min and the supernatant recentrifuged at $20,000 \times g$ for 30 min. The supernatant of the latter centrifugation was sedimented at $100,000 \times g$ for 30 min in a Beckman No. 40 rotor and the supernatant solution was discarded.[2] The crude, vesicle-containing pellet was resuspended gently in a volume of 130 mM potassium phosphate (pH 7.4) equal to that of the original ho-

[2] This sedimentation step is critical, as use of the $20,000 \times g$ supernatant as the source of vesicles interferes with the uptake measurement: uptake with the normal incubation procedure ($100,000 \times g$ pellet suspension in phosphate buffer) gave an uptake of 6.46 ± 0.10 pmols/g; uptake with the same volume of $20,000 \times g$ supernatant was only 3.99 ± 0.14 pmols/g. This decrement was not due to the presence of sucrose-Tris in the uptake incubation, as resuspension of the $100,000 \times g$ pellet in sucrose-Tris did not result in a reduced uptake. Four determinations were done with each preparation.

mogenate using 2 up-down strokes in a teflon-to-glass homogenizer, and this suspension was used for subsequent incubations. Although this microsomal fraction contains many particles and organelles, uptake of amines *in vitro* appears to occur primarily into the synaptic vesicles present in the preparation (18, 19, 23).

Determinations of amine uptake. For determinations of vesicular uptake, standard incubations contained 0.67 ml of the vesicle preparation (corresponding to 133 mg of original brain tissue), 0.83 ml of 2 mM ATP-Mg^{2+} in phosphate buffer, 17 μl of 1 mM ascorbic acid, 8.3 μl of 1 mM iproniazid, 0.38 μl of 220 μM [^{3}H]norepinephrine and phosphate buffer to make a final incubation volume of 1.7 ml. In some experiments, concentrations of ATP, Mg^{2+}, or [^{3}H]norepinephrine were varied, or other nucleotides, ions or drugs added to the incubation medium. Samples were incubated for 4 min at 30° while duplicate tubes were kept on ice to serve as blanks. Uptake was stopped by the addition of 1.7 ml of ice-cold phosphate buffer and the labeled vesicles trapped on cellulose acetate filter paper (Millipore type EG, pore size 0.2 μm, or equivalent Gelman paper) by rapid vacuum filtration. The paper was washed three times with ice-cold buffer and counted at an efficiency of 40%. Uptake was determined by subtracting the 0° blank from the 30° sample and expressed as pmols of norepinephrine taken up per gram of original tissue. Blanks at 1 mM ATP-Mg^{2+} and 0.05 μM [^{3}H]norepinephrine generally contained 200–400 CPM while 30° values were approximately 2000–3000 CPM. Binding of label to the filter paper in the absence of tissue averaged less than 200 CPM.

Results are reported as means and standard errors with levels of significance calculated by the two-tailed Student's *t*-test.

Materials. L-[7-^{3}H]norpinephrine (2.20 Ci/mmol) was obtained from New England Nuclear Corp. EDTA, N-ethylmaleimide, iproniazid phosphate, ATP, ADP, AMP, cyclic AMP, CTP, UTP, GTP and cyclic GMP were purchased from Sigma Chemical Co. Reserpine phosphate was obtained from Ciba Pharmaceuticals and cocaine hydrochloride from Merck, Sharp & Dohme.

RESULTS

In the presence of ATP, addition of Mg^{2+} to the incubation medium produced a concentration-dependent stimulation of [^{3}H]-norepinephrine (NE) uptake (Fig. 1). Maximal uptake was achieved at 1 mM Mg^{2+}, and the apparent affinity constant for Mg^{2+} was 1.3×10^4 liters/mole. Maximal stimulation also could be achieved with Co^{2+} or Mn^{2+}, although the affinity constants were lower than for Mg^{2+} (8.9×10^3 liters/mole for Co^{2+}; 5.6×10^3 liters/mole for Mn^{2+}). Zn^{2+}, Ni^{2+} and Ca^{2+} also caused slight stimulation of NE uptake, but none of them was as effective as Mg^{2+} and all three displayed much lower affinity constants. Ba^{2+}, Cr^{2+} and Sr^{2+} were totally ineffective. Some precipitate formation was observed with the highest concentrations of Zn^{2+} and Cr^{2+}.

The ability of divalent cations (1 mM) to block the uptake stimulation caused by Mg^{2+} (0.1 mM) also was assessed (Table 1). Significant inhibition was obtained only with Zn^{2+} and Ni^{2+}, and there was also slight inhibition by Ca^{2+} which was not

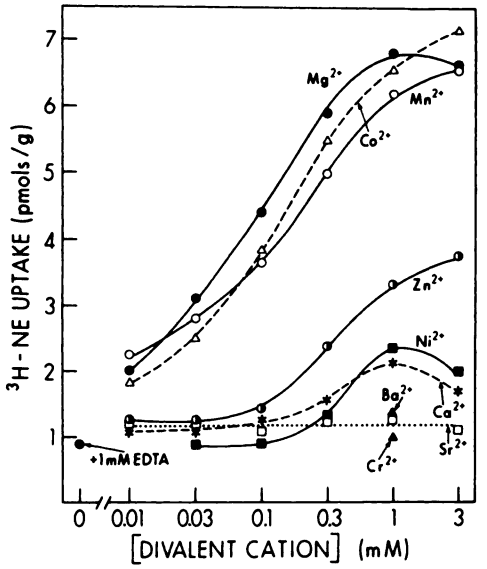

FIG. 1. *Stimulation of rat brain synaptic vesicle uptake of [^{3}H]norepinephrine (NE) by divalent cations*

The incubation medium contained 1 mM ATP and 0.05 μM [^{3}H]NE. Each point is the mean of 5–12 determinations; standard errors were less than 10% of the mean values. Abscissa is logarithmic.

TABLE 1

Inhibition of Mg^{2+}-dependent brain synaptic vesicle uptake of [^{3}H]norepinephrine by other divalent cations

Each value is the mean and standard error of the number of determinations in parentheses. The incubation medium contained 1 mM ATP and 0.05 μM [^{3}H]NE.

Cation added	[^{3}H]NE uptake
	pmols/g
Mg^{2+} (0.1 mM)	4.21 ± 0.20 (9)
Mg^{2+} + Ca^{2+} (1 mM)	3.94 ± 0.09 (3)
Mg^{2+} + Sr^{2+} (1 mM)	4.46 ± 0.14 (3)
Mg^{2+} + Ba^{2+} (1 mM)	4.20 ± 0.28 (3)
Mg^{2+} + Cr^{2+} (1 mM)	4.09 ± 0.19 (4)
Mg^{2+} + Zn^{2+} (1 mM)	3.22 ± 0.16 (4)[a]
Mg^{2+} + Ni^{2+} (1 mM)	3.12 ± 0.09 (4)[b]

[a] $p < 0.005$ vs. Mg^{2+} alone.
[b] $p < 0.001$.

statistically significant at this particular Mg^{2+} concentration. The kinetics of inhibition, assessed using 0.3 mM Ni^{2+} or 1 mM Ca^{2+}, appeared to be noncompetitive, with a reduction in the maximal stimulation by Mg^{2+} without alterations in the affinity for Mg^{2+} (Fig. 2). The apparent failure of 1 mM Ca^{2+} to reduce uptake at low Mg^{2+} concentrations probably results from the stimulatory effect of the 1 mM Ca^{2+} itself.

The actions of monovalent cations on brain synaptic vesicle uptake of NE is shown in Fig. 3. Replacement of K^+ in the medium by Na^+ did not result in a reduction in uptake; however, replacement by Li^+ markedly inhibited uptake, with an IC_{50} of about 10 mM Li^+. To ascertain the mechanism whereby Li^+ inhibited NE uptake, the type of inhibition was examined against Mg^{2+}, ATP and NE concentrations (Fig. 4). Inhibition by Li^+ of the Mg^{2+}-dependent component appeared to be competitive, as the inhibition could be overcome at high Mg^{2+} concentrations; the calculated affinity constant for Li^+ was 1×10^3 liters/mole. In contrast, noncompetitive inhibition was observed between Li^+ and ATP or Li^+ and NE.

In the presence of Mg^{2+}, stimulation of NE uptake by nucleotides appeared to be quite specific for ATP (Table 2). GTP was less effective, UTP even less so, and ADP, AMP, cAMP, cGMP and CTP were totally ineffective. In this experiment, the affinity constant for ATP was 1.3×10^4 liters/mole, and maximal uptake stimulation could be achieved at 1 mM ATP (Fig. 5). There was some variability in affinity for ATP from preparation to preparation with affinity constants sometimes as low as 3000 to 7000 liters/mole (Figs. 4, 6, 7). GTP was less potent and could not elicit maximal stimulation of NE uptake (Fig. 5).

Of the nucleotides which did not stimulate NE uptake, only ADP and AMP were capable of inhibiting the stimulation caused by ATP (Table 3). Addition of UTP produced a slight enhancement ($p < 0.05$) of the stimulation caused by 0.1 mM ATP. ADP reduced both the maximal effect and affinity for ATP, but the inhibition caused by AMP could be overcome by high ATP concentrations (Fig. 6).

Addition to the incubation medium of the sulfhydryl-reactive compound, N-ethylmaleimide (NEM) produced slight reductions in NE uptake which appeared to be noncompetitive against Mg^{2+} or ATP (Fig. 7). The inhibition of uptake could be en-

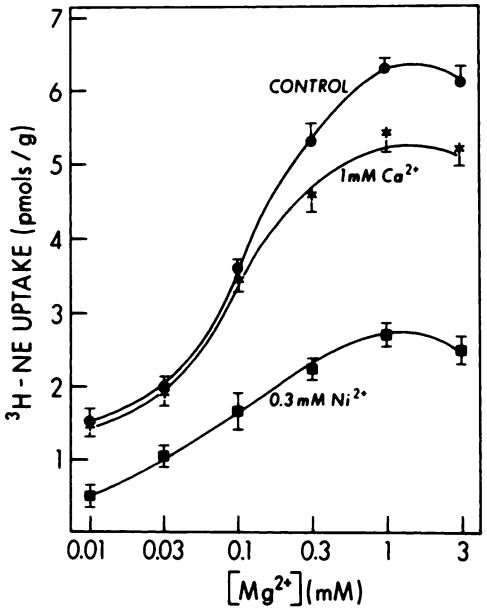

FIG. 2. *Inhibition of Mg^{2+}-dependent rat brain synaptic vesicle uptake of [^{3}H]norepinephrine (NE) by Ni^{2+} or Ca^{2+}*

The incubation medium contained 1 mM ATP and 0.05 μM [^{3}H]NE. Points and bars represent means and standard errors of 3–6 determinations. Abscissa is logarithmic.

hanced markedly by pre-incubating the vesicles with NEM prior to adding the ATP, Mg^{2+} and NE; the effects were non-competitive, with major reductions in maximal NE uptake without alterations in the apparent affinities for Mg^{2+}, ATP or NE. The inhibition of uptake by pre-incubation with NEM could be antagonized by inclusion of 1 mM ATP-Mg^{2+} in the pre-incubation mixture (Table 4); the protective effect was even more noticeable at 3 mM ATP-Mg^{2+}.

Replacement of the isotonic potassium phosphate medium with hypertonic or hypotonic media during resuspension of the 100,000 × *g* vesicle pellet, incubation and washing procedures produced marked alterations in NE uptake (Table 5). Uptake was inhibited in hypertonic potassium phosphate and enhanced in hypotonic potassium phosphate. Hypotonic medium

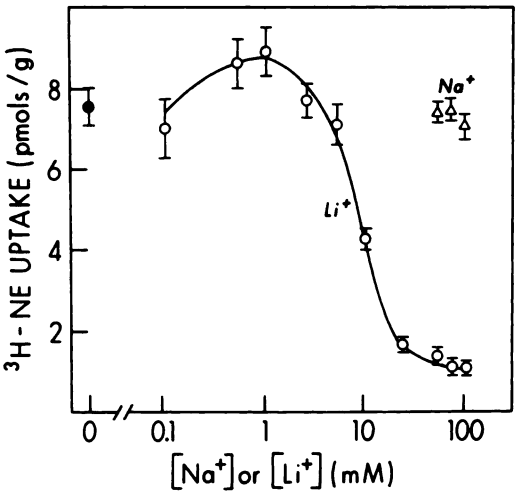

FIG. 3. *Inhibition of rat brain synaptic vesicle uptake of [^{3}H]norepinephrine (NE) by partial replacement of K^+ in the incubation medium with Na^+ or Li^+*

The incubation medium contained 1 mM ATP, 1 mM Mg^{2+} and 0.05 μM [^{3}H]NE. Points and bars represent means and standard errors of 6–24 determinations. Abscissa is logarithmic.

TABLE 2

Stimulation of rat brain synaptic vesicle uptake of [^{3}H]norepinephrine by nucleotides

Each value is the mean and standard error of the number of determinations in parentheses. The incubation medium contained 1 mM Mg^{2+} and 0.05 μM [^{3}H]NE.

Nucleotide added	[^{3}H]NE uptake
1 mM	*pmols/g*
None	1.28 ± 0.19 (4)
ATP	5.93 ± 0.25 (5)[a]
ADP	1.16 ± 0.04 (3)
AMP	1.06 ± 0.06 (3)
cAMP	1.01 ± 0.06 (3)
GTP	3.96 ± 0.08 (3)[a]
cGMP	1.70 ± 0.19 (3)
CTP	1.32 ± 0.05 (3)
UTP	1.84 ± 0.06 (3)[b]

[a] $p < 0.001$ vs. no nucleotide.
[b] $p < 0.05$.

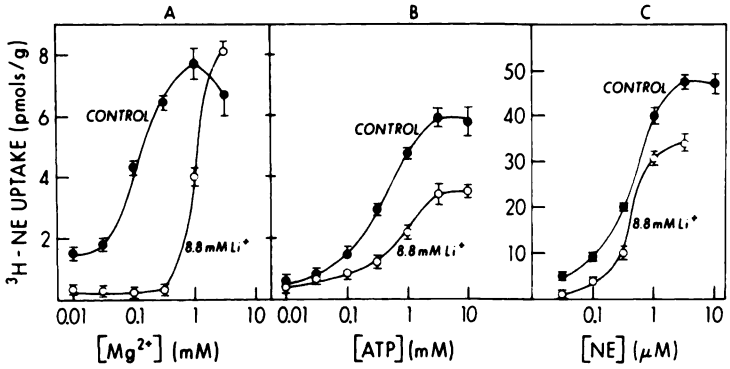

FIG. 4. *Inhibition of rat brain synaptic vesicle uptake of [^{3}H]norepinephrine (NE) by partial replacement of K^+ in the medium with Li^+*

In A, the incubation medium contained 1 mM ATP and 0.05 μM [^{3}H]NE; in B, 1 mM Mg^{2+} and 0.05 μM [^{3}H]NE; in C, 1 mM ATP and 1 mM Mg^{2+}. Blanks in C were run at 30° without ATP and Mg^{2+} to correct for non-specific uptake present at high NE concentrations (which can represent as much as 50–60% of total uptake at the highest concentrations). Points and bars represent means and standard errors of 4–12 determinations. Abscissa is logarithmic.

also increased the 0° blank, but there was still a marked effect on net uptake. These alterations were not irreversible in that adjusting the media back to isotonic conditions prior to beginning the incubation resulted in normal uptake. Freezing and thawing the isotonic preparation did not alter NE uptake (Table 5).

To determine whether the enhanced NE uptake seen in hypotonic medium actually

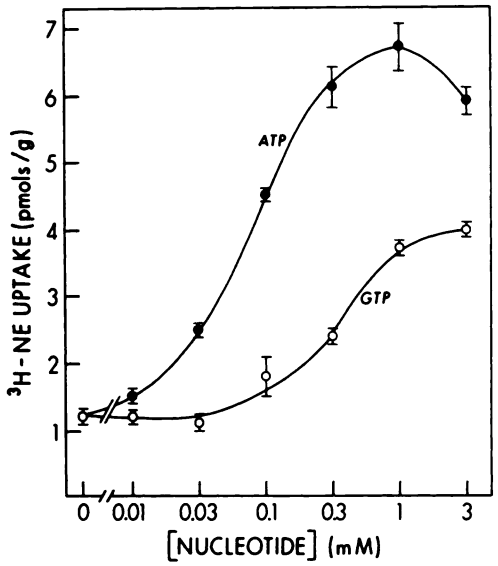

FIG. 5. *Stimulation of rat brain synaptic vesicle uptake of [^{3}H]norepinephrine (NE) by ATP and GTP*

The incubation medium contained 1 mM Mg^{2+} and 0.05 μM [^{3}H]NE. Points and bars represent means and standard errors of 4 determinations. Abscissa is logarithmic.

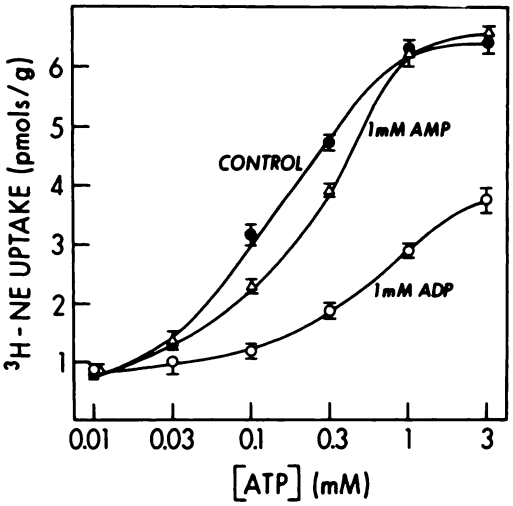

FIG. 6. *Inhibition of ATP-dependent rat brain synaptic vesicle uptake of [^{3}H]norpinephrine (NE) by ADP and AMP*

Points and bars represent means and standard errors of three determinations. Abscissa is logarithmic.

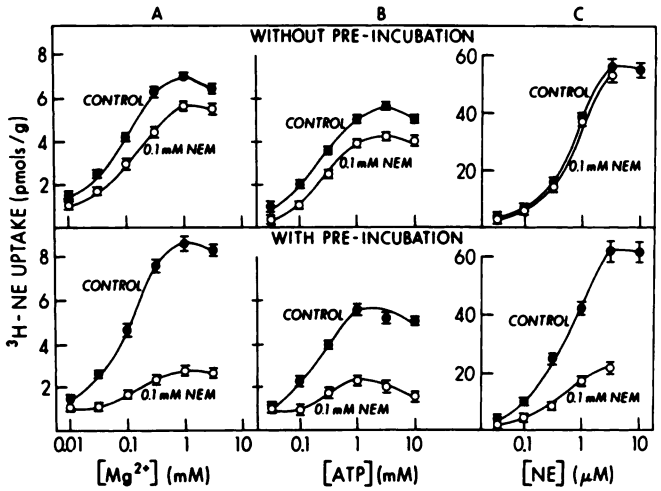

FIG. 7. *Inhibition of rat brain synaptic vesicle uptake of [^{3}H]norepinephrine (NE) by 0.1 mM N-ethylmaleimide (NEM)*

In A, the incubation medium contained 1 mM ATP and 0.05 μM [^{3}H]NE; in B, 1 mM Mg^{2+} and 0.05 μM [^{3}H]NE; and in C, 1 mM ATP and 1 mM Mg^{2+}. Blanks in C were run at 30° without ATP and Mg^{2+} to correct for nonspecific uptake present at high NE concentrations. Samples without pre-incubation had NEM added at the same time as ATP, Mg^{2+} and NE. Samples with pre-incubation were exposed to NEM (or buffer for controls) for 3 min at 30° prior to adding ATP, Mg^{2+} and NE. Points and bars represent means and standard errors of 4–20 determinations. Abscissa is logarithmic.

reflected ATP-Mg^{2+}-dependent vesicular transport, studies were conducted in which the ATP and Mg^{2+} requirements and drug sensitivities of uptake were evaluated in the hypotonic and isotonic preparations (Table 6). Omission of ATP from the medium reduced uptake in both isotonic and hypotonic preparations; however, the difference in uptake between the hypotonic and isotonic preparation was still present. Similarly, replacement of Mg^{2+} with the chelating agent, EDTA, reduced uptake but failed to eliminate the uptake difference between hypotonic and isotonic preparations. While addition of reserpine caused 95% inhibition of NE uptake in the isotonic preparation, inhibition was only 70% in the hypotonic preparation, again maintaining the uptake difference between the two osmotic conditions. Cocaine was totally ineffective in altering NE uptake into either preparation.

TABLE 3

Inhibition of ATP-dependent rat brain synaptic vesicle uptake of [^{3}H]norepinephrine by other nucleotides

Each value is the mean and standard error of the number of determinations in parentheses. The incubation medium contained 1 mM Mg^{2+} and 0.05 μM [^{3}H]NE.

Nucleotide added	[^{3}H]NE uptake
	pmols/g
ATP (0.1 mM)	3.84 ± 0.19 (4)
ATP + ADP (1 mM)	2.09 ± 0.07 (4)[a]
ATP + AMP (1 mM)	2.78 ± 0.14 (4)[b]
ATP + cAMP (1 mM)	3.36 ± 0.09 (4)
ATP + cGMP (1 mM)	4.46 ± 0.19 (4)
ATP + CTP (1 mM)	3.73 ± 0.05 (4)
ATP + UTP (1 mM)	4.45 ± 0.10 (4)[c]

[a] $p < 0.001$ vs. ATP alone.
[b] $p < 0.005$.
[c] $p < 0.05$.

DISCUSSION

In general, the cofactor requirements and specificities of rat brain synaptic vesicle norepinephrine uptake match those of the peripheral model systems nearly identically (1–5, 13). Mg^{2+} could be effectively replaced by Mn^{2+} or Co^{2+} but not by Ca^{2+}, Zn^{2+} or other divalent cations. The small degree of stimulation seen with Zn^{2+}, Ni^{2+} or Ca^{2+} may not even represent effects on the Mg^{2+} cation site, as the interaction between Ni^{2+} or Ca^{2+} and Mg^{2+} was noncompetitive. The affinity for Mg^{2+} in the rat brain vesicle preparation was similar to that seen in isolated bovine adrenomedullary vesicles, and in both systems maximal stimulation of uptake can be seen at 1 mM (1). The requirement for Mg^{2+} and the absence of a requirement for Na^+ also clearly distinguishes the vesicular uptake system from synaptic membrane uptake (11), an important distinction in the crude preparation used here. Further evidence that vesicular uptake is being measured is provided by the inhibition seen with reserpine (a specific inhibitor

TABLE 4

ATP-Mg^{2+} antagonism of inhibition of rat brain synaptic vesicle uptake of [^{3}H]norepinephrine caused by pre-incubation with N-ethylmaleimide (NEM)

Each value is the mean and standard error of the number of determinations in parentheses. Samples were pre-incubated for 3 minutes at 30° prior to adding material in uptake incubation.

Additions to medium		
Pre-incubation	Uptake incubation	[^{3}H]NE uptake
		pmols/g
(1) 1 mM ATP-Mg^{2+}	[^{3}H]NE	6.80 ± 0.25 (5)
(2) 0.1 mM NEM	1 mM ATP-Mg^{2+}; [^{3}H]NE	2.71 ± 0.16[a] (8)
(3) 1 mM ATP-Mg^{2+} + 0.1 mM NEM	[^{3}H]NE	4.54 ± 0.12[a, b] (5)
(4) 3 mM ATP-Mg^{2+}	[^{3}H]NE	7.10 ± 0.12 (6)
(5) 0.1 mM NEM	3 mM ATP-Mg^{2+}; [^{3}H]NE	3.68 ± 0.35[c] (10)
(6) 3 mM ATP-Mg^{2+} + 0.1 mM NEM	[^{3}H]NE	5.88 ± 0.29[d, e] (16)

[a] $p < 0.001$ vs. (1).
[b] $p < 0.001$ vs. (2).
[c] $p < 0.001$ vs. (4).
[d] $p < 0.001$ vs. (5).
[e] $p < 0.005$ vs. (4).

TABLE 5

Effects of osmolarity and freezing on rat brain synaptic vesicle uptake of [^{3}H]norepinephrine

Each value is the mean and standard error of the number of determinations in parentheses. Vesicles in the 100,000 × *g* pellet were resuspended and/or incubated and washed in 130 mM (isotonic), 300 mM (hypertonic) or 10 mM (hypotonic) potassium phosphate buffer. All incubations contained 1 mM ATP, 1 mM Mg^{2+} and 0.05 μM [^{3}H]NE.

Vesicle suspension	Incubation and wash condition	30° Value	0° Value	Net uptake (30° − 0°)
		pmols/g		
Isotonic	Isotonic	7.88 ± 0.36 (14)	1.83 ± 0.18 (6)	6.02 ± 0.36
Hypertonic	Hypertonic	3.90 ± 0.05 (4)[a]	1.63 ± 0.22 (2)	2.27 ± 0.05[a]
Hypertonic	Isotonic	8.49 ± 0.77 (4)	2.10 ± 0.18 (2)	6.39 ± 0.77
Hypotonic	Hypotonic	12.20 ± 0.70 (17)[a]	3.15 ± 0.55 (7)[b]	9.03 ± 0.70[c]
Hypotonic	Isotonic	7.24 ± 0.13 (8)	1.50 ± 0.06 (4)	5.74 ± 0.13
Isotonic, frozen and thawed twice	Isotonic	7.87 ± 0.46 (9)	2.22 ± 0.43 (5)	5.65 ± 0.46

[a] $p < 0.001$ vs. isotonic-isotonic.
[b] $p < 0.05$.
[c] $p < 0.005$.

TABLE 6

Effects of drugs, ATP and Mg^{2+} on rat brain synaptic vesicle uptake of [^{3}H]norepinephrine in isotonic and hypotonic incubation media

Each value is the mean and standard error of the number of determinations in parentheses. Vesicles in the 100,000 × *g* pellet were resuspended, incubated and washed in 130 mM (isotonic) or 10 mM (hypotonic) potassium phosphate buffer, containing additions as shown. All incubations contained 0.05 μM [^{3}H]NE.

Additions					[^{3}H]NE uptake	
ATP (1 mM)	Mg^{2+} (1 mM)	EDTA (1 mM)	Reserpine (50 nM)	Cocaine (10 μM)	Isotonic	Hypotonic
					pmols/g	
+	+	−	−	−	6.02 ± 0.36 (14)	9.03 ± 0.70 (17)[a]
−	+	−	−	−	0.49 ± 0.13 (6)	2.33 ± 0.20 (6)[b]
+	−	+	−	−	0.59 ± 0.28 (6)	1.90 ± 0.39 (6)[c]
+	+	−	+	−	0.40 ± 0.05 (6)	2.77 ± 0.18 (6)[b]
+	+	−	−	+	6.16 ± 0.36 (6)	8.85 ± 0.46 (6)[b]

[a] $p < 0.005$ vs. isotonic.
[b] $p < 0.001$.
[c] $p < 0.05$.

of vesicular uptake) but lack of effect of cocaine (a specific inhibitor of synaptic uptake).

The substitution of Na^+ for K^+ in the incubation medium did not affect norepinephrine uptake (again, consistent with vesicular and not synaptic uptake mechanisms). However, Li^+ was able to inhibit uptake substantially. The utility of Li^+ in management of manic-depressive psychosis has led to a plethora of hypotheses and data concerning effects on pre-synaptic catecholaminergic function (see reviews, 9, 10, 28). In the rat brain synaptic vesicles, Li^+ inhibited uptake by competing with Mg^{2+}, an effect which is consistent with the hypothesis that interference with storage mechanisms by Li^+ *in vivo* results in lessened effectiveness of central noradrenergic neurotransmission. It is difficult, however, to assess whether the *in vitro* effect is of significance *in vivo*. Mg^{2+} levels in whole brain or in cerebrospinal fluid range from 1 to 6 mM (29). While the effective plasma Li^+ level is about 1 mM, levels in cerebrospinal fluid are somewhat lower (30, 31), and in view of the ten-fold difference in vesicle affinity for Mg^{2+} vs. Li^+, an effect might not be expected to occur *in vivo*. However, the intracellular

level of Li^+ may be higher than that in the plasma (9, 32), and it is difficult to establish the intraneuronal concentration of Mg^{2+} due to the sizable contribution of non-neuronal (glial) cells in the brain. Thus, an effect of Li^+ on the Mg^{2+}-dependent component of vesicular amine uptake remains as a possible explanation of the known effects of Li^+ *in vivo* on brain catecholamine storage.

The nucleotide requirement for vesicular ^{3}H-NE uptake appeared to be fairly specific for ATP, although GTP was partially effective; other nucleotides were only slightly effective or totally ineffective. Again, these results are identical to those seen in adrenomedullary vesicles, and the affinities and maximally effective concentrations are comparable in the two preparations (1, 5). The partial effect of GTP could reflect either an intrinsically lower activity of this nucleotide, or could implicate involvement of another rate-limiting process, such as transphosphorylation of ADP by GTP, as an intermediate step in stimulation by GTP (5). The binding site for ATP clearly involves the purine nucleus, as ADP and AMP inhibit the stimulation of uptake caused by ATP. However, more than one effect may be present, since ADP reduced both the maximal effect and affinity for ATP. In addition to direct interaction of ADP with the ATP site, the presence of high concentrations of ADP may inhibit the uptake directly or may use up the pool of ATP available for uptake by diversion into transphosphorylation reactions involving other components of the crude preparation. The specific nature of the ADP effect cannot be established at this time due to the large number of ATPases and ATP-utilizing processes present in the microsomal fraction.

The studies involving the irreversible ATPase inhibitor, NEM, demonstrate that ATP is indeed being utilized as an energy source for vesicular uptake. Reaction with sulfhydryl groups appears to be retarded by the presence of ATP, effects which are specific to transport ATPases and which do not occur with nonspecific or mitochondrial ATPases (33–35). In the rat brain synaptic vesicle preparation, noncompetitive uptake inhibition by NEM was markedly enhanced by pre-incubation in the absence of ATP-Mg^{2+}, and the enhancement was antagonized by addition of ATP-Mg^{2+} during the pre-incubation, thus duplicating results obtained in peripheral catecholamine vesicles from large species (33–35). These data strongly implicate a similarity in the mechanisms of ATP utilization in the two types of vesicles.

A major difference between adrenomedullary vesicles and vesicles derived from peripheral sympathetic neurons lies in their susceptibilities to osmotic shock or freezing; while adrenal vesicles lyse in hypotonic medium, neuronal vesicles are fairly stable (36, 37). Rat brain synaptic vesicles appeared to retain their integrity (as assessed by uptake capability) after either freezing and thawing or after exposure to hypotonic medium, thus showing the same resistance to lysis which is characteristic of the peripheral nerve vesicles. Hypotonic medium did, however, produce an alteration in the uptake characteristic in that both 0° (blank) and 30° values were enhanced compared to the isotonic preparation. While the net temperature-dependent uptake also increased in hypotonic medium, the increase appeared not to be related to the specific vesicular uptake mechanism, as the uptake increment was not eliminated by reserpine or by omission of ATP or Mg^{2+} from the medium. This alteration could be of considerable importance in view of alternative methods of vesicle preparation and/or purification by hypoosmotic lysis of the synaptosomal fraction (review, 38). Since the additional nonspecific component in hypotonic medium represents between 30 and 50% of the value of the specific component, misleading results as to degree of cofactor requirement, drug inhibition or uptake capacity could be obtained if rat brain synaptic vesicles are incubated in hypotonic solutions and it is therefore essential that conditions be adjusted back to isotonicity prior to commencing uptake incubations.

The use of hypertonic medium strongly inhibited vesicular uptake of norepinephrine. Care must therefore be exercised in common isolation/purification techniques which utilize hypertonic sucrose density

gradients (38–40), in that isotonic conditions must be restored prior to measuring uptake. This problem could be avoided by the use of the isotonic sucrose-Ficoll-D_2O gradients which have been devised for isolation of adrenomedullary or peripheral nerve vesicles (38, 41, 42); however, these procedures do not readily lend themselves to measurements of numerous samples from individual brains (as would be needed for studies of effects of *in vivo* drug administration). While the crude preparation utilized here is ideal for rapid determination of vesicular uptake in numerous small tissue samples, it is essential to demonstrate that the uptake is indeed occurring into the synaptic vesicles present in the preparation. The cofactor studies reported here and previous work in this system (18–23) provide strong evidence that this is the case.

In conclusion, these studies with rat brain synaptic vesicles vindicate the historical use of adrenomedullary or peripheral nerve vesicles as models of central neuronal vesicles. Despite the use of the simple isolation procedure described here, the uptake cofactor characteristics of the crude preparation are consistent only with the vesicular uptake system. This can be of great utility in extending the capability of vesicle techniques to the central nervous systems of small animals, as is required for extensive studies of *in vivo* drug effects.

REFERENCES

1. Carlsson, A., Hillarp, N-Å & Waldeck, B. (1963) *Acta Physiol. Scand.* **59**, Suppl 215, 1–38.
2. Euler, U. S. v. (1972) *Handb. Exp. Pharmacol.*, **33**, 186–230.
3. Stjärne, L. (1972) *Handb. Exp. Pharmacol.*, **33**, 231–269.
4. Kirschner, N. (1974) *Life Sci.*, **14**, 1153–1167.
5. Kirschner, N. (1962) *J. Biol. Chem.*, **237**, 2311–2317.
6. Carlsson, A. (1965) *Handb. Exp. Pharmacol.*, **19**, 529–592.
7. Slotkin, T. A. (1974) in *Neuropoisons: Their Pathophysiological Actions*, vol. 2 (Simpson, L. L. & Curtis, D. R., eds.), pp. 1–60, Plenum Press, New York.
8. Muscholl, E. (1972) *Handb. Exp. Pharmacol.*, **33**, 618–660.
9. Davis, J. M. & Fann, W. E. (1971) *Ann. Rev. Pharmacol.*, **11**, 285–302.
10. Fieve, R. R. (1978) *Rev. Neurosci.*, **3**, 131–156.
11. Paton, D. M. (1976) in *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines* (Paton, D. M. ed.), pp. 49–66, Raven Press, New York.
12. Ross, S. B. (1976) in *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines* (Paton, D. M., ed.), pp. 67–93, Raven Press, New York.
13. Philippu, A. (1976) in *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines* (Paton, D. M., ed.), pp. 215–246, Raven Press, New York.
14. Philippu, A., Becke, H. & Burger, A. (1969) *Eur. J. Pharmacol.*, **6**, 96–101.
15. Philippu, A. & Beyer, J. (1973) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **278**, 387–402.
16. Philippu, A., Matthaei, H. & Lentzen, H. (1975) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **287**, 181–190.
17. Philippu, A. & Matthaei, H. (1975) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **287**, 191–204.
18. Seidler, F. J., Kirksey, D. F., Lau, C., Whitmore, W. L. & Slotkin, T. A. (1977) *Life Sci.*, **21**, 1075–1086.
19. Slotkin, T. A., Salvaggio, M., Lau, C. & Kirksey, D. F. (1978) *Life Sci.*, **22**, 823–830.
20. Kirksey, D. F., Seidler, F. J. & Slotkin, T. A. (1978) *Brain Res.*, **156**, in press.
21. Slotkin, T. A., Seidler, F. J. & Whitmore, W. L. (1978) *Eur. J. Pharmacol.*, in press.
22. Seidler, F. J., Whitmore, W. L. & Slotkin, T. A. (1978) *J. Pharmacol. Exp. Ther.*, in press.
23. Slotkin, T. A., Seidler, F. J., Whitmore, W. L., Lau, C., Salvaggio, M. & Kirksey, D. F. (1978) *J. Neurochem.*, in press.
24. Slotkin, T. A., Ferris, R. M. & Kirshner, N. (1971) *Mol. Pharmacol.*, **7**, 308–316.
25. Slotkin, T. A. & Kirshner, N. (1971) *Mol. Pharmacol.*, **7**, 581–592.
26. Slotkin, T. A., Anderson, T. R., Seidler, F. J. & Lau, C. (1975) *Biochem. Pharmacol.*, **24**, 1413–1419.
27. Slotkin, T. A. (1975) *Biochem. Pharmacol.*, **24**, 89–97.
28. Barchas, J. D., Patrick, R. L., Raese, J. & Berger, P. A. (1977) in *Depression: Clinical, Biological and Psychological Perspectives* (Usdin, E., ed.), pp. 139–165, Brunner/Mazel, New York.
29. Altman, P. L. & Dittmer, D. S. (1961) *Blood and Other Body Fluids*, Federation of American Societies for Experimental Biology, Bethesda, Md.
30. Platman, S. R. & Fieve, R. R. (1968) *Arch. Gen. Psychiat.*, **18**, 659–663.
31. Platman, S. R., Rohrlich, J. & Fieve, R. R. (1968) *Dis. Nerv. Sys.*, **29**, 733–738.
32. Prien, R. F., Klett, C. J. & Caffey, E. M. (1972) *Arch. Gen. Psychiat.*, **29**, 420–425.

33. Kirshner, N. (1965) in *Pharmacology of Cholinergic and Adrenergic Transmission* (Koelle, G. B., Douglas, W. W. & Carlsson, A., eds.), pp. 225–233, Czechoslovak Medical Press, Prague.
34. Hasselbach, W. & Seraydarian, K. (1966) *Biochem. Z.*, **345**, 159–172.
35. Taugner, G. & Hasselbach, W. (1966) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **255**, 266–286.
36. Thureson-Klein, Å, Klein, R. L. & Chen-Yen, S. H. (1975) *J. Neurocytol.*, **4**, 609–627.
37. Lagercrantz, H. and Thureson-Klein, Å. (1975) *Histochem.*, **43**, 173–183.
38. Morgan, I. G. (1976) *Neuroscience*, **1**, 159–165.
39. Lagercrantz, H., Pertoft, H. & Stjärne, L. (1970) *Acta Physiol. Scand.*, **78**, 561–566.
40. Viveros, O. H., Arqueros, L. & Kirshner, N. (1971) *Mol. Pharmacol.*, **7**, 434–443.
41. Yen, S. S., Klein, R. L. & Chen-Yen, S. H. (1973) *J. Neurocytol.*, **2**, 1–12.
42. Trifaró, J. M. & Dworkind, J. (1970) *Analyt. Biochem.*, **34**, 403–412.