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QUANTITATIVE STUDIES OF NOREPINEPHRINE UPTAKE BY SYNAPTOSOMES

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Abstract—Nerve-ending particles (synaptosomes) isolated from rat brain were studied as a model system for synaptic function with particular reference to the ionic conditions required for uptake of norepinephrine. For optimal uptake, Na^+ and K^+ were required at levels of 140 mM and 2–3 mM respectively, which are essentially physiological levels for these cations. The inhibition of norepinephrine uptake by reserpine and by higher concentrations of K^+ was linear with the logarithm of the inhibitor concentration. The V_{max} and K_m for norepinephrine uptake were 0·10 μg/min/g and $0{\cdot}56 \times 10^{-6}$ M respectively.

THE PREPARATION of pinched-off nerve endings (synaptosomes)[1–3] provides a convenient system *in vitro* for studies of the effects of ions on neurotransmitter uptake, since endogenous extracellular ions can be uniformly replaced by exogenous ions. In brains taken from animals receiving intraventricular injections of labeled norepinephrine (NE), the labeled NE is found concentrated predominantly in the synaptosomes.[4] There have been relatively few studies, however, of the uptake of NE by synaptosomes *in vitro*.[5] Recently, we have observed uptake of norepinephrine by synaptosomes in Krebs' bicarbonate buffer which was linear for 50 minutes.*[6] The amine was concentrated well above 200 times the concentration in the incubation media, and this accumulation was 20 times greater than a control sample held at 4°.* This is similar to norepinephrine uptake by tissue slices in the same buffer[7] and is 3–4 times greater than NE uptake by synaptosomes in isotonic sucrose media. In contrast to these results with *synaptosomes*, preparations of isolated synaptic *vesicles* and nerve granules take up NE best in isotonic sucrose, that is, the addition of even dilute solutions of ions markedly reduce the amine uptake.*

MATERIALS AND METHODS

The synaptosomes for the present experiments were isolated from whole rat brain (adult male, Sprague–Dawley, 170–220 g) by the method of Whittaker *et al.*[2] The animals were decapitated and the brains were quickly removed, chilled on ice, weighed, and then ground in 9 vol. of 0·32 M sucrose in a special homogenizer described by Whittaker *et al.*[2] The homogenate was centrifuged at 900 *g* for 15 min to remove the nuclei as a pellet, and the supernatant was recentrifuged at 17,000 *g* for 30 min,

* Data presented at the fall meeting of the American Society for Pharmacology and Experimental Therapeutics held in Washington, D.C. (28 August, 1967).

leaving the microsomes in the supernatant and the myelin, synaptosomes and mitochondria in the pellet. This pellet fraction was then resuspended in 8 ml of 0·32 M sucrose, layered on a discontinuous sucrose gradient with 8-ml layers of 1·2 M and 0·8 M sucrose, and centrifuged at 50,000 *g* for 90 min. The fractions recovered were myelin in the top layer, synaptosomes in the middle layer, and mitochondria in the bottom layer. (Electron microscopy indicated good homogeneity of the fractions.) The synaptosomal fraction was then recovered with a special tube-slicing device (Beckman Instruments). This fraction was not centrifuged to remove the sucrose but was divided directly into 8 equal aliquots of 0·8 ml, to which were added solutions of various salts to a final volume of 6·0 ml. One μc (19·2 mμg) of tritiated NE (DL-norepinephrine-7-H^3 acetate, New England Nuclear Corp.) was then added without carrier to the buffered samples. The incubations were carried out directly in the ultracentrifuge tubes in a constant temperature water bath (without shaking) for 10 min at 37°. The samples were centrifuged (15 min at 100,000 *g*), washed (Krebs' bicarbonate buffer), recentrifuged (15 min at 100,000 *g*) and suspended in 250 μl of 0·4 N perchloric acid. It is worthy of note that we found that after addition of the salt buffers and centrifugation the synaptosomes were found to form a compact hard pellet, which was unlike the softly packed friable pellet found after centrifugation directly after the sucrose gradient. After final centrifugation (10 min at 50,000 *g*) 200 μl of the supernatant were transferred to a counting solution for the radioactive assay (10 ml toluene, 4 ml ethanol containing 0·4% 2,5-diphenyloxazole and 0·01% 1,4-*bis*-2 (phenyloxazolyl) benzene).

Perchloric acid extracts from the optimal uptake system were chromatographed on paper in a butanol:acetic acid:water system (25:40:10), and it was found that 90 per cent of the radioactivity migrated with an authentic norepinephrine carrier. Quenching for each sample was followed with an external gamma emitter in a model 4000 Packard scintillation spectrometer. Perchloric acid extracts of our tissue samples had about 20 per cent quenching, which was essentially the same as that with perchloric acid alone. The range of quenching between individual samples was 3–4 per cent. The data presented here were not corrected for this 20 per cent quenching, except in the calculation of the kinetic constants. The average error between duplicate samples was generally about 5 per cent.

RESULTS

The net uptake of NE into synaptosomes was substantially (3–4 times) greater when the synaptosomes were incubated in Krebs' buffer rather than in sucrose media. In order to investigate which of the salts were most important in the increased net uptake of NE observed in Kreb's buffer, each salt was omitted individually from the buffer. The omitted salt may be seen along the diagonal line in the upper portion of Table 1. The omission of sodium bicarbonate reduces uptake to 2 per cent. This probably represents a pH effect, since uptake is restored by addition of phosphate buffer, as shown in the first line of the lower portion of the table. It is shown in the lower portion of Table 1 that both sodium and potassium are clearly required for optimal net uptake of NE. While with other ions there were several relatively minor effects, which will be commented on later, the sodium and potassium effects were of major importance and for this reason the amounts of sodium and potassium in the phosphate buffer were varied systematically.

TABLE 1. UPTAKE OF NOREPINEPHRINE BY SYNAPTOSOMES IN VARIOUS SALT MEDIA

Electrolyte medium								Per cent uptake†
NaCl 118*	$NaHCO_3$ 25	KCl 4·7	KH_2PO_4 1·2	$CaCl_2$ H_2O 2·54	$MgSO_4$ $7H_2O$ 1·18	Dextrose 11	NaH_2PO_4 10mM pH 7·0	
+	+	+	+	+	+	+	−	100
+	−	+	+	+	+	+	−	2
+	+	−	+	+	+	+	−	119
+	+	+	−	+	+	+	−	109
+	+	+	+	−	+	+	−	130
+	+	+	+	+	−	+	−	108
+	−	+	−	−	+	+	+	92
+	−	+	−	−	+	−	+	73
+	+	+	−	−	−	−	+	68
+	−	−	−	−	+	−	+	18
−	−	+	−	−	+	−	+	11

* mM/l. in each case.
† Samples in duplicate.

As shown in Figs. 1a and 1b the optimal levels for sodium and potassium are about 140 mM and 2–3 mM respectively, i.e. essentially physiological levels of these ions. Increasing the Na^+ concentration results in an increased NE uptake at all K^+

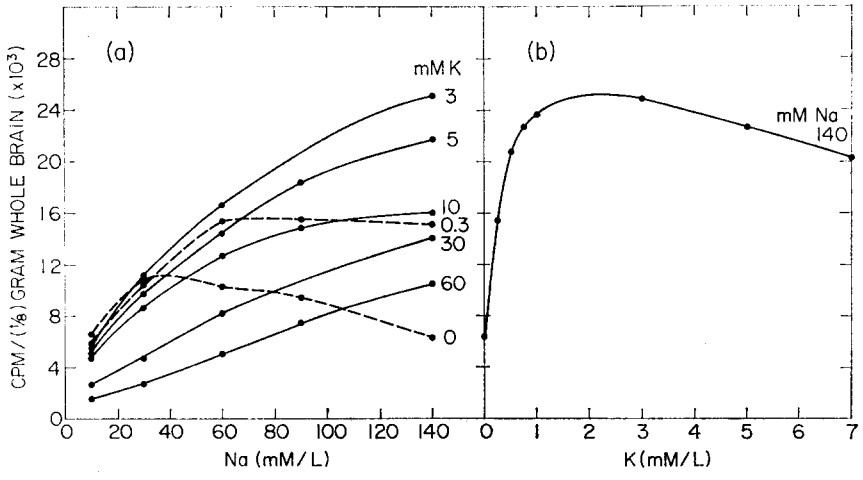

FIG. 1. Norepinephrine uptake by synaptosomes in a variety of salt media. The buffer consisted of 10 mM sodium phosphate at pH 7·0 and 11 mM glucose. In some cases osmolality was greater than isotonic, but sucrose added to the levels of highest osmolality (400 mM) did not suppress uptake.

levels between 3·0 and 60 mM. The presence of K^+ is clearly necessary for NE uptake, but high K^+ inhibits the uptake (Fig. 1b). With optimal Na^+ levels, the uptake is highest at a K^+ concentration of about 2–3 mM, decreasing rapidly as K^+ drops under 0·5 mM and gradually as K^+ increases above 5 mM.

The inhibition of uptake by high K^+ concentration is shown in Fig. 2. The concentration range of K^+ that reduced NE uptake from maximal to minimal levels is essentially the same as the concentration that is known to reduce membrane potential

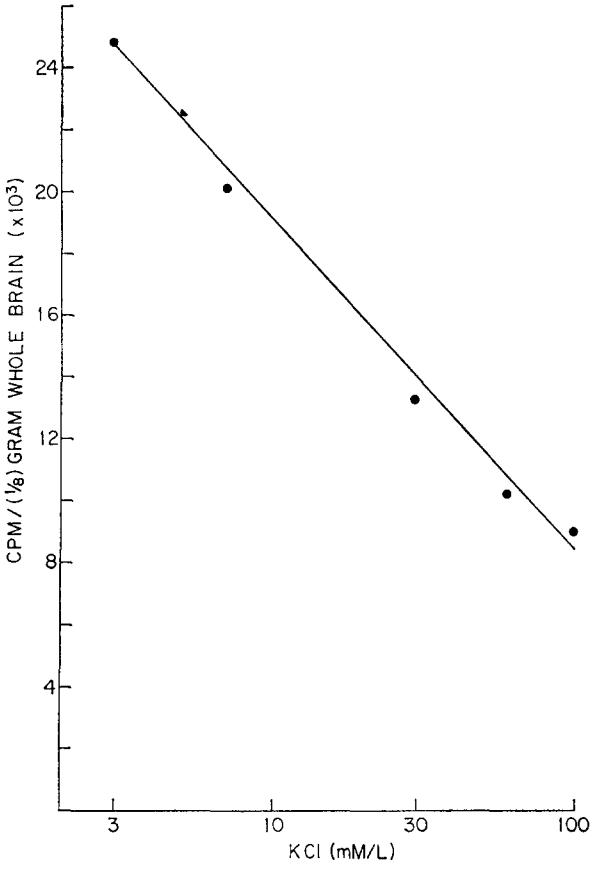

FIG. 2. Potassium inhibition of norepinephrine uptake by synaptosomes. Buffer consisted of 10 mM sodium phosphate at pH 7·0 and 11 mM glucose. Synaptosomes treated with the 60 mM K^+ buffer, when washed and resuspended in the Na^+ 140–K^+ 3 buffer, regain the maximal uptake capacity for the system.

from maximal to minimal value.[8] The relationship between K^+ concentration and uptake is represented by a straight line which is consistent with the interpretation of a single site of action. Similar effects of high potassium on tissue slices have been reported.[9]

To determine the reversibility of the high K^+ inhibition, synaptosomes were incubated in 1 ml of the regular (3 mM K^+) buffer and the 60 mM K^+ buffer without addition of isotope. They were then diluted to 10 cc with suitable buffer so that the electrolyte conditions during incubation were equal. Isotope was then added and the samples were incubated for the standard 10-min period. Under these conditions the

pretreatment with 60 mM K^+ caused a slight (14 $\pm$ 2 per cent) decrease in the net uptake of NE into the synaptosomes. Hence, the inhibitory effects of the 60 mM K^+ conditions could be largely, but not completely, reversed by dilution with low K^+ buffer. Thus the 60 per cent reduction by 60 mM K^+ could not be explained by irreversible damage to the synaptosomes by the 60 mM K^+, since uptake can recover to 86 per cent after the 60 mM K^+ treatment.

It was observed in Table 1 that the absence of calcium increased net NE uptake slightly and the omission of dextrose decreased net NE uptake moderately. In three additional animals, synaptosomes were washed in dextrose-free buffer and, after centrifugation, resuspended in dextrose-free buffer or in buffer with 1·18 mM dextrose, 36 mM sucrose, or both. The addition of dextrose increased net uptake (48 $\pm$ 2 per cent) above that seen in the dextrose-free buffer. Sucrose had a small effect (4 $\pm$ 2 per cent), perhaps due to small amounts of glucose formed by hydrolysis of sucrose. Three additional animals tested in duplicate with the omission of calcium from the Krebs' buffer resulted in an increase in net uptake by 31 $\pm$ 3 per cent.

In studies *in vivo*, reserpine has been found to release most of the intracellular NE.[10] Therefore, the effect of various concentrations of reserpine on the NE uptake was studied under the optimal salt conditions described above. As shown in Fig. 3, the inhibition of uptake by reserpine is clearly evident at all reserpine concentrations greater than 3×10^{-9}, and the relationship between the logarithm of the inhibitor concentration and NE uptake is linear between 3×10^{-9} and 1×10^{-7}. Moreover, a moderate uptake still occurs even in the presence of relatively high reserpine concentrations (Fig. 3, 10^{-7} to 10^{-5}) which is either consistent with the so-called "reserpine resistent uptake",[11] or with equilibration with the water spaces of the synaptosome.

Synaptosomes concentrated NE from the incubation media against a concentration ratio of approximately 200:1. The concentration inside the synaptosomes at the

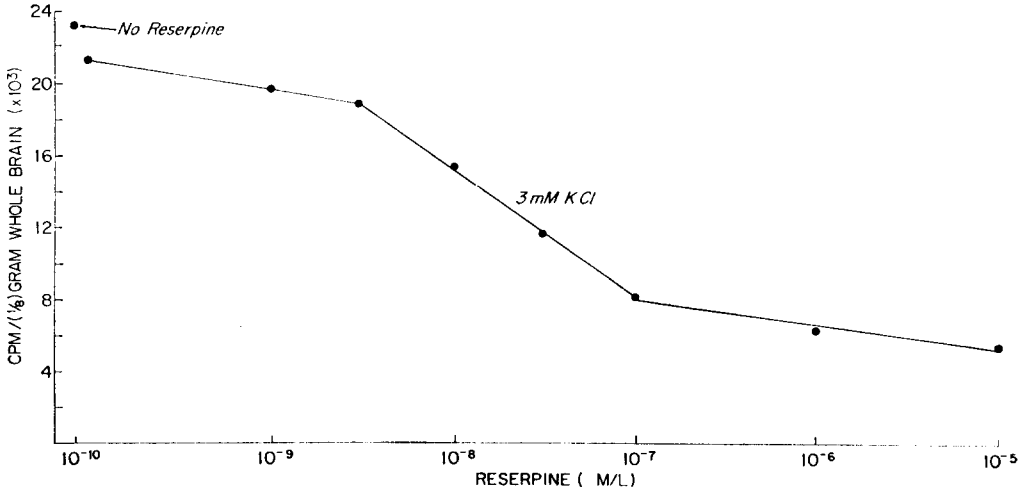

FIG. 3. Reserpine inhibition of norepinephrine uptake by synaptosomes. Buffer consisted of 10 mM sodium phosphate at pH 7·0, 11 mM glucose with 140 mM Na^+ and 3 mM K^+. Each point represents the average of 8 determinations. S.E.M. was 1200 cpm for low levels of reserpine and less than 500 cpm for high levels of the inhibitor.

period of maximal net uptake was approximately 16,000 cpm/μl. The volume estimated from the synaptosomal wet weight was 23 $\pm$ 1 μl, assuming a density of one. The NE concentration in the medium was 73 cpm/μl.

The uptake of NE was studied with increasing NE in the media. Kinetic data under these conditions indicate saturation at about 1–2 mμmole/ml. From a double reciprocal plot of the data (Fig. 4), the V_{max} for uptake was calculated to be 0·10 μg/min/g and the K_m was 0·56 $\times$ 10^{-6}M.

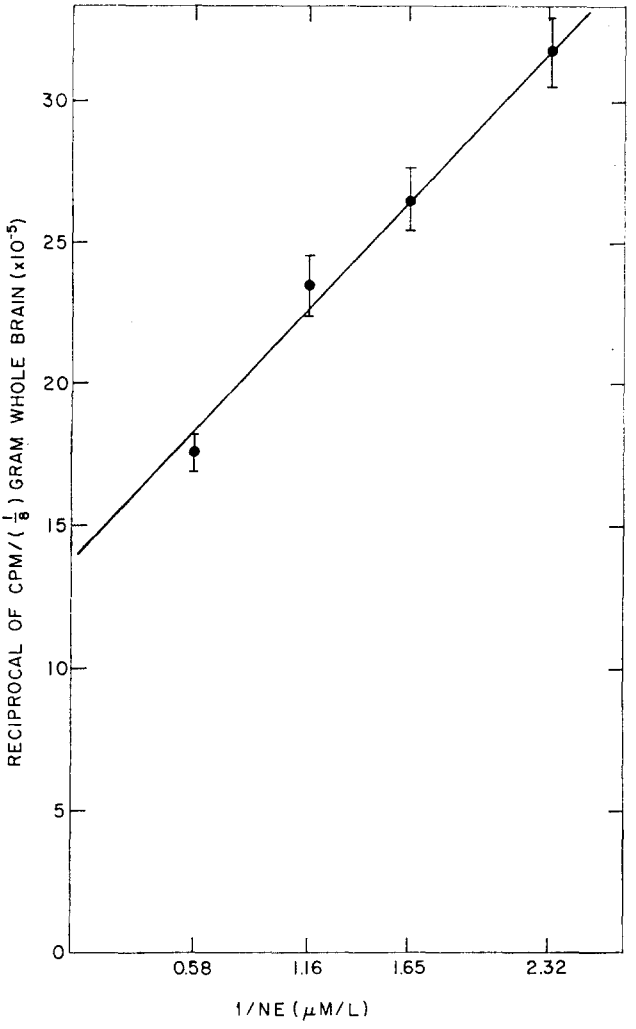

FIG. 4. Uptake of NE plotted as reciprocal of the uptake against the reciprocal of the NE concentration. The synaptosomes weighed 23 mg per test sample and the sp. act. of DL-norepinephrine-7-C^{14} was 48 mc/m-mole.

DISCUSSION

The present studies indicate that K^+ and Na^+ at essentially physiological levels (Na^+, 140 mM; K^+, 2–3 mM) are necessary for optimal NE uptake by nerve-ending preparations. Na^+ is known to be required for NE uptake by heart preparations,[7, 12–14]

but Na^+ and K^+ are required to prevent release of NE from such preparations.[14] The requirement for high Na^+ and low K^+ suggests that the uptake mechanism may in part be similar to those involved in the uptake of amino acids and glucose in other systems.[15] The observation that K^+ is required for net uptake would suggest that the release of NE from heart tissue[14] under conditions of low K^+ may be due to an impairment of re-uptake of the amine.

Since both cations are of major importance for net NE uptake, it would be reasonable to postulate that Na^+ and K^+ play a role in the transport of NE through membranes, a process which may be related to local flux of cations during nerve transmission. These ions may be involved in the function of ATP–metal–NE complexes which have been hypothesized as central to the mechanisms of NE uptake,[16, 17] or they may effect uptake through activation of an ATPase system, or both.

In spite of a wide interest in the function of biogenic amines in the brain, direct study *in vivo* has been difficult, since NE and other biogenic amines do not pass the blood–brain barrier. Isolated synaptosomes consisting of intact nerve membranes enclosing synaptic vesicles and some mitochondria may constitute essentially a model *in vitro* of the nerve ending. Several characteristics of the synaptosomal system which support its usefulness as a model for study of brain noradrenergic function are as follows: (1) most of the net uptake observed in fractions of brain tissue was found in the synaptosomal fraction; a small amount of the net uptake was found in the mitochondrial fraction possibly due to synaptosomal contamination, but there was essentially no uptake in the microsomal or myelin fractions; (2) the uptake obeys saturation kinetics, and the K_m and V_{max} are in reasonable agreement with that observed by Iversen for heart tissue;[18] (3) synaptosomes have been shown to synthesize serotonin *in vitro*;* (4) the net uptake is time- and temperature-dependent and is inhibited by ouabain, desmethylimipramine and reserpine in low concentrations. (see footnote p. 957) This evidence taken in total suggests that synaptosomes may indeed function in a manner similar to adrenergic systems *in vivo* and may serve as a useful system for investigation of central adrenergic tissue.

Even in this isolated system the uptake, storage and release mechanisms are complex, involving many functional components of the cell such as membranes, cell sap and storage vesicles. With techniques which will allow for the development of kinetic models, further studies are in progress to investigate the role of various salts and drugs on uptake and release of amines in different components of this system.

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