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MECHANISM OF TRANSPORT AND STORAGE OF BIOGENIC AMINES

III. EFFECTS OF SODIUM AND POTASSIUM ON KINETICS OF 5-HYDROXYTRYPTAMINE AND NOREPINEPHRINE TRANSPORT BY RABBIT SYNAPTOSOMES

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SUMMARY

The kinetics of biogenic amine transport by a process located in the membrane of synaptosomes were studied in order to determine the mechanism of the transport process and the effects of inorganic ions thereon. The following points were shown:

1. Exogenous 5-hydroxy[^{14}C]tryptamine is taken up by the sum of two processes; a saturable process predominating at low concentrations. At concentrations sufficient to saturate the first, a linear relationship between the velocity of uptake and substrate concentration suggests uptake occurs by passive diffusion.

2. The saturable processes for 5-hydroxy[^{14}C]tryptamine and [^{3}H]norepinephrine could be described by the Lineweaver–Burk representation of Michaelis–Menten kinetics, suggesting that uptake was mediated by a carrier mechanism.

3. The affinities of the carriers for 5-hydroxy[^{14}C]tryptamine and [^{3}H]norepinephrine were increased by Na^+.

4. The transport of 5-hydroxy[^{14}C]tryptamine in the presence of Na^+ was decreased by a high K^+ concentration.

5. These findings are presented in support of an ion gradient model for the transport mechanism located in the membrane of nerve endings.

INTRODUCTION

Biogenic amines released from nerve endings for the purpose of synaptic transmission are inactivated by a recapture mechanism located in the nerve ending[1]. Recent reports have shown that Na^+ is an essential requirement for the uptake and storage of norepinephrine in heart[2–6], and of norepinephrine and 5-hydroxytryptamine by pinched-off nerve endings (synaptosomes) from brain[7–11]. The Na^+-stimulated transport of the biogenic amines is facilitated by low K^+ concentration and is inhibited by high K^+ concentration[10–12], and by ouabain[11–13]. These characteristics of the transport process for amines are strikingly similar to those for other

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organic substances prompting us to propose a model of amine transport[11,12], based upon the models of sugar and amino acid transport proposed by CRANE[14], VIDAVER[15] and KIPNIS AND PARRISH[16]. According to this model, biogenic amines are transported across the neuronal membrane together with Na^+ by a carrier-mediated process, the activity of which is dependent upon the asymmetric distribution of Na^+ and K^+ across the membrane. Supporting evidence for this model was obtained in recent experiments from this laboratory demonstrating that the inhibition of amine transport by ouabain was Na^+ dependent and suggesting that ouabain acted indirectly by blocking ($Na^+ + K^+$)-ATPase[17], the enzyme generally thought to be responsible for maintaining the Na^+ and K^+ gradient in cells. The present work extends the knowledge gained from previous experiments with heart slices and synaptosomes and shows that the transport of biogenic amines into brain nerve endings is described by the Michaelis–Menten kinetics, in accord with the carrier concept for transport.

The present work also suggests that Na^+ increases the affinity of the carrier for amine and K^+ decreases that affinity.

MATERIALS AND METHODS

Synaptosomes were isolated from rabbit brain stem according to the method of RODRIGUEZ DE LORES ARNAIZ AND DE ROBERTIS[18]. The synaptosomes were recovered from the 1.0–1.2 M sucrose interphase. The material in this subfraction (mostly synaptosomes) retains more of the 5-hydroxytryptamine and norepinephrine injected into brain ventricle *in vivo* and takes up more amine *in vitro* than material in any other subfraction. Moreover, the amine accumulated *in vivo* and in synaptosomes incubated *in vitro* is lost from these synaptosomes at a rate corresponding to the turnover rate of amine *in vivo*. Other subfractions lost amine at a more rapid rate[11,19]. The synaptosome suspension was mixed with sufficient water to adjust the molarity of the sucrose solution to about 0.4 M. After equilibrating for 20 min on ice, the suspension was centrifuged at 10000 × *g* for 20 min.

The synaptosomes were then suspended in 16 ml of various media (averaging about 0.25 mg protein/ml solution) and incubated for 10 min at 37°; 5-hydroxy-[^{14}C]tryptamine (Nuclear Chicago, 56 mC/mmole) or (+, −)-[^{3}H]norepinephrine (New England Nuclear, 7 C/mmole) were then added to the incubation fluid and the synaptosomes were incubated for various periods of time as described in the figures and tables. 4-ml aliquots of the suspension were removed at various intervals of time, cooled immediately in an ice bath, with shaking, and centrifuged at 10000 × *g* for 5 min. The pellet was suspended in distilled water for the determination of protein and radioactivity. Additional details of the methods are described by TISSARI *et al.*[17]. The entrained supernatant volume in the pellet, estimated with [^{14}C]inulin, amounted to only 15 % of the water held in the particulate phase estimated as the total pellet water ([$^{3}H_2O$]) *minus* extracellular water (from [[^{14}C]inulin]).

The radioactivity contained in the particulate phase was reduced 10–20 % by each resuspension and wash in the cold, but this was undoubtedly due to breakage of fragile synaptosomes. Accumulated radioactivity was not nonspecific adsorption of amine since it was not reduced in the cold by 1 μg/ml unlabeled 5-hydroxytryptamine and norepinephrine, each ml containing about 50–200 times, respectively,

the smallest amount of labeled particulate amine. Moreover, adsorption is extremely unlikely to be the cause of accumulation because not only the accumulation but 75% of the metabolism of amine is blocked by ouabain or Na^+-free media[11,17], indicating that amine could not enter the cell.

The control medium was Krebs–bicarbonate solution (pH 7.4) equilibrated with O_2–CO_2 (95:5, by vol.). Na^+-deficient media were made isotonic with sucrose and contained normal concentrations of all cations except Na^+. The antagonism of Na^+-stimulated transport by K^+ was studied in media consisting of a modified Krebs–bicarbonate solution containing 50 mM Na^+ made isotonic with sucrose and also containing 6 or 100 mM of KCl. The concentrations of other cations were normal. The solution containing 100 mM K^+ was made hypertonic to prevent the tissue swelling that normally occurs with media containing high K^+ concentration.

Radioactive amines or metabolites were measured by scintillation counting. 5-Hydroxy[^{14}C]indoleacetic acid was assayed using the extraction procedure of UDENFRIEND *et al.*[20]. An additional deaminated product, probably 5-hydroxy[^{14}C]-tryptophol, was estimated according to TISSARI *et al.*[17]. Protein was assayed by the method of WARBURG AND CHRISTIAN[21].

The translocation of amine across the neuronal membrane was expressed in terms of counts/min 5-hydroxy[^{14}C]tryptamine or [^{3}H]norepinephrine per mg protein.

RESULTS

Accumulation and transport of 5-hydroxy[^{14}C]tryptamine by synaptosomes

Accumulation is the amount of unchanged exogenous amine inside the cell

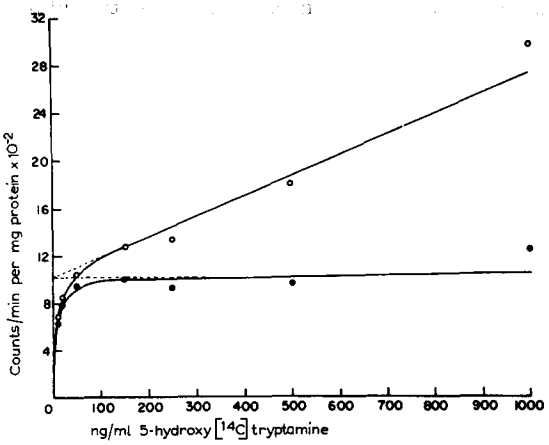

Fig. 1. Initial rates of accumulation of 5-hydroxy[^{14}C]tryptamine as a function of 5-hydroxy-[^{14}C]tryptamine concentration. A detailed explanation of this figure is given in the text. Various 5-hydroxy[^{14}C]tryptamine concentrations were incubated 6.5 min with synaptosomes suspended in Krebs–bicarbonate solution (pH 7.4) at 37°. ○, observed accumulation. The broken line is the extrapolation of the linear portion of the curve to the y-axis in order to obtain an equivalent to zero accumulation by a process linearly dependent upon 5-hydroxy[^{14}C]tryptamine concentration. ●, accumulation calculated as the difference between the observed accumulation and that accumulation which is linearly dependent upon 5-hydroxy[^{14}C]tryptamine concentration. Averages of two experiments in duplicate. For concentrations of 10–150 ng/ml, data from the experiments of Fig. 4 and two other experiments were incorporated.

after a period of incubation. Fig. 1 shows the initial rates of 5-hydroxy[^{14}C]tryptamine accumulation by synaptosomes after incubation for 6.5 min with various concentrations of amine in Krebs–bicarbonate solution. The data suggested that the accumulation of the amine was mediated by more than one process. The initial rates for Process 1 approached saturation at an external amine concentration of about 150 ng/ml. Process 2 showed a linear relationship to the concentrations of 5-hydroxy-[^{14}C]tryptamine used in these experiments, making its identity with Uptake 2 of IVERSEN[22] debatable. Rather, the data suggest that Process 2 represents simple diffusion of amine through the membrane. Extrapolating the linear segment of the curve back to the y-axis describes this diffusion process superimposed upon accumulation due to Process 1. Thus, the y-intercept actually represents zero accumulation by means of Process 2 at zero 5-hydroxy[^{14}C]tryptamine concentration. The difference between this line and the y-intercept represents the accumulation mediated by Process 2 at any given 5-hydroxy[^{14}C]tryptamine concentration. Subtracting the amount of 5-hydroxy[^{14}C]tryptamine accumulated by means of Process 2 from the total accumulation (observed line) indicates the accumulation mediated by Process 1 at any given 5-hydroxy[^{14}C]tryptamine concentration. Fig. 1 shows that the rate of accumulation mediated by Process 1 reaches a plateau of saturation at a concentration of about 150 ng/ml 5-hydroxy[^{14}C]tryptamine.

Since the 5-hydroxy[^{14}C]tryptamine that remains unchanged in the synaptosomes is only a fraction of the total amine that crosses the neuronal membrane[11,17] it was necessary to determine the total deaminated products in the particles and in the medium, as well as the amine retained by synaptosomes, in order to determine the total uptake of 5-hydroxy[^{14}C]tryptamine. Thus, some of the amine taken up is metabolized then lost from the synaptosome back into the medium. Fig. 2 shows that the total uptake of the amine increased with the concentration of extracellular

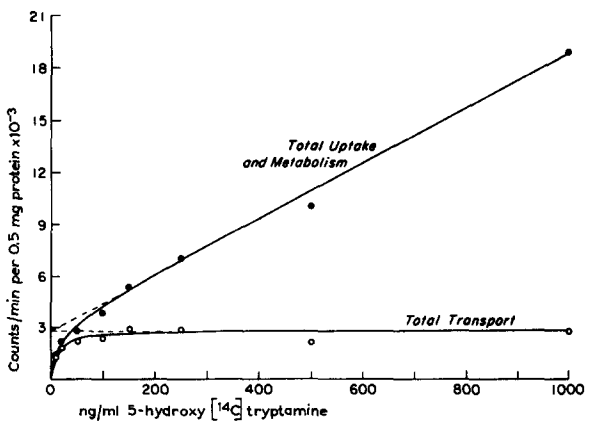

Fig. 2. Initial rates of uptake of 5-hydroxy[^{14}C]tryptamine as a function of 5-hydroxy[^{14}C]-tryptamine concentration. Definitions of terms are given in MATERIALS AND METHODS. A detailed explanation of this figure is given in the text. Various 5-hydroxy[^{14}C]tryptamine concentrations were incubated 6.5 min with synaptosomes suspended in Krebs–bicarbonate solution (pH 7.4) at 37°. ●, observed uptake. The broken line is an extrapolation of the linear portion of the curve as described for Fig. 1. ○, transport, calculated as the difference between observed uptake and the uptake which is linearly dependent upon 5-hydroxy[^{14}C]tryptamine concentration. Same experiments represented in Fig. 1.

5-hydroxy[^{14}C]tryptamine, approaching saturation when the external concentration was about 150 ng/ml. The curve then became linear. The extrapolation of the linear component of Fig. 2 back to the y-axis, as described for Fig. 1, revealed that total uptake of 5-hydroxy[^{14}C]tryptamine is also mediated by at least two processes. Process 1 is saturable, therefore fulfilling that requirement for a carrier-mediated process, and shall be hereafter designated as the transport process. The amount of amine transported by this process is called transport. Fig. 3, a Lineweaver–Burk plot of transport derived from the data of Fig. 2, shows that transport is represented by a straight line having an apparent K_m of 71 nM and a v_{max} of 1.1 nM 5-hydroxy-[^{14}C]tryptamine/mg protein per h.

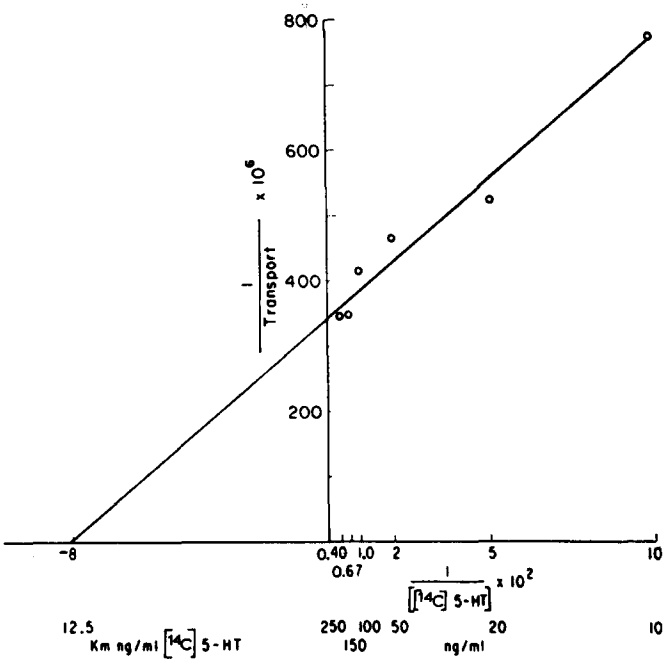

Fig. 3. A Lineweaver–Burk plot of transport. The values for the quantity of 5-hydroxy[^{14}C]-tryptamine (5-HT) taken up by the transport process were estimated and charted in Fig. 2. ○, reciprocals of the actual points representing Process 1 as estimated for Fig. 2. The actual 5-hydroxy[^{14}C]tryptamine concentration in ng/ml and reciprocals are given on the chart.

The effect of Na$^+$ concentration on accumulation of 5-hydroxy[^{14}C]tryptamine

Previous results from this laboratory have shown that Na$^+$ has a profound effect on the accumulation and metabolism of norepinephrine and 5-hydroxytryptamine by synaptosome. In an attempt to disclose the role of Na$^+$ on the transport process, we determined the effect of Na$^+$ on the kinetics of transport. When the reciprocal of the initial rate of accumulation (at 7 min) was plotted against the reciprocal of 5-hydroxy[^{14}C]tryptamine concentration in the presence of various Na$^+$ concentrations, a series of straight lines of different slopes indicated that the maximum increase in the K_m caused by variation in the Na$^+$ concentration was 270 %, but the increase of the v_{max} was only 37 % (Fig. 4). These findings suggest that 5-hydroxy-

tryptamine was transported by a carrier whose affinity for amine was controlled by Na^+. In other words, the lower the Na^+ concentration, the lower the affinity of 5-hydroxytryptamine for the transport mechanism. The v_{max} calculated from these data varied from 33 ng/ml (0.19 nM)/mg protein per h to 45 ng (0.26 nM)/mg protein per h; the apparent K_m decreased from 0.13 μM at a Na^+ concentration of 10 mM to 36 nM at a Na^+ concentration of 75 mM. The latter K_m is very similar to that calculated for transport from Fig. 2 (71 nM).

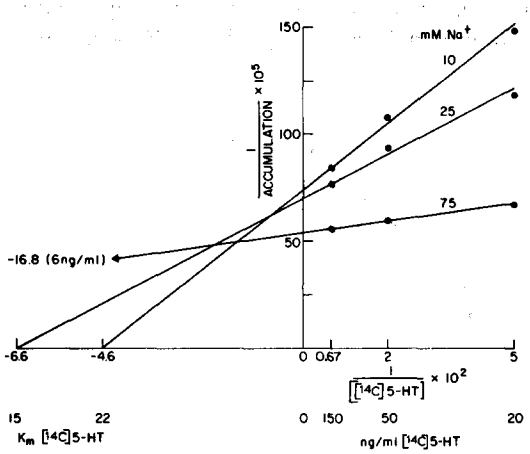

Fig. 4. Lineweaker–Burk plots of accumulation *vs.* 5-hydroxy[^{14}C]tryptamine ([5-HT]) as related to Na^+ concentration. Detailed explanation for this figure is given in the text. Various 5-hydroxy-[^{14}C]tryptamine concentrations were incubated 7 min with synaptosomes suspended in Krebs–bicarbonate solution (pH 7.4) at 37°. The actual 5-hydroxy[^{14}C]tryptamine concentration in ng/ml and reciprocals are given on the figure. Averages of four experiments. Each point was corrected for the difference between observed and calculated accumulation (Fig. 1).

Although accumulation accounts for only a fraction of the amine actually transported by the carrier mechanism, the treatment of the data as described above is justifiable as seen from inspection of Figs. 1 and 2. Taking into consideration the saturable transport process, the ratio of transport to accumulation is almost constant, ranging from 5.3 at 150 ng/ml to 4.7 at 20 ng/ml. The corrected v_{max} from Fig. 4, 75 mM Na^+ now becomes 225 ng/mg per h, or 1.3 nM which is very similar to the 1.1 nM calculated from Fig. 2.

Fig. 5 is a plot of the reciprocal of the amine accumulation against the reciprocal of Na^+ concentration at three external amine concentrations. A linear relationship would be expected if one Na^+ functions as a co-substrate with 5-hydroxytryptamine in the transport process[14,16] so that the carrier transports one molecule of amine together with one molecule of Na^+. Fig. 5 shows a linear relationship at Na^+ concentration 143–25 mM for 20 ng/ml 5-hydroxytryptamine. More 5-hydroxytryptamine enters the cell at 10 mM Na^+ than expected from a purely linear relationship. For 20 ng/ml serotonin, the apparent K_m was about 40 mM Na^+. The v_{max} was nearly the same as estimated in the presence of 75 mM Na^+ (Fig. 4).

Accumulation of [^{3}H]norepinephrine by synaptosomes

Fig. 6 shows the effects of Na^+ on the accumulation of [^{3}H]norepinephrin by synaptosomes. The data plotted according to Lineweaver–Burk showed that

accumulation of [^{3}H]norepinephrine also followed Michaelis–Menten kinetics and again indicated that an increase in Na^+ concentration did not affect the v_{max} but decreased the apparent K_m, indicating the affinity of the carrier for norepinephrine had been enhanced. The v_{max} for accumulation from these data was 7.98 ng (47 pM)/mg protein per h; the apparent K_m decreased from 0.27 μM at 40 mM Na^+ to 89 nM in the presence of 143 mM Na^+. The v_{max} for the accumulation of [^{3}H]norepinephrine was 0.64 nM, about 1/14 that for the accumulation of 5-hydroxy[^{14}C]tryptamine.

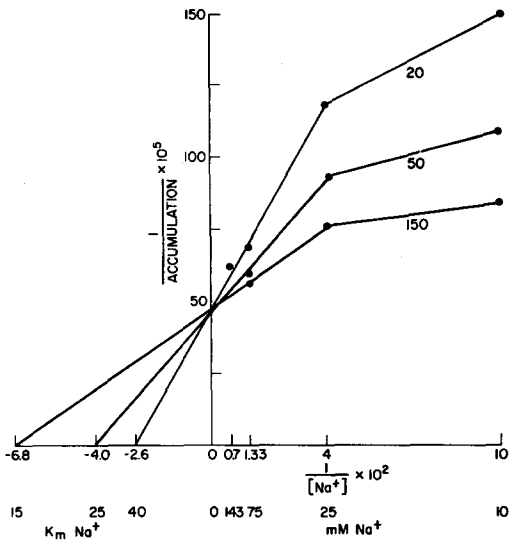

Fig. 5. Lineweaver–Burk plots of accumulation *vs.* Na^+ concentration as related to 5-hydroxy[^{14}C]-tryptamine concentration. These data are the same data plotted in Fig. 4 *plus* an additional point for accumulation at 20 ng/ml 5-hydroxy[^{14}C]tryptamine in 143 mM Na^+. The possible variation from the original series was corrected by comparison with a simultaneous run in 75 mM Na^+. The actual Na^+ concentration and reciprocals are given on the figure. The various 5-hydroxy[^{14}C]tryptamine concentrations employed are given on the figure. Each point was corrected for the difference between observed and calculated accumulation (Fig. 1).

When the reciprocal of the accumulation of [^{3}H]norepinephrine was plotted against the reciprocal of Na^+ concentration at three external amine concentrations (Fig. 7), a series of straight lines was produced. Changing the amine concentration produced little or no change in v_{max} but did affect the apparent K_m of the accumulation process. The linearity of these curves is strong evidence for a 1 to 1 transport of Na^+ and norepinephrine, suggesting that the metal activator is a co-substrate for the carrier.

The effect of high K^+ concentration on the K_m for 5-hydroxy[^{14}C]tryptamine transport

The studies described above indicate that the affinity of the biogenic amines for their carriers is a function of the Na^+ concentration in the external medium. In previous reports we showed that high K^+ concentration in peripheral sympathetic nerve endings reduced the affinity of the carrier for norepinephrine[12]. To test whether this result also applied to brain nerve endings we determined the effect of an increased K^+ concentration on the transport of 5-hydroxy[^{14}C]tryptamine.

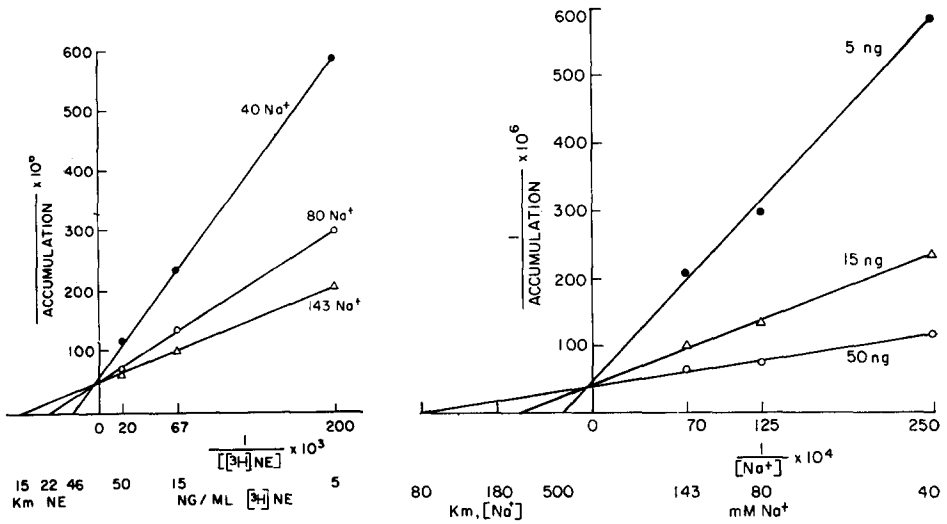

Fig. 6. Lineweaver–Burk plots of accumulation *vs.* [^{3}H]norepinephrine concentration ([NE]) as related to Na^+ concentration. Various [^{3}H]norepinephrine concentrations were incubated 7 min with synaptosomes suspended in Krebs–bicarbonate solution (pH 7.4) at 37°. The actual [^{3}H]norepinephrine concentration in ng/ml and reciprocals and the various Na^+ concentrations are given on the figure. Averages of four experiments.

Fig. 7. Lineweaver–Burk plots of accumulation *vs.* Na^+ concentration as related to [^{3}H]norepinephrine concentration. The data are the same data plotted in Fig. 6. The acutal Na^+ concentration and reciprocals and the various [^{3}H]norepinephrine concentrations are given in the figure.

TABLE I

TRANSPORT *vs.* 5-HYDROXY[^{14}C]TRYPTAMINE CONCENTRATION AND THE EFFECT OF HIGH K^+ CONCENTRATION

Transport was defined in METHODS. Various 5-hydroxy[^{14}C]tryptamine concentrations were incubated 7 min with synaptosomes suspended in modified Krebs–bicarbonate solution (pH 7.4) at 37°. The solutions contained 50 mM Na^+, isotonicity being maintained with 0.3 M sucrose. One set of experiments was run with solution containing 6 mM K^+ and the other set was run with solutions containing 100 mM K^+ in addition to sucrose (METHODS).

5-Hydroxy[^{14}C]tryptamine (ng/ml)	*Medium*	
	50 mM Na^+, 6 mM K^+	*50 mM Na^+, 100 mM K^+*
20	3273	1979
50	5883	3834
150	7227	4181

Table I shows that increasing the K^+ concentration from 6 to 100 mM at 50 mM Na^+ decreased the amount of 5-hydroxy[^{14}C]tryptamine taken up at all concentrations. The effect was slight, however, suggesting that the chief determinant of affinity at the outer membrane surface is Na^+, the concentration of which predominates *in vivo*.

DISCUSSION

The present studies indicate that norepinephrine and 5-hydroxytryptamine are transported across the neuronal membrane of isolated nerve endings of brain by a saturable process that is Na^+ dependent. In fact, the requirement for Na^+ is almost an absolute one since virtually no amine is taken up in its absence[10–12]. When analyzed according to the principles of kinetics, the present studies (Figs. 1–4) suggest that the main effect of Na^+ in biogenic amine transport is to increase the affinity between amine and a saturable carrier. On the other hand, K^+ lowers the affinity of the amine for the transport process without altering the v_{max} (ref. 12).

These results can be explained in terms of the mobile carrier concept of transport[23]. In accord with this view, the amine is transported by a mobile carrier[12,13,22], which has access to the inner and outer surfaces of the membrane. The carrier possesses two binding sites, one for the substrate and one for monovalent cation. Only the Na^+-complexed carrier can transport amine as shown by the fact that Li^+, choline$^+$ or K^+ cannot substitute for Na^+ (refs. 4, 12). Under physiological conditions the Na^+-loaded carrier predominates at the outer membrane surface. In this form the conformation of the amine binding site is such that affinity of the carrier for amine is high. The replacement of Na^+ by K^+ converts the carrier to the K^+-loaded variety in which form the conformation of the binding site is such that affinity is minimal. In the presence of low Na^+ concentration and high K^+ concentration, the reduced transport of amine by the carrier (Table I) could result from the competition of Na^+ and K^+ for the specific metal binding site on the carrier[14]. The other replacements for Na^+ previously mentioned are inert. Unlike K^+, they do not compete with Na^+ for the specific binding site[4,12].

Evidence that Na^+ is attached to the carrier is demonstrated by the linear relationship obtained when the reciprocal of the velocity of [^{3}H]norepinephrine uptake is plotted against the reciprocal of Na^+ concentration (Fig. 6). This relationship suggests that the Na^+, like the amine, is attached to the carrier according to the law of mass action and that Na^+ and amine are transported into the cell in a 1 to 1 ratio[16] in accord with the concepts developed by VIDAVER[15].

The deviation of the curves from linearity in Fig. 5 at 10 mM Na^+ might mean that some amines can be transported without the aid of Na^+, but this question is debatable on technical grounds. Distortion resulting from low levels of radioactivity taken up in the presence of low Na^+ concentration is magnified at the far ends of the curves. Despite the relative purity of the synaptosome preparations, the system does not approach the purity of solutions of purified enzymes used in classical descriptions of kinetics. Moreover, the estimate of transport of biogenic amines by synaptosomes involves factors such as translocation by a purely passive mechanism, retention of intracellular amine in another system containing endogenous amines (storage vesicles), and both intra- and extracellular metabolism of the transported molecule. Thus, several factors are involved which we minimize by measuring the corrected initial rate of transport (unidirectional flux). Theoretically, the initial rate is not influenced by the various other factors mentioned.

The results of the present studies are in accord with the extracellular portion of our model[12] of a mobile carrier having an affinity for amine that is increased by Na^+ and decreased by low Na^+ concentration or by high K^+ concentration.

Outside the cell the attachment of amine to the carrier would be favored by a high Na^+ concentration and a low K^+ concentration. The amine–Na^+–carrier complex then transfers to the inner side of the membrane where ionic conditions tend to decrease the affinity of the carrier for amine. In this environment, the carrier releases the amine, which is then stored or metabolized, and the Na^+ is returned to the outside of the cell by means of the Na^+ pump. The rate of transport is determined by the absolute Na^+ concentration, which controls the rate of amine attachment by the carrier outside the cell.

Low K^+ concentration facilitates amine transport although high K^+ concentration antagonizes it[10–12]. Evidence that low K^+ concentration acts indirectly through ($Na^+ + K^+$)-ATPase and the Na^+ pump, as does ouabain[17], is now in the press[24].

It is worthy of note that amines are transported in far lower concentrations than some amino acids and sugars[14,16]. Thus, the K_m for amine transport is expressed in nmoles rather than mmoles. The K_m for amines may be less sensitive to changes in Na^+ concentration than the K_m for amino acids. For example, when the Na^+ concentration is raised from 40 to 150 mM, the K_m for the transport of amino acids increases by a factor of about 30 (ref. 16). The factor for [^{3}H]norepinephrine transport is about 3. The relative changes in the K_m for the transport of 5-hydroxytryptamine reported above are similar to those reported for sugars by CRANE *et al.*[25]. A possible reason for the large difference in the K_m for amino acids may be related to the fact that the amine, but not amino acids, are stored in vesicles inside the neuron, obviating the need for large changes in affinity across the membrane. Thus, transport of amines is essentially unidirectional, since they can be bound in synaptic vesicles[26–28], or metabolized[11,29], hence do not move as readily in reverse direction. By contrast, amino acids can move in either direction (but not necessarily[16]), unless metabolized or incorporated into larger molecules.

Accumulation of amine is not the result of binding of amine that has entered the neuron by passive diffusion. Previous experiments have shown that ouabain blocks accumulation and intracellular deamination indicating that amine did not enter the nerve ending. Reserpine destroys binding sites, but does not block the accumulation of amine by synaptosomes prepared from animals previously given a monoamine oxidase inhibitor[11]. Moreover, uptake of amines by brain synaptosome is competitive[30], further evidence for the existence of a carrier-mediated amine transport.

An estimate of the concentration of amine present in the synaptic cleft during synaptic transmission is the K_m, since the transport mechanism must terminate synaptic transmission by removing the amine from the synaptic cleft. The values for 5-hydroxytryptamine and norepinephrine are 100 and 89 nM, respectively.

While this report was in preparation, SUGRUE AND SHORE[31] reported that Na^+ increased the v_{max} for the uptake of metaraminol by rabbit heart slices but had no effect on the K_m. The discrepancy between our results and theirs may possibly be attributed to their use of larger concentrations of a nonphysiological substrate for a different organ.

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