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PHYSICO-CHEMICAL PROPERTIES OF PHENETHYLAMINES AND THEIR UPTAKE INTO SYNAPTIC VESICLES OF THE CAUDATE NUCLEUS*

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Abstract—Synaptic vesicles isolated from the caudate nucleus of the pig were incubated with labelled (±)-, (−)-noradrenaline, dopamine, tyramine, (±)-octopamine, (+)-amphetamine or β-phenylethylamine in the presence or in the absence of ATP–Mg^{2+}. After incubation the vesicles were separated by filtration through membrane filters. In the absence of ATP–Mg^{2+} the phenethylamines used were taken up at different rates showing direct proportionality to the amine concentration in the incubation medium. The rate of uptake of the undissociated amines (at pH 7.4) depended on their lipophilic character. ATP–Mg^{2+} enhanced the uptake of (±)-, (−)-noradrenaline, dopamine, tyramine and (±)-octopamine; the uptake of amphetamine and β-phenylethylamine was not influenced. The ATP–Mg^{2+}dependent uptake was saturable and obeyed Michaelis–Menten kinetics. ATP–Mg^{2+} enhanced the uptake of compounds, which possessed at least one OH-group at the benzene ring. Amines with one OH-group in *para*-position or an additional one to that of the benzene ring and no OH-group in the side-chain showed similar affinities. OH-substitution in β-position of the side-chain decreased the affinity. The affinity to the transport system increased with increasing lipophilic character of the amine.

The uptake of some biogenic amines (e.g. noradrenaline, dopamine, serotonin) into synaptic vesicles of the caudate nucleus is increased by ATP–Mg^{2+} [1, 2].

An ATP–Mg^{2+}-dependent uptake of tyramine was also found, when microfiltration through membrane filters was used for the immediate separation of the vesicles after their incubation instead of the common centrifugation procedure [3]. The same procedure was now used to study the uptake of several phenethylamines into the synaptic vesicles of the caudate nucleus.

Furthermore, the partition coefficients of the phenethylamines were determined and correlated with the slopes (uptake of amines in the absence of ATP–Mg^{2+}) and with the kinetic parameter K_m (uptake of amines in the presence of ATP–Mg^{2+}). Finally, the significance of the position of hydroxyl-groups in the phenethylamine molecule for the ATP–Mg^{2+}-dependent uptake was investigated.

MATERIALS AND METHODS

Isolation of synaptic vesicles from the caudate nucleus of the pig. Incubation and separation of the incubated vesicles by membrane filtration. Synaptic vesicles of the caudate nucleus were isolated as previously described [2]. Briefly, the caudate nuclei were removed in the slaughter-house and immediately immersed in ice-cold sucrose solution (0.3 M), which contained pargyline (10^{-4} M). After homogenization with a Potter–Elvehjem homogenizer and centrifugation at 600 *g* for 15 min the supernatant was centrifuged at 20,000 *g* for 30 min. The pellet was discarded, the supernatant centrifuged at 80,000 *g* for 30 min and the pellet (vesicle fraction) suspended in ice-cold potassium phosphate buffer (0.13 M, pH 7.4).

Incubation and separation of the incubated vesicles by membrane filtration were carried out as previously described [3]. Briefly, the 5 ml incubation sample contained vesicle suspension (0.4 ml), various concentrations of amine (0.5 ml), $MgSO_4$ (3 mM; 0.5 ml), ATP (5 mM; 0.5 ml), phosphate buffer pH 7.4 (3.1 ml). In some samples, ATP was replaced by buffer. Incubation was stopped by adding ice-cold phosphate buffer pH 7.4 and the sample (9.6 ml) was transferred into a chamber of gas-operated (nitrogen) filtration apparatus. The filtration (Sartorius cellulose nitrate membrane filters No. 11309, 0.1 μm middle pore size) took place at 4° under stirring. After washing with phosphate buffer, the filter was removed, dried and transferred into scintillation vials [3]. Na^+-free ATP was used for the incubation experiments. It was prepared according to Schwartz *et al.* [4].

To study initial rates of uptake of the amines, the vesicles were incubated for 0.5 min (tyramine, noradrenaline, octopamine) or 2 min (dopamine, phenylethylamine, amphetamine) at 25° [3].

For characterization of the uptake in the absence of ATP–Mg^{2+}, slope b (the ratio of the amount of the amine taken up as pmoles × mg $protein^{-1}$ × min^{-1}/the concentration of the amine in the incubation medium as pmoles × ml^{-1}) was calculated. The ATP–Mg^{2+}-dependent uptake of the amines was calculated by subtracting the uptake found in the absence of ATP–Mg^{2+} from that in its presence.

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Determination of partition coefficients of phenethylamines. Octanol saturated buffer and buffer saturated octanol were used. To saturate them, equal volumes of potassium phosphate buffer (0.13 M, pH 7.4), which contained 10^{-3} M Na_2SO_3 and 4×10^{-5} M EDTA, and *n*-octanol were mixed over a period of 60 min with a shaking apparatus at room temperature. 15 min after end of shaking the phases were separated, transferred into centrifugation tubes and centrifuged at 600 *g* for 15 min.

0.3–80.0 nmoles of labelled amine were added to 10 ml phosphate buffer saturated with octanol. Aliquots of 3 ml of this mixture were transferred to shaking flasks, 1 ml was used for scintillation counting. According to the lipophilic character of the amine, 30 to 150 ml octanol saturated with buffer were added (dopamine and tyramine 90 ml, noradrenaline and octopamine 150 ml, amphetamine 30 ml, β-phenylethylamine 60 ml octanol). The mixture was shaken for 2 min, centrifuged at 600 *g* for 15 min, and the octanol phase removed by using a thin cannula connected to a vacuum pump. Shaking of the mixture for periods longer than 2 min did not change the partition coefficients. The concentration of the amine was expressed at pmoles per 3 ml.

For the determination of the partition coefficients, purified [^{3}H]tyramine, [^{3}H]octopamine [5], [^{3}H]dopamine [6], and [^{3}H]noradrenaline were used. Noradrenaline was purified by combination of the procedures of Atack and Magnusson [6] and Graefe *et al.* [7]. [^{3}H]Noradrenaline was added to an alumina column in a sample containing 0.2 N acetic acid: ethanol (9:1; 5 ml), 10 mg/ml of ascorbic acid (0.5 ml), 1.25 mg/ml of Na_2SO_3 (0.5 ml), 1 mg/ml of EDTA (0.5 ml), and adjusted to pH 8.4 with 1 M Tris (2.5 ml). The amine was eluted with 10×0.5 ml of 0.2 N acetic acid and passed through a Dowex 50 W × 4 column. Subsequently, noradrenaline was eluted with 5 ml of 1 N HCl.

Additionally, the determination of partition coefficients of nonradioactive dopamine, noradrenaline, tyramine, and octopamine was carried out. The concentration of amine was 9×10^{-6} M. (Excitation wavelengths: for dopamine, noradrenaline and tyramine 280 nm, for octopamine 275 nm. Emission wavelengths: for dopamine, noradrenaline and tyramine 320 nm, for octopamine 616 nm).

The calculation of the coefficients was carried out according to the equation

$$P = \frac{p}{q} \times \frac{V_u}{V_o}$$

where P = partition coefficient, p = amount of compound in the octanol phase/total amount of the compound, q = amount of compound in the buffer phase/total amount of the compound, V_u = volume of buffer phase, V_o = volume of octanol phase.

Substances used. (+)-Amphetamine sulphate ([^{3}H(G)), sp. act. 4.6 Ci/mmole, 3,4-dihydroxyphenylelthylamine (ethyl-2-[^{3}H(N)), sp. act. 7.5 Ci/mmole, octopamine (2-[^{3}H(N)), sp. act. 7.4 Ci/mmole, (−)-noradrenaline (7-[^{3}H(N)), sp. act. 5.9 Ci/mmole, (±)-noradrenaline (7-[^{3}H(N)), sp. act. 10.3 Ci/mmole, β-phenylethylamine hydrochloride (ethyl-1-[^{14}C) sp. act. 51.0 mCi/mmole, tyramine ([^{3}H(G)), sp. act. 10.8 Ci/mmole (NEN Chemicals, Dreieichenhain), pargyline hydrochloride (Abott, Chicago), ATP as disodium salt (Boehringer, Mannheim), (±)-octopamine hydrochloride (Calbiochem, San Diego/Cal.), (−)-noradrenaline hydrochloride, (+)-amphetamine sulphate (Merck, Darmstadt), dopamine hydrochloride (Nutritional Biochemicals Corporation, Cleveland/Ohio), 2-phenylethylamine hydrochloride, reserpine (Roth, Karlsruhe), tyramine hydrochloride (Serva, Heidelberg), (±)-noradrenaline bitartrate (Sterling, Winthrop, Rensselaer/N.Y.).

Reserpine was dissolved in a small amount of distilled water and 0.5 ml acetic acid. 200 mg ascorbic acid were added as antioxidant agent. The solution was filled up to 1000 ml with distilled water. For the incubation experiments the stock solution was diluted with potassium phosphate buffer (0.13 M, pH 7.4).

RESULTS

Uptake of phenethylamines in the absence and in the presence of ATP–Mg^{2+}

Incubation of the vesicles with various concentrations of [^{3}H]tyramine at 25° in the absence of ATP–Mg^{2+} led to a concentration-dependent uptake of the amine. The uptake was linear with increasing concentration of the amine in the incubation medium. The slope of the uptake was 0.009.

Table 1. Uptake of phenethylamines into synaptic vesicles of the caudate nucleus in the absence (*b*) and in the presence (K_m) of ATP–Mg^{2+}

Amine	*b** (uptake of total amine)	*n*	log *b*	*b'*† (uptake of non-protonated amine)	log *b'*	K_m × 10^{-6} M	*n*
(+)-Amphetamine	0.007 ± 0.0003	3	−2.15	2.48	0.39	—	3
β-Phenylethylamine	0.020 ± 0.009	4	−1.70	4.15	0.62	—	3
Tyramine	0.009 ± 0.001	5	−2.05	0.62	−0.21	0.14 ± 0.04	18–22
(±)-Octopamine	0.020 ± 0.004	5	−1.70	0.48	−0.32	1.79 ± 0.16	3–5
Dopamine	0.001 ± 0.0003	4	−3.00	0.04	−1.44	0.39 ± 0.07	5–8
(−)-Noradrenaline	0.002 ± 0.001	4	−2.70	0.03	−1.46	1.72 ± 0.53	3–6
(±)-Noradrenaline	0.003 ± 0.001	4	−2.52	0.04	−1.35	1.78 ± 0.55	3–6

* Slope *b* is the ratio of the amount of the amine taken up in the absence of ATP–Mg^{2+} as pmoles × mg protein^{-1} × min^{-1} to the concentration of amine in the incubation medium as pmoles × ml^{-1} (mean values ± S.D.).

† *b'* is the slope *b* calculated from the part of non-protonated amine at pH 7.4 in the incubation medium. K_m-values were calculated according to Wilkinson [23]. For the calculation of the values the ATP–Mg^{2+}-dependent uptake was considered (mean values ± S.E.M.).

Table 2. Determined and calculated physico-chemical data of phenethylamines

Amine	Partition coefficient* (P)	log P	n	log P'†	π-value‡	pK_1¶	pK_2¶	% Amine‖ protonated	% Amine‖ non-protonated
Amphetamine	0.1152 ± 0.0025	−0.94	8	1.54	0.16	9.88	—	99.7	0.3
β-Phenylethylamine	0.0663 ± 0.0022	−1.18	6	1.16	−0.19	9.74	—	99.5	0.5
Tyramine	0.0086 ± 0.0004	−2.07	12	−0.24	−0.86	9.23	10.6	98.5	1.5
Dopamine	0.0042 ± 0.0005	−2.38	13	−0.97	−1.53	8.81	10.5	96.3	3.7
Octopamine	0.0042 ± 0.0004	−2.39	12	−1.06	−2.25	8.73	9.9	95.5	4.5
Noradrenaline	0.0011 ± 0.0001	−2.97	8	−1.84	−2.92	8.53	9.8	93.1	6.9

* Partition coefficients were calculated from the distribution of amine between phosphate buffer phase (0.13 M, pH 7.4) and *n*-octanol phase (mean values ± S.E.M.).
† Logs of partition coefficients were corrected according to Scherrer and Howard [8].
‡ Calculated substituted group constants (π-values) (see Results).
¶ pK_1 and pK_2 values were taken from Armstrong and Barlow [22], pK_1 value of amphetamine from Mack and Bönisch [24].
‖ The parts of protonated and non-protonated amine at pH 7.4 were calculated by means of the Henderson–Hasselbalch equation.

Incubation of the vesicles with various concentrations of [^{3}H]dopamine in the absence of ATP–Mg^{2+} revealed a slope that was one ninth (0.001) of that of [^{3}H]tyramine (Table 1), while the slopes for the uptake of [^{3}H]-(±)-noradrenaline and [^{3}H]-(−)-noradrenaline were 0.003 and 0.002, respectively. The slope for the uptake of [^{3}H]-(+)-amphetamine (0.007) was comparable with that of [^{3}H]tyramine, while those of [^{14}C]-β-phenylethylamine and [^{3}H]octopamine were very high (0.02), when compared with the slopes for the uptake of the other amines.

Incubation of the vesicles in the presence of ATP–Mg^{2+} increased the uptake of [^{3}H]tyramine, [^{3}H]-(±)-octopamine, [^{3}H]-(±)-noradrenaline, [^{3}H]-(−)-noradrenaline and [^{3}H]dopamine. The K_m-value for [^{3}H]dopamine was very similar to that found previously when the vesicles were separated by centrifugation instead of microfiltration [5]; moreover, the kinetic constant of [^{3}H]dopamine was comparable to that of [^{3}H]tyramine. K_m-values of [^{3}H]-(±)-octopamine, [^{3}H]-(±)-noradrenaline and [^{3}H]-(−)-noradrenaline were almost identical with each other.

ATP–Mg^{2+} did not influence the uptake of [^{3}H]-(+)-amphetamine and [^{14}C]-β-phenylethylamine into the vesicles (Table 1).

The lipophilic character of phenethylamines

Since phenethylamines are protonated to a great extent at pH 7.4, the partition coefficient depends on the pK_a-value(s) of the molecule. The partition coefficients (P) experimentally determined in our study were corrected according to the non-protonated part [8] from the equation

$$\log P' = \log P + pK_1 - \text{pH}$$

(only for basic compounds)

$P-$, log $P-$ and log P'-values are shown in Table 2.

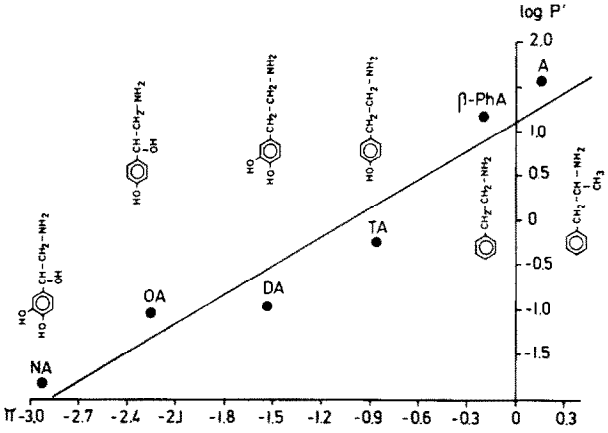

Fig. 1. Correlation between log P' and substituted group constants (π-values) of phenethylamines. Ordinate: log of corrected partition coefficients (log P'), abscissa: π-values of the substituted groups on the benzene ring of amphetamine (A), β-phenylethylamine (β-PhA), tyramine (TA), dopamine (DA), octopamine (OA), and noradrenaline (NA). For number of experiments see Table 2.

Substituted group constants (π-values) were calculated from the following equation

$$\pi_x = \log P_x - \log P_H.$$

In this equation P_x is the partition coefficient of a compound with the substituted group x, P_H is the coefficient of the same compound in which x is replaced by H, π is the log of the partition coefficient of the substituted group x.

The π-value of the benzene ring (common for all compounds used in this work) was taken as nought. π-values for the single substituted groups were taken from Leo *et al.* [9].

The partition coefficients were plotted against π-values. The statistically significant correlation ($r = +0.9660$; $P < 0.01$) shows that the determined partition coefficients of the non-protonated amines agree with the calculated π-values (Fig. 1).

Correlations between amine uptake processes and the lipophilic character of the amines

To calculate the slopes (b) of the uptake of phenethylamines in the absence of ATP–Mg^{2+}, the total amine concentration in the incubation medium was considered. Additionally, we calculated the concentrations of non-protonated free bases in the medium from the pK_1-value of the compounds. In this way, the slopes b' were obtained (Table 1). pK_1-values and parts of protonated and non-protonated phenethylamines in per cent are shown in Table 2.

There was no correlation between log P' and log b ($r = +0.5260$) (Fig. 2, upper panel). Similarly, there was no correlation between π-values and log b (not shown). However, a significant correlation ($r = +0.9054$; $P < 0.01$) was found, when log P'-values were plotted against log b'-values (Fig. 2, lower panel).

Likewise, a significant correlation ($r = -0.9510$; $P < 0.05$) was found between π-values and log K_m-values for the ATP–Mg^{2+}-dependent uptake of tyramine, (±)-octopamine, dopamine, (±)- and (−)-noradrenaline (Fig. 3). No correlation was found, when π-values were plotted against log V_{max}-values.

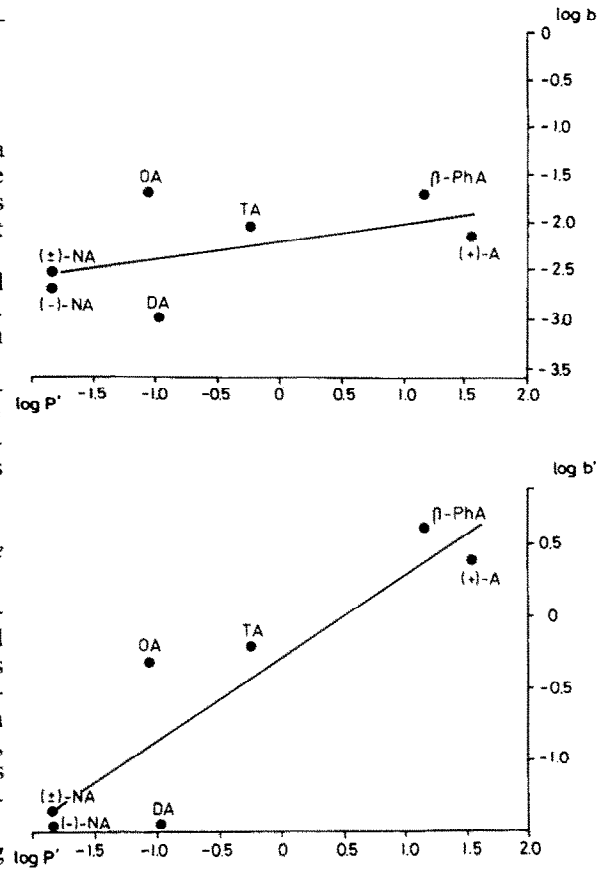

Fig. 2. Correlation between uptake of amines in the absence of ATP–Mg^{2+} and log of the corrected partition coefficients. Ordinates: log of the slopes b or b', abscissa: log of the corrected partition coefficients (P'). Upper panel: log b values represent the uptake of amines as calculated from the total amine concentration in the incubation medium in pmoles × mg protein^{-1} × min^{-1} ($r = +0.5260$; $P > 0.05$), lower panel: log b' values represent the uptake of amines calculated from the non-protonated amine concentration in the incubation medium at pH 7.4 ($r = +0.9054$; $P < 0.01$). For number of experiments see Table 1 and Table 2.

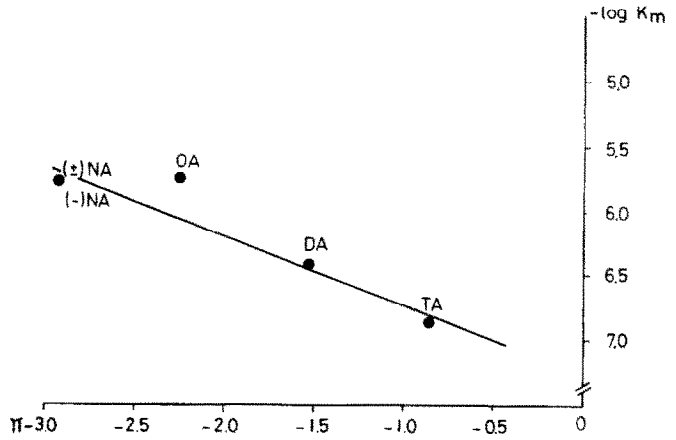

Fig. 3. Correlation between substituted group constants (π-values) and K_m-values. Ordinate: -log K_m-values, abscissa: π-values ($r = -0.9510$; $P < 0.05$). For number of experiments see Table 1.

DISCUSSION

To study the physico-chemical properties of the uptake of biogenic amines into synaptic vesicles, a vesicle-enriched fraction (80,000 *g*) was used, which was isolated by differential centrifugation. Previous studies have shown that the vesicles of this fraction contain mainly dopamine (approximately 250 pmoles × mg protein^{-1}), while the concentrations of noradrenaline and serotonin are about one-tenth of that of dopamine [1, 2]. Determination of marker enzymes revealed that this fraction is slightly contaminated with other subcellular particles [1]. However, only synaptic vesicles seem to take up biogenic amines by an ATP–Mg^{2+}-dependent process [11]. The main advantage of this fraction is that it is rapidly prepared in sufficient amounts for incubation experiments. For these reasons, this fraction is useful for studying initial rates of uptake of amines into synaptic vesicles [1, 25].

In this investigation a microfiltration method for immediate separation of the vesicles after incubation was used to investigate the uptake of (+)-amphetamine, β-phenylethylamine, tyramine, dopamine, (±)-octopamine, (±)- and (−)-noradrenaline into synaptic vesicles isolated from caudate nucleus.

In the absence of ATP–Mg^{2+}, the amounts of amines taken up were directly proportional to the concentrations of the amines in the incubation medium. According to their slopes, the amines we investigated can be classified as following:

1. dopamine, (±)- and (−)-noradrenaline with slopes of 0.001–0.003;
2. tyramine and (+)-amphetamine with slopes of 0.009 and 0.007, respectively;
3. (±)-octopamine and β-phenylethylamine with slopes of 0.02.

When the total amine concentration was considered, there was no correlation between the lipophilic character of the compounds and the slopes of their uptake. However, a correlation existed between lipophilic character and slopes of the nonprotonated amines indicating that the permeation of undissociated phenethylamines into synaptic vesicles is dependent on the lipophilic character of the amines.

This type of uptake into the vesicles of the caudate nucleus is not inhibited by drugs such as reserpine and prenylamine, which inhibit the ATP–Mg^{2+}-dependent uptake into synaptic vesicles [1, 5]. Moreover, it occurs also at 0°, though at a considerably lower rate [1] than at 25°. The diminished uptake at low temperatures agrees with findings of Euler and Lishajko [10], who studied uptake of catecholamines into splenic nerve granules, as well as findings of Philippu *et al.* [11], who investigated the uptake of noradrenaline into synaptic vesicles of the hypothalamus. Very probably, the ATP–Mg^{2+}-independent uptake of the amines is mainly due to diffusion.

As in the storing vesicles of the peripheral nervous system [10, 12, 13], neurotransmitters are taken up into synaptic vesicles of the central nervous system by an ATP–Mg^{2+}-dependent process. Indeed, synaptic vesicles of the pig brain take up dopamine, noradrenaline and other biogenic amines in an ATP–Mg^{2+}-dependent way [1, 5, 11].

Using the microfiltration method it is now shown, that tyramine, dopamine, octopamine, (−)- and (±)-noradrenaline are taken up by an ATP–Mg^{2+}-dependent mechanism that is saturable and obeys Michaelis–Menten kinetics.

Comparison of the chemical structure of the amines revealed that only compounds are taken up by the ATP–Mg^{2+}-dependent mechanism, which have at least one OH-group at the benzene ring. Tyramine with one OH-group in *para*-position showed the highest affinity (K_m: 0.14×10^{-6} M) to the transport system. An additional hydroxyl group at the benzene molecule tended to diminish the affinity (dopamine K_m: 0.39×10^{-6} M). OH-substitution in β-position of the side-chain in addition to one hydroxyl group in para-position at the ring, as in case of octopamine, led to a decrease in the affinity. The comparison of the uptake of tyramine with that of noradrenaline led to similar conclusions. Indeed, (±)- and (−)-noradrenaline had lower affinities than tyramine. The uptake of amines without any OH-substitution, such as amphetamine and β-phenylethylamine, was not stimulated by ATP–Mg^{2+}.

It is known, that with increasing lipophilicity (expressed as π-values) hydrophobic interactions are enhanced. It is of interest to note that in our experiments a negative correlation existed between K_m-values for ATP–Mg^{2+}-dependent uptake of amines and their π-values.

Hence, the affinity of an amine to the transport system seems to depend on the lipophilic character of the amine, provided that at least one OH-group is present at the benzene ring.

These results support the view, that OH-substitution of the benzene ring is a prerequisite for the transport of arylalkylamines by the ATP–Mg^{2+}-dependent system. Supposing that this transport is a carrier-mediated process, the binding of the amines to the carrier seems to result by hydrophobic interactions. This type of binding permits the transported molecule to be quickly released from the carrier inside the vesicle, perhaps by protonation of the base. If this were in fact the case, dissipation of the transmembrane ΔpH should reduce the amount of catecholamine taken up into vesicles. Indeed, transport of catecholamines into isolated chromaffin granules and synaptic vesicles is markedly inhibited by uncouplers of oxidative phosphorylation [14, 15], which are known to dissipate transmembrane pH gradients [16, 17]. Moreover, a pH gradient is known to exist across granule membranes (internal pH of 5.5) [18, 19] and there is evidence that this gradient can be generated by a proton-translocating Mg^{2+}-ATPase situated on the granule membrane [20, 21].

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