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EFFECT OF DRUGS ON THE ATP-INDUCED AND pH-GRADIENT-DRIVEN MONOAMINE TRANSPORT BY BOVINE CHROMAFFIN GRANULES

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Abstract—Reserpine, tetrabenazine and the neuroleptics chlorpromazine and haloperidol blocked the ATP-dependent uptake of noradrenaline and tyramine by ghosts derived from bovine chromaffin granules. The drugs did not affect chromaffin granules energization since they were without any effect on the membrane ATPase activity and on the transmembrane potential and pH-gradient generated by the ATP-dependent H^+-translocase. Differences were observed in the inhibitory effect of the drugs on the monoamine uptake by ghosts acidic with respect to the external medium. These differences were accounted for by the existence under these conditions of two mechanisms of uptake, as shown by kinetic experiments. Noradrenaline was taken up by a carrier-mediated process which was blocked by all drugs, whereas tyramine transport involved non-specific diffusion of its unprotonated form, a process which was sensitive to neuroleptics and high doses of reserpine. From the kinetic and pharmacological studies of tyramine uptake, it is concluded that the ATP-dependent active transport of monoamines requires a carrier-mediated process even for amines which are rapidly transported by non-specific diffusion through the membrane.

Reserpine and tetrabenazine are known to be inhibitors of the *in vitro* uptake of monoamines by chromaffin granules, the catecholamine storage organelles [1–3]. This process is also blocked by the neuroleptics chlorpromazine and haloperidol [3]. The uptake is ATP-dependent [1, 4] and is thought to be carrier-mediated since it shows stereospecificity and saturation kinetics [2, 5]. Although much effort has been devoted to the study of these drugs, their mechanism of action on monoamine uptake is not fully understood. Progress should be expected since our knowledge of bioenergetic processes in chromaffin granules is increasing rapidly [6]. A Mg^{2+}-activated ATPase (EC 3. 6. 1. 3) of the membrane has been shown to be an inward proton pump. H^+-translocation results in generation of a transmembrane potential positive with respect to the external medium [7, 8] and, when permeant anions are added, in acidification of the vesicle interior [9]. In terms of Mitchell's chemiosmotic hypothesis, it has been proposed that the transport of catecholamines is driven either by the pH gradient [10–12] or by the potential difference [6, 13]. In the absence of ATP, chromaffin granules or 'ghosts' with an acidic internal medium accumulate catecholamines which become distributed berween the inside and the outside of the vesicle according to the pH gradient [11, 12, 14].

In the present communication, the effects of drugs on the ATP-dependent chromaffin granule energization and on the ATP-induced and ΔpH-driven uptakes of noradrenaline and tyramine have been investigated. Two different types of transport have been distinguished on pharmacological and kinetic grounds and the sites of action of the different drugs have been defined. Our observations also give insight on the mechanism of active transport.

MATERIALS AND METHODS

Materials. [Side chain-2-^{14}C]-tyramine hydrochloride (50 mCi/mmole), *l*-[7-^{3}H] noradrenaline hydrochloride (15 Ci/mmole), [^{14}C]thiocyanate (59 mCi/mmole) and D-[U-^{14}C]sorbitol (191 mCi/mmole) were obtained from the Radiochemical Centre (Amersham, Bucks, U.K.); [^{14}C]methylamine (38 mCi/mmole) and ^3H_2O were from CEA, France.

Reserpine (free base) was obtained through Sigma, tetrabenazine (free base) and chlorpromazine chlorhydrate were gifts of Hoffman–La Roche (Basle, Switzerland) and Rhône Poulenc (Paris, France), respectively. Tetrabenazine (4 mM) and haloperidol (free base) (4 mM) were dissolved in 90% ethanol, chlorpromazine (10 mM) in water; reserpine was made 10 mM in 2 M acetic acid and was diluted ten times in 0.1 M phosphate buffer (pH 6.5).

Preparation of chromaffin granule 'ghosts'. Chromaffin granules were prepared by successive differential centrifugation at 27,000, 21,000 and 16,300 *g* for 30 min in 0.3 M sucrose, 10 mM Hepes,* pH 7.0 [7]. Such preparations were homogenous on electron micrographs. Lysis was performed by dropwise addition of the granules (15–20 mg protein/ml) to an hypo-osmotic buffered solution containing 2 mM $MgSO_4$, 0.1 mM dithiothreitol, 10 μM $CaCl_2$ and either 5 mM Tris-succinate (pH 5.9) or 5 mM Hepes (pH 7.0) [15]. The solution was stirred for 10 min at

* Abbreviations used: Mes, 2 (*N*-morpholino) ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazin-*N*′-2-ethanesulfonic acid.

4°C and then centrifuged (37,000 *g* for 30 min). The pellet was washed in the same medium, centrifuged and resuspended in half the volume of the granule preparation. The resuspension medium was similar to the lysis buffer but contained 0.3 M sucrose and no $CaCl_2$. 'Ghosts' lost, respectively, more than 99.5 per cent and 70–75 per cent of their catecholamines and their proteins. The internal specific volumes of 'ghosts' preparations were estimated to be 2.3 ± 0.3 μl/mg of protein. Their monoamine oxidase activity assayed as in [5] with 50 μM [^{14}C]-tyramine hydrochloride as a substrate was less than 0.25 nmole tyramine oxidized/min/mg protein. Adrenal mitochondria, when assayed under the same conditions, had an activity of 1.5 nmoles tyramine oxidized/min/mg protein.

Preparation of purified chromaffin granule membranes. ATPase activity was measured on membranes derived from granules purified by discontinuous 0.3 M–1.8 M sucrose gradients [16]. Lysis was performed as above in the pH 7.0 buffer, but the membranes were collected by centrifugation at 140,000 *g* for 45 min in a Spinco ultracentrifuge.

ATP-induced amine uptake. Granules (approximately 1 mg protein/ml) or 'ghosts' prepared at pH 7.0 (2 mg protein/ml) were added to a medium containing 0.3 M sucrose, 5 mM ATP, 2.5 mM $MgSO_4$, 10 mM Hepes (pH 7.0), drug where indicated, and the radioactive amine. The final volumic activities were usually: *l*-[7-^{3}H]noradrenaline (4 μCi/ml), [side chain-2-^{14}C]-tyramine (2 μCi/ml) and [^{14}C]-methylamine (1.4 μCi/ml). Where indicated, the granules or 'ghosts' were preincubated with the same drug concentration at 4° for 4 min. The complete mixture was incubated for various times at 37°; 100 μl aliquots were then withdrawn, diluted in 0.3 M ice-cold buffered sucrose (1 ml), and rapidly filtered through 0.45 μm millipore filters (HAWP) [17]. The filters were washed twice with the same buffer (2 ml), dried, and counted in toluene containing PPO (5 g/l) and POPOP (0.3 g/l). The results were corrected for amine adsorption on the membrane by subtracting control values obtained in the absence of ATP and in the presence of 10 μM reserpine. Such controls were in general less than 5 per cent of the corresponding experiment.

ΔpH-driven amine uptake. 'Ghosts' prepared at pH 5.9 (which were preincubated with drugs as above, where indicated) were added at a final concentration of 2 mg protein/ml to a solution containing 0.3 M sucrose, 20 mM Hepes (pH 8.5) and amines as above. 'Ghosts' were added in the minimal volume in order not to perturb the pH of the incubation mixture. After incubation for various times, the samples were treated as previously described for the ATP-induced uptake. Controls were performed at an external pH of 5.9 and in the presence of 10 μM reserpine. The controls were always less than 10 per cent of the corresponding experiment.

Transmembrane potential measurements. The procedure of Casey *et al.* [9] was applied. For each measurement, four 0.25-ml aliquots of chromaffin granules (2 mg of protein) preincubated with drugs were mixed with the same volume of 0.3 M sucrose, 40 mM Mes (pH 6.6) containing 5 mM ATP and 2.5 mM $MgSO_4$. After a 3 min incubation at 20°, isotopes (20 μl) were added: two samples were used to estimate the internal exchangeable water space (sorbitol exclusion volume) and two for the transmembrane potential. The first used ^3H_2O and D-[U-^{14}C] sorbitol and the latter [^{14}C] SCN^- and ^3H_2O. Final isotope activities were (in μCi/ml): ^3H_2O, 2; D-[U-^{14}C] sorbitol 0.1 mM, 1; [^{14}C] SCN^- 25 μM, 1.5. The samples were equilibrated for 2.5 min at 20° and centrifuged for 10 min at 27,000 *g* at the same temperature. Both supernatant and pellet fractions were treated as in [9], their radioactivity measured in 10 ml of scintillation mixture (NE 260, Nuclear Enterprise, Edinburgh, U.K.) or alternatively the pellets dissolved in 0.5 ml of 2% Triton X-100 and the samples counted in a Triton–toluene scintillation fluid. Internal water space, and internal to external concentration ratios of SCN^- were derived as in [9] from the relative activities of the isotopes in pellets and supernatants.

Granule internal water spaces ranged from 1.8 to 2.5 μl/mg of vesicle protein. Transmembrane potentials were calculated as:

$$\psi_{in} - \psi_{out}\ (mV) = 57.6 \log \frac{(SCN)\ in}{(SCN)\ out}.$$

pH-gradient measurements. For each measurement, four 0.25-ml aliquots of granules (2 mg of protein) were preincubated with drugs and mixed with an equal volume of hypertonic 40 mM Mes, 0.3 M KCl (pH 6.6) buffer containing 5 mM ATP and 2.5 mM $MgSO_4$ and, after 30 min of incubation at 37°, isotopes (20 μl) were added: ^3H_2O (2 μCi/ml) and D-[U-^{14}C]-sorbitol (0.1 mM, 1 μCi/ml) for internal water space measurements and ^3H_2O and [^{14}C] methylamine (36 μM, 1.35 μCi/ml) for methylamine partition which was derived as above from activities of the isotopes in pellets and supernatants. The pH gradient was calculated as:

$$pH_{out} - pH_{in} = \log \frac{(MeNH_2)_{in}}{(MeNH_2)_{out}}.$$

ATPase activity. Purified membranes (50–100 μg protein/ml) were incubated with drugs or an equivalent volume of ethanol (less than 1 per cent) in 25 mM Tris–HCl buffer (pH 7.5) for 4 min at 37°. ATP (2 mM, final concentration) and $MgSO_4$ (1 mM) were then added; aliquots (50 μl) were withdrawn at intervals and assayed for inorganic phosphate by the method of Anner and Moosmayer [18].

Liposome preparation. Liposomes I were prepared according to Nichols and Deamer [19]. A 2.5 ml solution in ether of freshly prepared egg lecithin (3.5 μmoles/ml) was slowly injected with an infusion pump into 5 ml of citrate–phosphate buffer, pH 5.0 (200 mM $PO_4H\ Na_2$ titrated by 100 mM citric acid), at 55°. Liposomes were filtered through 1.2 μm Millipore filters, but phospholipid assays showed that no multilamellar liposomes were retained on the filter.

Liposomes II were obtained by sonication under argon at 22° for 30 min of a suspension of lecithin (2 μmoles/ml) in pH 5.0 citrate phosphate buffer. The clarified solution was then centrifuged for 1 hr at 140,000 *g* at 8°.

Δ pH-induced amine uptake by liposomes [19]. Liposomes (1 ml) in pH 5.0 buffer were incubated for 2 min at 22° with the drugs. They were titrated

to pH 8 with 2 N NaOH and *l*-[7-^{3}H] noradrenaline (150,000 c.p.m./ml) or [2-^{14}C] tyramine (350,000 c.p.m./ml) was then rapidly added. After incubation at 22°, the mixture was layered onto a Sephadex G-50 column (1 × 18 cm) which was washed by citrate–phosphate buffer, pH 8.0. Fractions (0.5 ml) were collected and their radioactivity measured in 10 ml of Bray scintillation mixture [20].

Protein and catecholamine assays. Proteins were estimated according to Lowry *et al.* [21] with bovine serum albumin as standard, following precipitation in 5% trichloacetic acid and redissolution in 2% deoxycholate, 3% NaOH. Catecholamines were assayed according to Von Euler *et al.* [22] without separation of the various amines.

RESULTS

Effect of drugs on ATP-induced amine uptake. The concentration of drug causing 50 per cent inhibition (EC_{50}) of the ATP-induced uptake of *l*-noradrenaline (100 μM) by bovine chromaffin granules has been derived from dose–inhibition curves. Tetrabenazine, reserpine, haloperidol and chlorpromazine gave figures of 0.1, 0.1, 6 and 35 μM, respectively. These values are comparable to those previously reported for 'ghosts' [3]. Tetrabenazine and reserpine, which are specific blockers of this process, were the most potent inhibitors with EC_{50} values two orders of magnitude lower than haloperidol and chlorpromazine.

With chromaffin granules the interpretation of uptake experiments is often obscured by the presence of high ATP and catecholamine concentrations in the vesicles. This difficulty has been circumvented by the use of resealed membranes ('ghosts') as proposed by Taugner [23] and Phillips [17]. This preparation accumulated *l*-noradrenaline efficiently and, at 10 μM amine, 20 min were required to reach a plateau of 3 nmoles noradrenaline/mg protein (Table 1). This plateau was not a result of noradrenaline deamination by monoamine oxidase since addition to the incubation mixture of 1 mM pargyline did not change the time course of the uptake reaction (data not shown). Tyramine was taken up at a similar initial rate but its incorporation stopped more rapidly, leading to a 3-fold decrease of the plateau. The reaction was not affected by 1 mM pargyline. Its lower plateau value did not originate in oxidation of tyramine by dopamine-β-hydroxylase, since diethyldithiocarbamate, which at 10 μM inhibited dopamine-β-hydroxylase (data not shown) did not affect the time course of uptake up to 100 μM concentration (data not shown). With the same preparation and under the same conditions, only a limited ATP-dependent transport of methylamine was observed. This transport was slow and did not reach a defined plateau, as in the noradrenaline and tyramine case. The lack of methylamine accumulation could not be attributed to technical problems since addition of 30 mM thiocyanate to the incubation mixture, which allowed electroneutral transport of protons and thus rapid acidification of the interior of the 'ghosts', resulted in an increase of methylamine uptake (data not shown).

Inhibition by drugs of noradrenaline uptake in

Table 1. ATP-induced amine uptake by 'ghosts'

	Uptake in absence of drug*			Drug inhibition (%)†				
Amine	Initial rate of uptake (pmoles/min/mg protein)	Time for reaching plateau (min)	Total amount accumulated (pmoles/mg protein)	Tetrabenazine (10 μM)	Reserpine (1 μM)	Reserpine (10 μM)	Haloperidol (40 μM)	Chlorpromazine (100 μM)
Methylamine (10 μM)	5 ± 3	‡	‡					
Tyramine (10 μM)	420 ± 45	4	1060 ± 80	75 ± 5	80 ± 6	85 ± 5	80 ± 7	70 ± 15
Noradrenaline (10 μM)	400 ± 38	15–20	3050 ± 370	94 ± 3	92 ± 4	97 ± 2	92 ± 4	80 ± 8
Noradrenaline (100 μM)	1900 ± 130			95 ± 3	92 ± 2	96 ± 2	80 ± 3	96 ± 2

* 'Ghosts' (2 mg protein/ml) prepared at pH 7.0 were incubated, at the same pH, as described in Materials and Methods. Time courses of methylamine, tyramine and noradrenaline uptakes were derived from measurement of the radioactivity bound to the vesicles on aliquots withdrawn at intervals. Initial rates were derived from the linear period of the uptake reaction: 2 and 5 min for tyramine and noradrenaline, respectively, and *more than 8 min for methylamine.*

† The time course of uptake in presence of drugs was measured under the same conditions and inhibition was determined at the plateau. Controls without drug were adjusted for ethanol. Results are means ± S.E. of at least three experiments.

‡ Methylamine uptake was slow and gave an ill defined plateau, preventing measurement of drug inhibition.

granules and 'ghosts' were comparable (Table 1). The effect of the same concentration of drugs on tyramine uptake by 'ghosts' has also been studied (Table 1). The transport was inhibited by all drugs tested and inhibitions were similar to those observed for noradrenaline uptake.

Effect of drugs on chromaffin granule energization. To understand the mechanism(s) of inhibition, we investigated the effects of the drugs on various components of the uptake process. In agreement with previous observations [3, 24], drugs at doses which blocked the ATP-dependent catecholamine uptake were without any significant effect on the ATPase activity (Table 2). Chlorpromazine inhibited the enzyme by 12, 32 and 55 per cent at concentrations of 100, 200 and 300 μM, respectively, but the 96 per cent inhibition of the noradrenaline uptake observed at the lowest drug concentration cannot be accounted for by the slight ATPase inhibition.

The ATPase activity of chromaffin granule membrane has been shown to be associated with an electrogenic H^+-translocase which polarizes positively the granule interior [7, 8]. The effect of the drugs on the transmembrane potential is shown in Table 2. None of them affected the potential difference (interior positive) observed in presence of ATP, and tetrabenazine, reserpine and haloperidol even slightly enhanced it. This enhancement might originate in the inhibition by the drug of the continuous reuptake of catecholamines by the granules. Under appropriate conditions [9], the H^+-translocating ATPase induced an acidification of the granule interior (Table 2). Drugs did not significantly affect this property.

Effect of drugs on the ΔpH-driven amine uptake by 'ghosts'. The driving force for catecholamine uptake is thought to be either the ΔpH resulting from the ATP-dependent H^+-translocase activity or to involve also the transmembrane potential generated by this enzyme. Since neither the ΔpH nor the potential difference were affected by the drugs, the site of action of these compounds should reside in the mechanism of amine entry, presumably at the level of the specific carrier. To further substantiate this hypothesis, the energization process was bypassed and the effect of drugs on the ΔpH-driven amine uptake was studied. 'Ghosts' resealed at pH 5.9 and suspended at pH 8.5 accumulated amines in absence of any added ATP (Table 3). Noradrenaline, tyramine and methylamine were transported under these conditions and total amounts accumulated were very similar, as expected if the ΔpH was the only driving force.

Differences were observed in the effect of drugs on the Δ pH-driven uptake of the three amines (Table 3). Tetrabenazine inhibited noradrenaline but not methylamine or tyramine uptakes, which at 10 μM were completely unaffected by this drug. Inhibition of noradrenaline transport by reserpine was considerable and similar at 1 and 10 μM drug concentrations, but the drug had only a limited effect on tyramine and methylamine uptakes at 1 μM. Neuroleptics were also more potent inhibitors of noradrenaline uptake but had nevertheless an appreciable blocking effect on the transport of methylamine and tyramine. These results differ from those obtained on the ATP-induced process (Table 1) where tyramine and noradrenaline were equally well blocked by the drugs. It should also be noted that, even with noradrenaline, inhibitions were less marked in the case of the ΔpH-driven process.

Kinetics of noradrenaline and tyramine uptakes by the ATP-dependent and ΔpH-driven processes. The resistance of the ΔpH-driven tyramine uptake to tetrabenazine and reserpine is somewhat surprising. A possible interpretation is that in this type of experiment tyramine did not enter the granule membrane through the catecholamine carrier as it did in the ATP-driven process. Since a carrier mediated process is defined as having saturation kinetics, this hypothesis was tested by comparing the kinetics of tyramine and noradrenaline uptakes by the ATP-dependent and ΔpH-driven processes. In the ATP-induced experiments, both tyramine and noradrenaline had saturation kinetics, whereas with the ΔpH-driven processes, only noradrenaline uptake was saturable (Fig. 1). The initial rate of the ΔpH-driven tyramine uptake increased linearly with amine concentration. Since this reaction was very rapid and thus difficult to follow accurately, it was repeated at 20° and with a ΔpH of only 1.0 pH unit (using 'ghosts' prepared at pH 7.0 and incubated at pH 8.0). Again, non-saturable kinetics were observed (data not shown), thus suggesting that in the presence of a ΔpH tyramine simply diffused through the membrane.

Table 2. Effect of drugs on chromaffin granule energization*

Drug	Concentration (μM)	ATPase activity (% of control)	Transmembrane potential (mV)	$pH_{out} - pH_{in}$
Control without ATP			−35.4 ± 2.0	0.75 ± 0.05
Control with ATP		100	64.4 ± 0.5	1.06 ± 0.06
Tetrabenazine	10	100 ± 5	70.4 ± 1.2	1.10 ± 0.03
Reserpine	10	100 ± 3	72.7 ± 1.6	1.00 ± 0.03
Haloperidol	40	100 ± 5	68.3 ± 0.4	1.08 ± 0.02
Chlorpromazine	100	88 ± 8	61.4 ± 0.3	1.10 ± 0.03

* The effects of drug concentrations 3- to 100-fold higher than EC_{50} have been tested on the ATPase activity of purified membranes and the ATP-induced granule transmembrane potential and pH gradient in absence of monoamine. The untreated ATPase control had an activity of 235 nmoles P_i/min/mg protein. $\Delta\psi$ and ΔpH were measured at pH 6.6 and ATPase activity at pH 7.5. Results are means ± S.E. of duplicate measurements on two similar experiments.

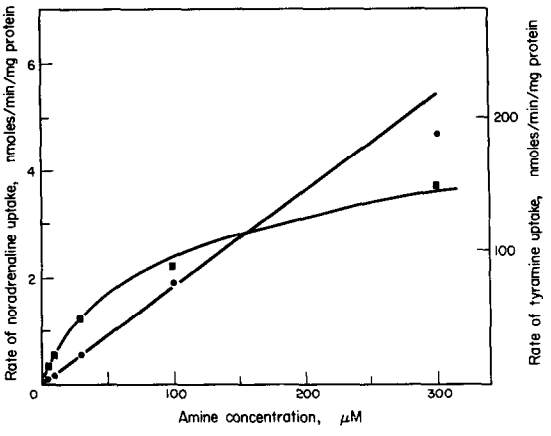

Fig. 1. Dependence of the ΔpH-driven initial rate of uptake on amine concentration. The same 'ghost' preparation was used to determine the kinetics of noradrenaline (■) and tyramine (●) uptake. Incubation mixtures were as described in Materials and Methods with a 'ghost' concentration of 2.2 mg protein/ml. Aliquots were withdrawn at 20, 40, 60 and 90 sec for noradrenaline uptake and at 5, 10, 15 and 30 sec for tyramine uptake. Uptake was linear for 40 sec and 10 sec for noradrenaline and tyramine uptake, respectively.

Where observed, the saturation kinetics have been further characterized. The three kinetics were non-Michaëlian and showed a concave deviation from linearity on Eadie plots. Hill plots of the three kinetics drawn using maximal initial velocities were shown to be linear. Moreover, the three sets of data gave rise to very close lines and to very similar kinetic parameters (Table 4). Thus noradrenaline ATP-induced and ΔpH-driven uptakes, but also tyramine ATP-induced uptake, had very close Hill numbers (h) and substrate concentrations at half saturation ($S_{0.5}$). Tyramine and noradrenaline ATP-dependent transport kinetics differed mainly by their maximal velocity. With tyramine, it should be noted that the ATP-dependent uptake, which is presumably carrier-mediated, is considerably slower than its Δ pH-driven counterpart.

ΔpH-driven uptake of amines by liposomes. The neuroleptics haloperidol and chlorpromazine had inhibitory effects on the ΔpH-driven uptake of tyramine (Table 3). They thus appeared to affect not only the carrier-mediated process but also non-specific diffusion of amines through the membrane. Additional evidences supporting this view were provided by the effect of these drugs on the uptake of amines by liposomes [19]. Liposomes in which the interior was acidic with respect to the external medium took up amines by diffusion of their neutral form through the phospholipid bilayer since in this case no specific carrier was involved. Noradrenaline (Fig. 2) and tyramine were accumulated into liposomes, but it may be noted that tyramine was taken up at faster rates than noradrenaline (Table 5).

Chlorpromazine, at doses which blocked the ΔpH-driven uptake of catecholamines by chromaffin granule membranes, efficiently blocked tyramine and noradrenaline uptake by liposomes (Table 5). High concentrations (10 μM) of reserpine had also an

Table 3. ΔpH-driven amine uptake by 'ghosts'

Amine (10 μM)	Uptake in absence of drug*			Drug inhibition (%)†					
	Rate of uptake (pmoles/min/mg protein)	Time for reaching plateau (sec)	Total amount accumulated (pmoles/mg protein)	Tetrabenazine (1 μM)	Tetrabenazine (10 μM)	Reserpine (1 μM)	Reserpine (10 μM)	Haloperidol (40 μM)	Chlorpromazine (100 μM)
Methylamine		<5	810 ± 90		3 ± 3		46 ± 3	48 ± 5	60 ± 6
Tyramine	6700 ± 1500	10–15	830 ± 36	0 ± 2	5 ± 4	14 ± 5	42 ± 8	36 ± 10	45 ± 3
Noradrenaline	560 ± 105	240	855 ± 27	80 ± 5	80 ± 5	63 ± 4	63 ± 6	73 ± 15	86 ± 5

* 'Ghosts' (2 mg protein/ml) prepared at pH 5.9 were incubated at pH 8.5 as described in Materials and Methods. Aliquots were withdrawn at 5, 10, 15 and 30 sec for methylamine and tyramine uptakes and at 20, 40, 60, 90, 180 and 240 sec for noradrenaline uptake. Methylamine uptake was at the plateau at 5 sec and was too fast to allow rate determination. Tyramine uptake reached a plateau at 10–15 sec and the value at 5 sec was used to determine the rate of uptake. For noradrenaline the uptake was linear for 40–60 sec. The final concentrations of the accumulated amines were estimated to be 350, 360 and 370 μM for methylamine, tyramine and noradrenaline, respectively.

† The time course in the presence of drugs was measured under the same conditions and inhibition values determined at the plateau. Controls without drugs were adjusted for ethanol. Up to 100 μM, no chlorpromazine precipitation was observed at pH 8.5. Results are means ± S.E. of at least three experiments.

Table 4. Kinetic parameters of catecholamine uptake*

Nature of uptake	Catecholamine	V (pmoles/min/mg protein)	$S_{0.5}$ (μM)	h
ATP-induced	Noradrenaline	4300	140	0.85
ΔpH-driven	Noradrenaline	5650	140	0.85
ATP-induced	Tyramine	3000	160	0.70

* 'Ghosts' concentration was 2.2 mg protein/ml. The kinetic parameters were derived from Hill and Eadie plots of initial velocities of uptake. V is maximal uptake velocity; $S_{0.5}$, substrate concentration at half maximal uptake velocity; h, Hill coefficient. The correlation coefficients for the Hill plot were 0.999, 0.998 and 0.990 for noradrenaline ATP-induced, noradrenaline ΔpH-driven and tyramine ATP-induced uptakes, respectively.

inhibitory effect which was nevertheless less marked than that of chlorpromazine. Tetrabenazine had no effect either on noradrenaline or on tyramine transport.

DISCUSSION

Involvement of a specific carrier in noradrenaline and tyramine uptake. The present communication describes the uptake of monoamines by chromaffin granule 'ghosts' under different and well-defined conditions. In the ATP-dependent reaction there is no initial ΔpH between the two sides of the membrane. Addition of ATP induced only a limited and slow acidification, as shown by limited methylamine accumulation. On the other hand, during the ΔpH-driven uptake, granule energization was by-passed and noradrenaline and tyramine accumulations were similar to that of methylamine and were thus related only to the magnitude of the imposed ΔpH. With these two model systems, the mechanism of amine permeation has been shown to be dependent not only on the nature of the amine but also on that of the driving force. Thus tyramine was transported through a specific carrier during the ATP-driven process but this carrier was not involved in the ΔpH-driven process. We define a carrier-mediated uptake as being slow (about 400 pmoles/min/mg protein at 10 μM substrate concentration), saturable with respect to amine concentration and inhibited by tetrabenazine and reserpine at 1 μM. The similar values of h, $S_{0.5}$ and V obtained for tyramine and noradrenaline suggest a unique carrier for the two amines.

Our measurement of V for ATP-driven noradrenaline uptake gave results similar to the figures of 7.7 and 6.0 nmoles/min/mg protein obtained by Phillips [5] and Da Prada *et al.* [2], whereas $S_{0.5}$ values agree with Da Prada *et al.* and Slotkin [25] but are higher than the 18 μM figure noted by Phillips. Phillips has also reported a positive cooperativity for noradrenaline uptake, which contrasts with the negative effect that we have observed. Such discrepancies might be due to differences in the vesicle preparation, which have been resealed in different media. In contrast with tyramine, noradrenaline ΔpH-driven uptake is totally carrier-mediated. The differences observed between the two amines can be attributed to the lower passive permeability of lipidic membranes to noradrenaline. This hypothesis is supported by our experiments with liposomes (Table 5) which indicate a difference of at least one order of magnitude in the passive permeabilities towards the two amines. The lack of passive permeability may be related to the presence of two hydroxyl groups on the phenyl ring of noradrenaline since this amine shares this property with other catecholamines such as dopamine and adrenaline which also show an ATP-dependent uptake completely blocked by reserpine [2].

The fast tetrabenazine-resistant uptake of tyramine observed after a pH jump is thought to be the result of non-specific diffusion of the neutral form of tyramine across the membrane and of its accumulation inside the vesicle in accordance with the pH gradient. Under different conditions, such a non-specific ΔpH-driven transport might be superimposed on the ATP-dependent uptake. With intact chromaffin granules and in the case of tyramine and metaraminol, the pH-difference existing between the granule interior and the medium would induce a non-specific process which might explain the limited

Table 5. ΔpH-driven catecholamine uptake by liposomes*

Liposomes	Catecholamine (10 μM)	Uptake (pmoles/min/mg phospholipid)	Inhibition (%) Tetrabenazine (10 μM)	Reserpine (10 μM)	Chlorpromazine (100 μM)
I	Noradrenaline	15.7	0	26	72
II	Noradrenaline	8	0	43	78
II	Tyramine	61	0	64	79

* Liposomes I (obtained by vaporization of an ether solution of phospholipid) and liposomes II (obtained by sonication of an aqueous solution of phospholipid) were prepared at pH 5.0 and titrated to pH 8.0 as described in Materials and Methods. Uptake was measured after 20 min of incubation with noradrenaline or 4 min with tyramine and is expressed as pmoles/min/mg phospholipid for comparison, but these values are not intended to be rate measurements.

or lack of stimulation by ATP and the partial inhibition by reserpine observed in this case [26, 27].

Differences in the drug effects. Our studies clearly showed that the drugs used in the present communication did not affect, at concentrations which blocked monoamine uptake, chromaffin granule energization. They were neither inhibitors of the H^+-translocase activity nor uncouplers (H^+-ionophores) since ΔpH and $\Delta\psi$ generations were unaffected. It was assumed, therefore, that the drugs only altered the passage of catecholamines through chromaffin granule membrane.

Differences have been noted in the effects of the various drugs. Throughout this study tetrabenazine (1–10 μM) inhibited only the carrier-mediated process. Reserpine at low doses (1 μM) was also a specific inhibitor of the catecholamine carrier. At higher doses (10 μM), this drug also affected processes not mediated by the catecholamine carrier, such as the ΔpH-driven uptake of tyramine by 'ghosts' or liposomes or of noradrenaline by liposomes. In this regard, reserpine bears resemblance to the neuroleptics haloperidol and chlorpromazine. This effect may be interpreted as a decrease of the rate of passive amine fluxes. It may also result from a perturbation of the imposed pH gradient since the total amount of accumulated amine was decreased. The drugs would thus perturb the transient ΔpH imposed on weakly buffered 'ghosts', but would not destroy the pH gradient continuously generated by the proton pump in the well buffered granules. Neuroleptics also blocked the catecholamine carrier, since they inhibited the ATP-driven uptake of tyramine and noradrenaline without affecting energization. This effect is not surprising since numerous reports [28, 29] have described inhibition of facilitated fluxes by neuroleptics, detergent and anesthetics.

Inhibition by chlorpromazine of the ATP-induced adrenaline uptake by 'ghosts' has recently been explained by an uncoupling effect of the drug [30]. Nevertheless, it has to be noted that the concentration of chlorpromazine reported to affect the $\Delta\psi$ involved in the mechanism of uptake [13], i.e. that observed after addition of ATP, was higher than the concentration which blocked the active transport. We thus suggest that chlorpromazine presents not only a non-specific effect which increases the permeability of the membrane to protons, as proposed in [30] and as indicated by its effect on monoamine uptake by liposomes or ΔpH-driven uptake of methylamine and tyramine by 'ghosts', but also inhibits the carrier-mediated process. The fact that 'ghost' ΔpH-driven uptake of noradrenaline is more sensitive to chlorpromazine than tyramine is further evidence of an inhibition of the carrier.

Mechanism of catecholamine uptake. The basic question is the relative importance of the ΔpH and the $\Delta\psi$ generated by the H^+-translocase as driving forces of catecholamine uptake. Although not intended in that purpose, the present communication gives some insight on that problem. We have shown that methylamine, which is a pH probe and which is not transported through the catecholamine carrier, is not significantly accumulated by the ATP-dependent process (Table 2). This result suggests that, in the absence of an added permeant anion, the pH gradient generated in 'ghosts' by the proton pump is weak and cannot induce by itself an accumulation of methylamine comparable to that of specifically-transported amines. The uptake of tyramine is also interesting in this respect. As noted before, in response to an imposed ΔpH this amine was transported by the same non-specific route as methylamine. But when 'ghosts' were energized by addition of ATP, tyramine was transported by the specific

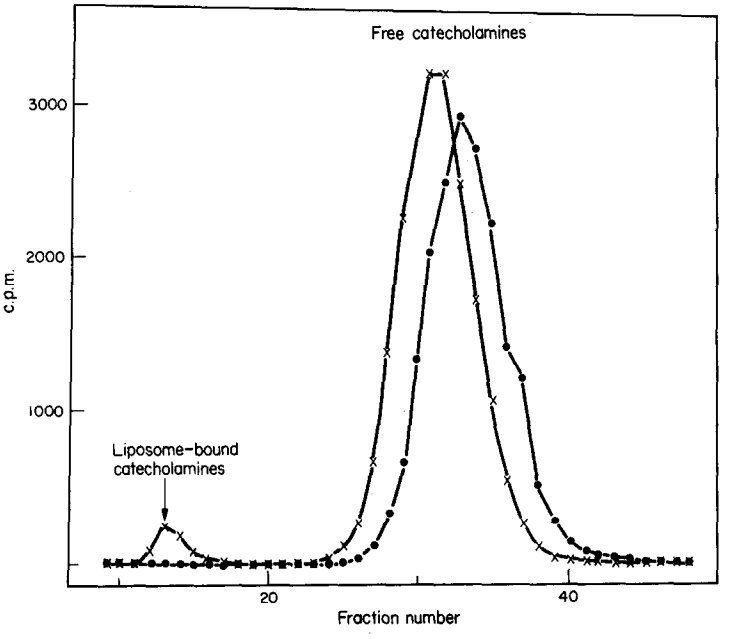

Fig. 2. Separation of liposome-bound and free [^{3}H] noradrenaline on Sephadex G-50 columns. Type I liposomes prepared at pH 5.0 were incubated with [^{3}H]noradrenaline, either at pH 8 (×) or at pH 5 (●).

carrier and this process was efficient whereas the non carrier-mediated uptake of methylamine was very limited. Under these conditions the carrier-mediated process thus appears to be an absolute requirement for active transport. This observation is consistent with the hypothesis involving the potential difference as a driving force in the mechanism of uptake [6, 13] since such an hypothesis assumes net exit of positive charges coupled with catecholamine influx, an exchange mechanism which implies participation of the catecholamine carrier [6].

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