5-Hydroxytryptamine Transport by the Bovine Chromaffin-Granule Membrane

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5-Hydroxytryptamine is accumulated by resealed chromaffin-granule 'ghosts' if a pH gradient (acid inside) is imposed across their membranes by preincubating them at low pH. This uptake, like that driven by MgATP, is reserpine- and uncoupler-sensitive. This strongly suggests that catecholamines are taken up by intact granules in response to a pH gradient. In line with this, it is shown that 5-hydroxytryptamine decreases the pH gradient generated in the presence of MgATP, an effect that is inhibited by reserpine; nigericin, which discharges the pH gradient in the presence of K^+ , inhibits uptake. Permeant anions, however, also inhibit uptake. It is suggested that this may be because they permit equilibration of amine cations directly across the membrane, down concentration gradients.

The membranes of chromaffin granules, the catecholamine-storage organelles of the adrenal medulla, transport biogenic amines, such as the catecholamines and 5-hydroxytryptamine, against large concentration gradients in the presence of MgATP (Phillips, 1974*a,b*). Furthermore they contain a proton-translocating ATPase (Casey *et al.*, 1977; Phillips & Allison, 1978). In the present paper I consider whether these two activities are linked, as suggested by Radda and his colleagues (Bashford *et al.*, 1976; Casey *et al.*, 1977).

There is no simple stoichiometric relationship between catecholamine transport and ATP hydrolysis (Taugner, 1972*a*). The processes have different pH optima, as might be expected if the membrane contains an amine-specific permease. Attempts to modify the rate of ATP hydrolysis and to compare this with catecholamine uptake depend on there being a single ATPase in the membrane, and on the system being tightly coupled. Although it is likely that the membranes contain a single ATPase, this has not been proved (Phillips & Allison, 1978).

In the present work I have investigated the problem by using resealed chromaffin-granule 'ghosts', membranes that have been resealed in the absence of the components of the granule matrix. I show that pH gradients imposed across the membrane can drive amine uptake, that reagents affecting the proton gradient across the membrane affect catecholamine transport in a predictable way, and that the rate of

proton translocation across the membrane is similar to the rate of amine accumulation.

In this work I have used 5-hydroxytryptamine as a substrate for the transport process. The catecholamine transporter accepts this as a substrate with high affinity (Phillips, 1974*b*), making it a useful model compound for investigating catecholamine uptake.

Experimental

Materials

All materials used have been described previously (Phillips, 1974*a*, 1977; Phillips & Allison, 1978).

Methods

The preparation of chromaffin-granule 'ghosts', and other methods were described in the preceding paper (Phillips & Allison, 1978). Incorporation of 5-hydroxytryptamine by suspensions of 'ghosts' was measured by filtration of samples through cellulose nitrate filters (0.45 μ m pore size) as described by Phillips (1974*a*). Details of incubations are given in the Figure legends.

Measurement of pH gradient and membrane potential. The method used has been described in the preceding paper (Phillips & Allison, 1978). The incubation medium for the 'ghosts' (0.34 mg in 2 ml) contained 0.3 M-sucrose, 30 mM-Hepes, pH 7.1, 6 mM-ATP, 3 mM-MgSO₄, 25 μ M-KCNS, 12 μ M-methylamine hydrochloride, 0.5 mM-choline chloride and [³H]inulin (2.5 μ g/ml, 140 μ Ci/mg). Incubation was continued for 5 min at 37°C. All measurements were made in duplicate.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid; ATPase, adenosine triphosphatase; Δp , protonmotive force; $\Delta\psi$, membrane potential; ΔpH , pH gradient.

Results

Uptake of 5-hydroxytryptamine in response to an imposed pH gradient

If amine movement is linked to the generation of a transmembrane pH gradient, it should be possible to demonstrate that an imposed gradient can be utilized in place of ATP hydrolysis. Nichols & Deamer (1976) have shown that egg phosphatidylcholine liposomes prepared at pH 5.0 will accumulate catecholamines on being transferred to a medium at pH 8.0; this accumulation, corresponding to the distribution of a weak amine in response to the imposed pH gradient, is relatively slow, continuing for up to 90 min, with a time for half-maximal accumulation of about 5 min. In this case the permeability of the membrane is presumably rather low, and decay of the pH gradient occurs with a half-time of about 30 min.

Fig. 1 shows some results obtained in a series of experiments with chromaffin-granule 'ghosts'. Granules were lysed on a hypo-osmotic Sephadex

column and purified by centrifugation through 0.4M-sucrose (Phillips, 1974a). This removes mitochondria and provides a preparation of resealed membranes which accumulate 5-hydroxytryptamine in the presence of ATP, and are osmotically active (Phillips, 1977). These 'ghosts' were incubated at 37°C for 15 min in a sucrose medium buffered at pH 5.5. They were then transferred to media buffered at higher pH containing 5-hydroxy[¹⁴C]tryptamine. Samples were withdrawn at intervals for assessment of amine uptake by the standard catecholamine-transport assay (Phillips, 1974a).

5-Hydroxytryptamine was rapidly accumulated (half-maximal within about $\frac{1}{2}$ min) in response to the imposed pH gradient. It is likely that this is because the preincubation of the 'ghosts' results in a decrease in their internal pH, probably by a slow exchange of protons for Na⁺ ions, since the membrane is likely to be impermeable to the zwitterionic buffers used (Good *et al.*, 1966). The exact value of the internal pH reached is not known; it will not be as low as pH 5.5, since the 'ghosts'

Fig. 1. 5-Hydroxytryptamine uptake by 'ghosts' in response to an imposed pH gradient

Chromaffin-granule 'ghosts' (0.5 ml, 0.56 mg/ml) were incubated for 15 min at 37°C in a medium containing 0.3M-sucrose and 23 mM-Mes, pH 5.52. Samples were then transferred to media of different pH, buffered with 44 mM-Hepes and containing 22 μ M-5-hydroxy[¹⁴C]tryptamine (25 μ Ci/ μ mol) at 37°C. Uptake was assayed in five media with the pH values shown in the Figure. (b) Each medium was supplemented with either 10 μ M-reserpine or 10 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Results are shown for uptake into the medium at pH 8.26: control medium (●); plus reserpine (○); plus carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Δ). A blank value obtained by lysing the 'ghosts' with water before filtration has been subtracted from all experimental values (point shown at zero time; the first experimental sample was taken from each assay 0.5 min after transfer).

originally contain about 50 mM-Hepes buffer at pH 7.0 as discussed in the preceding paper (Phillips & Allison, 1978). However, taking the internal water space of the 'ghosts' to be $4 \mu\text{l}/\text{mg}$ of protein, an internal pH of approx. 6.0–6.3 can be calculated from the data for 5-hydroxytryptamine accumulation at the three highest pH values in Fig. 1(a). This seems plausible; the fact that the amount of accumulation increases with an increasing pH gradient supports the overall picture of transport of the amine in response to such a gradient.

The incorporation is inhibited by the uncoupler carbonyl cyanide *p*-trifluoromethoxy phenylhydrazide (Fig. 1b). The presence of the uncoupler increases the background of 5-hydroxy[^{14}C]tryptamine trapped on the filters, however. This may be because the uncoupler, a hydrophobic weak acid, promotes the binding of the amine to the lipid-rich membranes. Although not shown in Fig. 1, the presence of uncoupler inhibited incorporation at all pH values tested, giving background values similar to that shown in Fig. 1(b).

The incorporation was also partially inhibited by reserpine at all pH values tested. Reserpine was used at a concentration of $10 \mu\text{M}$, which effectively completely inhibits incorporation into 'ghosts' in the presence of ATP. Incorporation in the present experiment is of course very much less than that found in the presence of ATP (Phillips, 1974b), so that uptake in the presence of reserpine is a greater fraction of the control uptake in this case. It is generally thought that reserpine blocks the catecholamine carrier (see the Discussion section), but it is likely that the chromaffin-granule membrane has a fairly high permeability to 5-hydroxytryptamine, and that the amine is crossing the membrane by passive diffusion in this case. A similar phenomenon may explain the relative insensitivity to reserpine of tyramine transport (Slotkin & Kirshner, 1971; Phillips, 1974b).

This experiment establishes the plausibility of reserpine-sensitive catecholamine transport across chromaffin-granule membranes in response to a pH gradient; this would be generated in intact granules by the activity of the proton-translocating ATPase.

Effect of anions on 5-hydroxytryptamine transport

Taugner (1972b) investigated the effects of a variety of anions on adrenaline uptake by intact chromaffin granules. The experiments were very difficult to interpret, since the anions have a variety of effects on the granules themselves, owing to the granules' osmotic fragility. I therefore investigated the effect of adding a variety of potassium salts on the uptake of 5-hydroxytryptamine by resealed 'ghosts'. The results, presented in Fig. 2, clearly show that the best medium is the simplest, namely that in which the only components (apart from any trace

Fig. 2. *Effect of potassium salts on 5-hydroxytryptamine incorporation by chromaffin-granule 'ghosts'*

Chromaffin-granule 'ghosts' (0.13 mg of protein/ml) were incubated with 5 mM-ATP, 2.5 mM-MgSO₄ and $46 \mu\text{M}$ -5-hydroxy[^{14}C]tryptamine ($20 \mu\text{Ci}/\mu\text{mol}$) at pH 7.0 in buffered sucrose at 37°C. The media were supplemented with 40 mM solutions of potassium salts, as shown. Samples (100 μl) were removed at intervals for assay of 5-hydroxytryptamine incorporation.

contaminants adsorbed to the 'ghosts') are 0.3 M-sucrose, 10 mM-Hepes (sodium salt), 5 mM-ATP (sodium salt) and 2.5 mM-MgSO₄. Addition of 40 mM-potassium salts decreases the incorporation to an extent that depends on the permeability of the membrane to the anion.

Altering the anion has little effect on uptake in the medium of low ionic strength; 2 mM-MgSO₄, -MgCl₂ and -magnesium acetate in the presence of 6 mM-ATP lead to similar rates of 5-hydroxytryptamine uptake.

5-Hydroxytryptamine is a weak base, and most molecules will therefore be protonated at the pH of both the medium and the interior of the 'ghost'. Since it is actively concentrated within the 'ghosts', its uptake must be accompanied by the establishment of a potential difference across the membrane (inside positive). Alternatively, uptake must be accompanied by anion uptake or cation efflux. The latter seems unlikely, since the passive permeability of the membrane to Na⁺ (the only internal cation) is very low (Phillips, 1977), but a catalysed exchange mechanism is hard to rule out. This would have to be linked to a novel electrogenic sodium pump, since Na⁺/H⁺ exchange cannot be demonstrated.

Effect of inhibitors on 5-hydroxytryptamine transport

The effect of a variety of potential inhibitors of 5-hydroxytryptamine uptake by 'ghosts' is shown in Table 1. As pointed out by Bashford *et al.* (1975), mitochondrial uncoupling agents, such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide and

pentachlorophenol (which have lipophilic anions), are potent inhibitors. The mode of action of these compounds is discussed below.

Measurements of proton gradients

In the preceding paper (Phillips & Allison, 1978) measurements were made of pH and potential gradients generated across the chromaffin-granule membrane by ATP hydrolysis. In the present experiments, 'ghosts' were incubated in the basic sucrose medium as used for amine incorporation experiments. The effects of 5-hydroxytryptamine,

reserpine and uncoupler on such gradients are shown in Table 2.

Under conditions optimal for 5-hydroxytryptamine uptake, ΔpH is decreased by adding amine to the medium. By contrast, in the presence of reserpine, a potent inhibitor of uptake, ΔpH is increased.

Leakage of 5-hydroxytryptamine from 'ghosts'

To investigate the permeability of the 'ghost' membrane to 5-hydroxytryptamine, resealed 'ghosts' were loaded with radioactive amine and then diluted into warm buffered sucrose. Leakage of amine from the 'ghosts' was followed by filtering samples (Fig. 3).

Little amine leaks from controls. Inclusion of excess Mg^{2+} in the leakage medium, which completely inhibits amine uptake (Phillips, 1974b) but has no effect on proton translocation by the membrane ATPase (Phillips & Allison, 1978), slightly increases the leakage rate: the lower rate in the control is presumably due to continuation of uptake at a very low rate.

Inhibition of ATP hydrolysis by inclusion of EDTA, discharge of ΔpH by inclusion of an uncoupler, or immediate loss of ΔpH by acidification of the medium, lead to loss of amine at similar rates. As the rate of decay of ΔpH must vary greatly in these cases, this suggests that the rate of amine loss is determined by the rate at which the amine can cross the membrane. This rate is very much lower than is observed if the 'ghosts' are lysed osmotically by dilution into 10mM-Hepes (sucrose-free).

In Fig. 3(b) are shown the effects of a low concentration (30 μM) of the lipophilic anion tetraphenylboron, of a high concentration (40mM) of thiocyanate, and of reserpine. The two anions are discussed below; why leakage should be so rapid in the presence of reserpine, which does not affect the pH gradient or the ATPase is unknown.

Discussion

The experiment presented in Fig. 1 demonstrates that 5-hydroxytryptamine transport can result from a pH gradient imposed across chromaffin-granule

Table 1. *Effect of inhibitors on 5-hydroxytryptamine incorporation*

Incorporation of 5-hydroxytryptamine into 'ghosts' was measured by using 46 μM -5-hydroxy[^{14}C]-tryptamine (15 $\mu\text{Ci}/\mu\text{mol}$), 6mM-ATP and 2mM- MgSO_4 (10min incubations at 37°C). Three different preparations of 'ghosts' were used, with a mean ($\pm\text{s.d.}$) rate of incorporation of $89 \pm 16 \text{ nmol}/10 \text{ min}$ per mg of protein. All incorporation values are expressed as percentages of an appropriate control: controls contained ethanol (1%) when the inhibitor was added in this solvent, and 40mM-KCl for valinomycin and nigericin. Standard deviations refer to two different preparations of 'ghosts'.

Addition to medium	5-Hydroxytryptamine incorporation (% of control)
Ethanol (1%)	92.0 $\pm$ 0.6
Reserpine (10 μM)	2.5 $\pm$ 0.1
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazon (10 μM)	4.5 $\pm$ 0.6
Pentachlorophenol (20 μM)	3.6 $\pm$ 0.4
Atractyloside (100 $\mu\text{g}/\text{ml}$)	90.3 $\pm$ 1.2
Valinomycin (0.1 μM) + KCl (40mM)	110.0 $\pm$ 10
Nigericin (70 nM) + KCl (40mM)	8.8 $\pm$ 2.7
Quercetin (20 $\mu\text{g}/\text{ml}$)	19.4 $\pm$ 1.8
Dicyclohexylcarbodi-imide*	3.1 $\pm$ 0.3
Sodium tetraphenylboron (30 μM)	3.0 $\pm$ 2.0

* 'Ghosts' (0.4 mg of protein/ml) were preincubated for 30 min at 22°C with dicyclohexylcarbodi-imide (0.5mM).

Table 2. *Measurements of ΔpH , $\Delta\psi$ and Δp across the chromaffin granule 'ghost' membrane*
'Ghosts' were incubated for 5 min at 37°C in the medium described under 'Methods', with the additions shown below.

Additions	ΔpH (pH units)	$\Delta\psi$ (mV)	Δp (mV)
None	1.26	74	150
5-Hydroxytryptamine (100 μM)	1.13	78	146
5-Hydroxytryptamine (100 μM) + reserpine (10 μM)	1.49	64	153
5-Hydroxytryptamine (100 μM) + carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazon (10 μM)	1.01	26	87

Fig. 3. Leakage of 5-hydroxytryptamine from chromaffin-granule 'ghosts'

Chromaffin-granule 'ghosts' (0.07 mg of protein/ml) were incubated at 37°C in buffered 0.3M sucrose, pH 7.0, containing 62 μM-5-hydroxy[¹⁴C]tryptamine (10 μCi/μmol), 5.3 mM-ATP and 2.7 mM-MgSO₄, and uptake was followed (●). After 16 min (arrowed), 0.1 ml samples were transferred to 1 ml of buffered 0.3M sucrose at 37°C containing the following additions: (a) No addition (●); 4 mM-MgSO₄ (○); 10 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide (Δ); 6 mM-Mes, final pH 6.2 (■); 4 mM-EDTA (▲); 10 mM-Hepes (no sucrose) (□); (b) same experiment, but this part of the Figure is displaced for clarity: 40 mM-KCNS (▽); 10 μM-reserpine (⊙); 30 μM-sodium tetraphenylboron (▼).

membranes with inside acid. This is uncoupler- and reserpine-sensitive, in line with the idea that the catecholamine porter in the membrane is inhibited by reserpine; the action of reserpine is discussed below.

Uptake of the amine is not accompanied by the generation of large potential gradients (Table 2). This suggests that amine uptake is accompanied by anion uptake. It was therefore surprising to find that, in media of high ionic strength, permeant anions were inhibitory to uptake (Fig. 2), and furthermore that uptake is optimal in a low-ionic-strength medium in which the only anions are 6 mM-ATP and 2 mM-sulphate. Replacement of sulphate by 4 mM-acetate or -chloride has essentially no effect on the rate of uptake. The accompanying anion does not, however, seem to be ATP, since atractyloside, an inhibitor of ATP uptake by the 'ghosts' (Kostron *et al.*, 1977), has no effect on amine uptake in a medium in which the only other anion is sulphate (Table 1).

The explanation appears to be that the activated 'ghosts' have a mechanism for anion uptake linked to proton translocation; thus it is possible to demonstrate sulphate uptake by the ghosts, and swelling experiments suggest that the mechanism for anion

uptake is of very broad specificity (Phillips & Allison (1978). A possible explanation for the inhibitory effect of permeant anions is that the ions may act as ionophores for the positively charged 5-hydroxytryptamine molecule, permitting this to leak from the 'ghosts' down its concentration gradient. Tetraphenylboron is a potent inhibitor of uptake (Table 1) and leads to very rapid release of amine from loaded 'ghosts' (Fig. 3); thiocyanate and other permeant anions may have a similar effect, albeit much less potent. Thus, in spite of the large pH gradients generated in the presence of permeant anions (Phillips & Allison, 1978), we explain the inhibitory effects of these ions on uptake in terms of a specific interaction with the rather hydrophobic 5-hydroxytryptamine molecule, permitting the cation to equilibrate across the membrane. It is possible that uncouplers have a similar mode of action to some extent, since they also have hydrophobic anions: this might explain why uncouplers are potent inhibitors of uptake (Table 1), even though they fail to discharge the pH gradient completely (Phillips & Allison, 1978).

Dicyclohexylcarbodi-imide inhibits the chromaffin-granule ATPase, and amine uptake (Bashford *et al.*,

1976), presumably by reaction with a component of the enzyme. Most interesting among the compounds in Table 1, however, are valinomycin and nigericin. Although the former is without effect on amine uptake, the latter is completely inhibitory in the presence of high K^+ concentrations. Under these conditions the pH gradient is abolished, although the potential gradient remains, or is increased (Phillips & Allison, 1978). This again strongly suggests that amine uptake is linked to the pH gradient, and indeed direct measurements of ΔpH (Table 2) show that the gradient is partially discharged during amine uptake.

Mechanism of amine uptake

Amine uptake is linked to proton movements. In the chromaffin-granule membrane, proton movements are linked to anion movements either by passive anion entry in response to electrogenic proton uptake, or by direct coupling (Phillips & Allison, 1978). This solves the problem of why accumulation of a weak base by the 'ghosts' does not lead to generation of large potential gradients (Table 2). We cannot at the moment distinguish between a mechanism in which an uncharged (or zwitterionic) amine molecule is transported through the membrane, and that in which a proton is exchanged for a positively charged amine ion. Both mechanisms suggest that the extent of amine accumulation by 'ghosts' should depend on the pH gradient generated, but a simple relationship based on this would only be true if all the amine is present inside the 'ghosts' in free solution.

However, very rough calculations show the following.

1. Maximal rates of 5-hydroxytryptamine uptake are generally in the range 20–40 nmol/mg of protein in 5 min, corresponding to internal concentrations of 5–10 mM, when the external concentration is about 50 μM .

2. The pH gradient across the membrane is about 1.5 pH units after 5 min incubation in the presence of reserpine. During 5-hydroxytryptamine uptake this decreases to 1.1. Titration of 50 mM-Hepes (the probable concentration inside the 'ghosts') with HCl shows the latter value to be equivalent to about 15 mg-ions of H^+ /litre inside the 'ghosts', a decrease of about 2 mg-ions of H^+ /litre compared with the value in the presence of reserpine.

3. The maximal possible sulphate uptake over 5 min that can be deduced from experimental observations is about 15 mg-ions of SO_4^{2-} /mg of protein, or 4 mg-ions/litre (Phillips & Allison, 1978), an amount roughly equivalent to the uptake of amine.

4. During amine uptake a potential difference of about 80 mV (inside positive) can be measured across the membrane.

Although the sulphate uptake measured is not sufficient to account completely for the low $\Delta\psi$ measured, these values are consistent with the idea that uptake is dependent on protons, since the amine flux over 5 min is less than the proton flux, and can be roughly accounted for by the difference in proton concentration within the 'ghosts' in the presence and absence of reserpine.

In view of the link between the pH gradient and amine uptake, it is reasonable to ask what the role of electrogenic proton translocation may be. It is possible that this is linked with ATP transport, since this has been shown to be uncoupler-sensitive (Kostron *et al.*, 1977). ATP transport is insensitive to reserpine and is not coupled to amine translocation.

Intact chromaffin granules have a matrix maintained at pH 5.5 (Johnson & Scarpa, 1976; Casey *et al.*, 1977). Although accumulation of catecholamines by granules is greatly stimulated by addition of MgATP, there is nevertheless considerable reserpine-sensitive uptake in the absence of exogenous ATP, and in the presence of EDTA to inhibit hydrolysis of ATP released by granule lysis (Kirshner, 1962). These old observations suggest, therefore, that in intact granules, as in 'ghosts', a pH gradient alone is sufficient to give some measure of amine uptake.

Reserpine inhibition of amine uptake

Although it is attractive to think of reserpine as a tightly binding competitive inhibitor of the catecholamine porter [K_i about 0.3 μM (Phillips, 1974b)], the experiments in this paper suggest that its action may be more complex. If the pH-gradient-induced amine uptake in the presence of reserpine shown in Fig. 1(b) were merely due to passive diffusion through the membrane, one might expect uptake to continue for longer, and its initial rate to be lower. The curve resembles instead a resultant between a fairly rapid uptake and a high rate of amine leakage.

The experiment presented in Fig. 3 supports this interpretation, showing a very high rate of loss of amine from preloaded 'ghosts' when reserpine is added. This effect of reserpine, causing a high rate of loss of amine down its concentration gradient from the 'ghost' interior to the medium (rather than merely inhibiting its uptake) is not due to the alkaloid simply making the 'ghosts' generally leaky. It has no effect on swelling experiments with 'ghosts' and does not lead to loss of the ATP-induced proton gradient (Table 2). There is no evidence that reserpine or its analogue, harmine, is accumulated within 'ghosts' or granules (Green & Slotkin, 1973), and the mechanism of action of these inhibitors clearly merits further investigation.

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