Stoichiometry of catecholamine/proton exchange across the chromaffin-granule membrane

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Catecholamines are accumulated by bovine chromaffin-granule 'ghosts' in the presence of MgATP at 25°C. With low concentrations of catecholamine, ratios of internal to external amine concentration of up to 20 000 were obtained. These values fit well with a transport model in which amine accumulation is both electrogenic and dependent on a pH gradient across the membrane.

It has been known for many years that the secretory granules (chromaffin granules) of the bovine adrenal medulla contain the catecholamines adrenaline or noradrenaline at internal concentrations of about 0.5 M (Hillarp, 1959). Kirshner (1962) and Carlsson *et al.* (1963) demonstrated that these amines could be accumulated by the granules in an ATP-dependent process, and Radda and his colleagues (Bashford *et al.*, 1976; Casey *et al.*, 1976) suggested that ATP hydrolysis was linked to the transport function through a proton gradient. The ATPase has since been purified from chromaffin-granule membranes (Apps & Schatz, 1979) and has been shown to catalyse electrogenic proton movement across resealed membranes (Flatmark & Ingebretsen, 1977; Phillips & Allison, 1978; Schuldiner *et al.*, 1978).

The demonstration that amine transport is linked to the proton gradient depends on the sensitivity of the transport to inhibitors such as uncouplers that prevent proton gradient formation (Bashford *et al.*, 1976; Apps *et al.*, 1980b) and on the fact that H⁺ gradients imposed across resealed 'ghost' membranes lead to amine accumulation in the absence of ATP (Phillips, 1978; Schuldiner *et al.*, 1978). In the case of intact granules, however, which have a matrix buffered at pH 5.5 (Johnson & Scarpa, 1976a,b; Casey *et al.*, 1977) and therefore maintain a large pH gradient even in the absence of ATP, amine accumulation is markedly stimulated by adding exogenous ATP. This led some workers to propose that amine accumulation is primarily dependent on the membrane potential ($\Delta\psi$) generated by proton translocation (Holz, 1978; Njus & Radda, 1979). In a recent series of experiments with intact granules, however, Johnson & Scarpa (1979)

showed that both the proton concentration gradient (ΔpH) and the membrane potential are involved, and they proposed the mechanism shown in Scheme 1(a).

The negatively-charged carrier (X⁻) shown in Scheme 1(a) is not an essential part of the model, which, as pointed out by Njus & Radda (1978), is functionally equivalent to a protein catalysing exchange of a protonated catecholamine for two protons (CAH⁺/2H⁺ antiport; Scheme 1b). Qualitative support for these mechanisms comes from experiments showing that valinomycin-induced membrane potentials promote H⁺-gradient-driven amine uptake by resealed 'ghosts' (Scherman & Henry, 1979; Kanner *et al.*, 1980; Apps *et al.*, 1980a). In the present paper we report quantitative data that support this idea.

In recent papers Booth *et al.* (1979) and Mitchell *et al.* (1979) have examined the way in which metabolite accumulation by *Escherichia coli* is linked to a proton gradient. By using their notation, eqn. (1) can be derived from Mitchell (1973):

$$Z \log \left(\frac{[S]_i}{[S]_o} \right) = (m - n) \Delta\psi + nZ\Delta\text{pH} \quad (1)$$

where $[S]_i$ and $[S]_o$ are internal and external metabolite concentrations (assuming that no internal metabolite is bound), n is the proton/substrate stoichiometry, m is the charge on the metabolite, and Z (equal to $2.3 RT/F$, which has the value of 59 at 25°C) converts concentration ratios into mV. Both $\Delta\psi$ and ΔpH in eqn. (1) are measured as 'outside' values minus 'inside'. For the case of the chromaffin granule, the metabolite is catecholamine (CAH⁺), with $m = +1$. Protons are pumped to the interior [the stoichiometry of 2H⁺/ATP shown in Scheme 1 is suggested by the Data of Flatmark & Ingebretsen

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Scheme 1. Models for catecholamine transport into chromaffin granules

ATP hydrolysis is shown linked to translocation of two protons across the membrane (Flatmark & Ingebreetsen, 1977). In (a), the carrier, X^- , is shown as catalysing exchange of an unprotonated catecholamine (designated CA^0) for a proton (Johnson & Scarpa, 1979). In (b), a protonated catecholamine (CAH^+)/ $2H^+$ antiporter is shown.

(1977)], giving a negative value for $\Delta\psi$ and a positive value for ΔpH . Following Scheme 1(b), n takes the value +2, so that at equilibrium the catecholamine accumulation ratio is given by eqn. (2):

$$Z \log \left(\frac{[CA]_i}{[CA]_o} \right) = 2Z\Delta pH - \Delta\psi \quad (2)$$

where $[CA]$ is the total concentration of free catecholamine (the symbol CA^0 is used in Scheme 1 to denote unprotonated catecholamine). A similar expression has been derived by Kanner *et al.* (1980); it differs from that of Njus & Radda (1978) in that the proton concentration term given in their review has been squared to take account of the involvement of two protons in Scheme 1(a) and 1(b).

The application of eqn. (2) cannot be tested with intact granules, in which the activity coefficient of the internal catecholamine is far from unity, and the free concentration ($[CA]_i$) is unknown. In resealed 'ghosts', however, the bulk of the accumulated amine is believed to be free in solution, so that it is possible to measure the amine accumulation ratio and the values of ΔpH and $\Delta\psi$ under identical conditions.

Experimental

Materials

Radiochemicals were obtained from The Radio-

chemical Centre, Amersham, Bucks., U.K. Hepes and catecholamines were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. and other chemicals were from BDH Chemicals, Poole, Dorset, U.K. Membrane filters (cellulose nitrate) were from Sartorius, Göttingen, Germany. The pH of 1 M-Hepes was adjusted with 4 M-NaOH.

Methods

Resealed chromaffin granule 'ghosts' were prepared by lysis of crude bovine chromaffin granules in 10 mM-Hepes, pH 7.0; after adjustment of the sucrose concentration to 0.3 M, the 'ghosts' were purified by density-gradient centrifugation as described previously (Apps *et al.*, 1980a).

Accumulation experiments were performed as follows. The ghosts were incubated in a medium containing 0.3 M-sucrose, 30 mM-Hepes, pH 7.2, 10 mM-ATP (sodium salt; pH 7.2) and 5 mM-MgSO₄. Parallel incubations were supplemented with 3,4-dihydroxyphenyl[1-¹⁴C]ethylamine ([¹⁴C]dopamine) (0.12 μ M, 61 mCi/mmol), [¹⁴C]methylamine (2.2 μ M, 41 mCi/mmol), or K¹⁴CNS (2.7 μ M, 27 mCi/mmol). Incubations were terminated by chilling in ice for 5 min, followed by addition of non-radioactive dopamine, methylamine or KCNS to 80 μ M. Six replicate samples were then filtered through 13 mm-diameter cellulose nitrate filters (0.45 μ m pore size);

to eliminate possible losses of material on drying, the wet filters were transferred to scintillation vials and dissolved in 2 ml of a mixture (1:2, v/v) of Triton X-100 and scintillation fluid {5 g of PPO (2,5-diphenyloxazole) and 0.3 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre in toluene}. Total radioactivity in the incubation mixtures was determined by adding 0.5 ml of incubation medium to 3 ml of the scintillation mixture.

Catecholamines were determined fluorimetrically by the method of von Euler & Lishajko (1961), with adrenaline and noradrenaline bitartrate standards; protein was determined by the method of Bradford (1976). Catecholamine-distribution ratios were determined as follows. The catecholamine concentration inside the 'ghosts' ($[CA]_i$, in nmol/ μ l) is given by:

$$[CA]_i = [CA]_{\text{total}} \times \frac{\text{d.p.m.}_i}{\text{d.p.m.}_{\text{total}}} \times \frac{1}{v_i} \quad (3)$$

where $[CA]_{\text{total}}$ is the catecholamine concentration (nmol/ml) of the incubation, arising from catecholamines in the 'ghost' preparation plus added radioactive dopamine; d.p.m._i is the radioactivity (d.p.m.) trapped after filtering a suitable volume of 'ghosts', less the radioactivity trapped after filtering a similar volume of an unincubated control; d.p.m._{total} is the total radioactivity of the same volume of incubation medium; and v_i is the internal volume of the 'ghosts' in μ l/ml of incubation mixture. This value was derived from the protein concentration of the incubation and the previously determined internal volume of the 'ghosts' (3.65 μ l/mg of protein; Phillips & Allison, 1978), a value very close to that of 3.9 μ l/mg found for a rather similar 'ghost' preparation by Schuldiner *et al.* (1978).

The external catecholamine concentration at equilibrium ($[CA]_o$, also in nmol/ μ l) is given by:

$$[CA]_o = \frac{[CA]_{\text{total}}}{1000} \times \left(\frac{\text{d.p.m.}_{\text{total}} - \text{d.p.m.}_i}{\text{d.p.m.}_{\text{total}}} \right) \quad (4)$$

so that, by dividing eqn. (3) by eqn. (4):

$$\frac{[CA]_i}{[CA]_o} = \frac{\text{d.p.m.}_i}{\text{d.p.m.}_o} \times \frac{1000}{v_i} \quad (5)$$

where d.p.m._o is equal to (d.p.m._{total} - d.p.m._i). Methylamine and thiocyanate concentration ratios were derived similarly. They were assumed to distribute according to the H⁺ gradient and the membrane potential respectively (Phillips & Allison, 1978). Approx. 95% confidence limits for values in mV in Table 1 were obtained from the differences between the mean d.p.m. of experimental samples and controls ($\pm$ twice the standard error of the difference).

Results

Effect of 'ghost' concentration

In the cases of lactose and glutamate accumulation by *E. coli*, eqn. (1) was found to give a poor approximation of equilibrium substrate concentrations when suitable values of m and n were fitted. This was attributed to metabolite leakage through the membrane by a mechanism independent of the proton-linked permease (Booth *et al.*, 1979; Mitchell *et al.*, 1979). Catecholamines are known to permeate through phospholipid membranes (Nichols & Deamer, 1976). In order to decrease such leakage as far as possible we have carried out our experiments at 25°C rather than at 37°C. We have also used relatively low concentrations of 'ghosts'; this is because the 'ghost' preparation contains endogenous catecholamine carried over from the chromaffin-granule-lysis step (about 0.1–0.2 μ mol/mg of protein; intact granules contain about 10 μ mol of catecholamine/mg of membrane protein). Decrease of the 'ghost' concentration leads to a decrease in equilibrium internal amine concentration, thus decreasing the rate of amine efflux through the membrane by permease-independent diffusion.

The experimental design involves equilibration of the endogenous catecholamine across the 'ghost' membrane in the presence of MgATP. The values of the equilibrium concentrations are obtained by adding a very small concentration (relative to endogenous amine) of [¹⁴C]dopamine, the uptake of this by the 'ghosts' being measured by filtration. In preliminary experiments conducted under a variety of conditions, we found that radioactivity was taken up by the 'ghosts' at 25°C, with an equilibrium value being reached after about 2 h incubation (Fig. 1). In the experiment shown in Fig. 1, Δ pH was varied by addition of (NH₄)₂SO₄; this decreases the equilibrium concentration attained in the 'ghosts'. In other control experiments, omission of MgATP was shown to decrease the equilibrium concentration by more than 80%, and inclusion of uncoupler (10 μ M-carbonyl cyanide p-trifluoromethoxyphenylhydrazone) eliminated uptake completely.

We investigated the effect on the distribution ratio (after 3 h at 25°C) of varying the 'ghost' concentration; there is thus concomitant variation of the endogenous catecholamine concentration. The distribution ratio is plotted as a function of the external catecholamine concentration at equilibrium in Fig. 2(a) and of the equilibrium concentration inside the 'ghosts' in Fig. 2(b). The amine concentration ratio achieved is inversely proportional to the internal concentration over the range investigated, suggesting that there is indeed a 'leak' pathway that is dependent on the concentration of amine within the 'ghosts'. In subsequent experiments we therefore used as low a 'ghost' concentration as possible. By

contrast, we found that measurements of ΔpH and $\Delta\psi$ (calculated from the distribution ratios of [^{14}C]methylamine and K^{14}CNS respectively) were independent of 'ghost' concentration. In all experiments we used high concentrations of ATP, so that this was not significantly depleted during the incubations.

Fig. 1. Accumulation of dopamine by 'ghosts' 'Ghosts' ($7\mu\text{g/ml}$) were incubated at 25°C in a medium which was supplemented with $0.8\mu\text{M}$ -[^{14}C]dopamine. Accumulation of dopamine was monitored by filtering 0.2ml samples and measuring radioactivity. The basic medium (●) was modified by addition of $(\text{NH}_4)_2\text{SO}_4$ (○, 0.4mM ; △, 2.0mM).

Catecholamine-accumulation ratios

Results obtained with two different preparations of 'ghosts' are presented in Table 1. In the first experiment, values for $[\text{CA}]_i/[\text{CA}]_o$, ΔpH and $\Delta\psi$ were measured in parallel incubations with all conditions identical, apart from the radioactive tracers. Because the accumulation ratios for methylamine and thiocyanate are low, however, the protocol was varied in the second experiment; in this case the 'ghost' concentration was decreased for measuring catecholamine accumulation, but increased for measuring methylamine and thiocyanate accumulation, in order to improve accuracy.

In control incubations, catecholamine-distribution ratios of over 10000 were found. The contributions of ΔpH (about 1.5pH units) and $\Delta\psi$ are approximately equal at equilibrium, and the value of $(2Z\Delta\text{pH} - \Delta\psi)$, derived from eqn. (2), is $20\text{--}30\text{mV}$ in excess of the catecholamine-distribution ratio, a reasonable agreement in view of the errors in the method.

We investigated the effect of decreasing ΔpH or $\Delta\psi$. This can be done by adding an ammonium salt or a permeant anion (Johnson & Scarpa, 1979). Such treatment had a considerable effect on the equilibrium catecholamine-distribution ratio (Table 1), but it then became difficult to measure the new values of ΔpH and $\Delta\psi$. This is because the control values, at about $90\text{--}100\text{mV}$, are not very much in excess of 60mV (corresponding to methylamine and thiocyanate accumulation ratios of about 10), which is the limit of reliable measurement in this system. This is reflected in the wide range of the standard errors shown for Expt. 1. These are decreased

Fig. 2. Catecholamine distribution ratio at equilibrium

'Ghosts' ($0.5\text{--}80\mu\text{g/ml}$) were incubated for 3h at 25°C with $0.12\mu\text{M}$ -[^{14}C]dopamine. Catecholamine-distribution ratios ($[\text{CA}]_i/[\text{CA}]_o$) are plotted against (a) the external and (b) the internal catecholamine concentration at equilibrium.

Table 1. *Measurements of catecholamine distribution, ΔpH and $\Delta\psi$ at equilibrium*

Chromaffin-granule 'ghosts' were incubated for 3 h at 25°C. Protein concentrations were 7 $\mu\text{g}/\text{ml}$ (Expt. 1), 3 $\mu\text{g}/\text{ml}$ (Expt. 2, catecholamine accumulation) and 30 $\mu\text{g}/\text{ml}$ (Expt. 2, ΔpH and $\Delta\psi$ determinations). Values in parentheses are 95% confidence limits; in the case of $Z \log([CA]_i/[CA]_o)$, these were always within ± 1 mV of the values given.

Expt. no.	Additions to medium	$10^{-3} \times [CA]_i/[CA]_o$	$Z \log \frac{[CA]_i}{[CA]_o}$ (mV)	ΔpH (mV)	$\Delta\psi$ (mV)	$2Z\Delta pH - \Delta\psi$ (mV)
1	None	15.1	247	88 (81–93)	–92 (79–101)	267
	5 mM-NaI	22.9	257	97 (91–102)	–45 (0–92)	239
	0.4 mM- $(\text{NH}_4)_2\text{SO}_4$	8.9	233	56 (5–72)	–88 (74–97)	199
2	None	20.3	254	92 (85–97)	–102 (98–106)	285
	5.5 mM-NaI	20.3	254	103 (100–106)	–32 (0–66)	238
	0.2 mM- $(\text{NH}_4)_2\text{SO}_4$	11.3	239	86 (77–93)	–103 (98–108)	275
	0.6 mM- $(\text{NH}_4)_2\text{SO}_4$	8.6	232	70 (60–78)	–103 (99–107)	243

somewhat in Expt. 2, by using a higher concentration of 'ghosts' for these measurements.

Addition of iodide is seen to decrease $\Delta\psi$, but to increase ΔpH . This latter increase presumably accounts for the slight increase found in $[CA]_i/[CA]_o$, in view of the great influence of ΔpH in eqn. (2). By contrast, addition of NH_4^+ decreases ΔpH , has little effect on $\Delta\psi$ and decreases $[CA]_i/[CA]_o$.

Conclusion

The remarkably high values for the equilibrium distribution of catecholamine found in the present study are compatible with models for transport such as those shown in Scheme 1, in which the permease is considered as an electrogenic exchanger, catalysing protonated amine/ 2H^+ antiport or free amine/ H^+ antiport. As pointed out by Johnson & Scarpa (1979) and Johnson *et al.* (1979), the effects of NH_4^+ and permeant anions at high concentrations implicate both ΔpH and $\Delta\psi$ in the transport mechanism, and the experiments reported here support their model, which was derived to account for this.

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References

- Apps, D. K. & Schatz, G. (1979) *Eur. J. Biochem.* **100**, 411–419
 Apps, D. K., Pryde, J. G. & Phillips, J. H. (1980a) *FEBS Lett.* **111**, 386–390

- Apps, D. K., Pryde, J. G., Sutton, R. & Phillips, J. H. (1980b) *Biochem J.* **190**, 273–282
 Bashford, C. L., Casey, R. P., Radda, G. K. & Ritchie, G. A. (1976) *Neuroscience* **1**, 399–412
 Booth, I. R., Mitchell, W. J. & Hamilton, W. A. (1979) *Biochem J.* **182**, 687–696
 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
 Carlsson, A., Hillarp, N.-Å. & Waldeck, B. (1963) *Acta Physiol. Scand.* **59**, Suppl. **215**, 1–38
 Casey, R. P., Njus, D., Radda, G. K. & Sehr, P. A. (1976) *Biochem J.* **158**, 583–588
 Casey, R. P., Njus, D., Radda, G. K. & Sehr, P. A. (1977) *Biochemistry* **16**, 972–977
 Flatmark, T. & Ingebretsen, O. C. (1977) *FEBS Lett.* **78**, 53–56
 Hillarp, N.-Å. (1959) *Acta Physiol. Scand.* **47**, 271–279
 Holz, R. W. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5190–5194
 Johnson, R. G. & Scarpa, A. (1976a) *J. Biol. Chem.* **251**, 2189–2191
 Johnson, R. G. & Scarpa, A. (1976b) *J. Gen. Physiol.* **68**, 601–631
 Johnson, R. G. & Scarpa, A. (1979) *J. Biol. Chem.* **254**, 3750–3760
 Johnson, R. G., Pfister, S., Carty, S. E. & Scarpa, A. (1979) *J. Biol. Chem.* **254**, 10963–10972
 Kanner, B. I., Sharon, I., Maron, R. & Schuldiner, S. (1980) *FEBS Lett.* **111**, 83–86
 Kirshner, N. (1962) *J. Biol. Chem.* **237**, 2311–2317
 Mitchell, P. (1973) *J. Bioenerg.* **4**, 65–91
 Mitchell, W. J., Booth, I. R. & Hamilton, W. A. (1979) *Biochem J.* **184**, 441–449
 Nichols, J. W. & Deamer, D. W. (1976) *Biochim. Biophys. Acta* **455**, 269–271
 Njus, D. & Radda, G. K. (1978) *Biochim. Biophys. Acta* **463**, 219–244
 Njus, D. & Radda, G. K. (1979) *Biochem J.* **180**, 579–585
 Phillips, J. H. (1978) *Biochem J.* **170**, 673–679

- Phillips, J. H. & Allison, Y. P. (1978) *Biochem J.* **170**, 661–672
- Scherman, D. & Henry, J.-P. (1979) *C.R. Acad. Hebd. Séances Sci. Ser. D* **289**, 911–914

- Schuldiner, S., Fishkes, H. & Kanner, B. I. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3713–3716
- von Euler, U. S. & Lishajko, F. (1961) *Acta Physiol. Scand.* **51**, 348–356