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Stoichiometry of H^+-Linked Dopamine Transport in Chromaffin Granule Ghosts†

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ABSTRACT: A proton-translocating adenosinetriphosphatase in adrenal medullary chromaffin granule ghosts can generate either a membrane potential (inside positive) or a pH gradient (inside acid). Dopamine uptake occurs in response to both the membrane potential and the pH gradient. The natural logarithm of the dopamine concentration gradient [ln (D_{in}/D_{out})] is linearly related to the membrane potential with a slope of $F/(RT)$. This dependence is not affected by the pH of the medium. ln (D_{in}/D_{out}) is linearly dependent on ln ($[H^+]_{in}/[H^+]_{out}$) with a slope of 2. These results indicate that dopamine is taken up via an exchange diffusion or antiport mechanism. The stoichiometry of this exchange is two H^+/dopamine cation and is independent of pH.

Catecholamines in the adrenal medulla are stored at high concentration (0.55 M) in the chromaffin granules. Consequently, the chromaffin granule membrane must maintain an enormous catecholamine concentration gradient. Catecholamine accumulation into the granules is driven by an inwardly directed proton-translocating adenosinetriphosphatase (ATPase).[1] Protons are pumped into the granules and then exchanged for catecholamines via an antiport or exchange dif-

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[1] Abbreviations used: ATPase, adenosinetriphosphatase; ATP, adenosine 5′-triphosphate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenyl hydrazone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential gradient; D_{in}/D_{out}, transmembrane dopamine concentration gradient.

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fusion mechanism (Njus & Radda, 1978; Njus et al., 1981). Uptake of catecholamines is an electrogenic process; two or more H^+ are released for each amine taken up (Holz, 1978; Njus & Radda, 1979; Johnson et al., 1979; Knoth et al., 1980; Apps et al., 1980; Kanner et al., 1980; Scherman & Henry, 1980).

The catecholamine gradient achieved depends on the membrane potential ($\Delta\psi$), the pH gradient (ΔpH), and the stoichiometry of the H^+/catecholamine exchange (Njus & Radda, 1978; Knoth et al., 1980; Phillips & Apps, 1980; Njus et al., 1981). If the stoichiometry is n protons/catecholamine cation, the theoretical catecholamine gradient (D_{in}/D_{out}) at equilibrium is

$$\frac{D_{in}}{D_{out}} = \left(\frac{[H^+]_{in}}{[H^+]_{out}}\right)^n e^{(n-1)F\Delta\psi/(RT)} \quad (1)$$

To eliminate complications caused by internal binding and exchange, catecholamine gradients are best measured in chromaffin granules that have been lysed and resealed to form ghosts. Depending on the medium in which the ghosts are suspended, one can correlate the catecholamine gradient with either $\Delta\psi$ or ΔpH. If the ghosts are suspended in an anion-free medium (sucrose), ATP addition will generate a membrane potential but not a pH gradient (Knoth et al., 1980). ln (D_{in}/D_{out}) should, therefore, be linearly dependent on $F\Delta\psi/(RT)$ with a slope of $n - 1$. If, however, the ghosts are suspended in a KCl medium, ATP addition will create a pH gradient, but $\Delta\psi$ remains constant (Knoth et al., 1980). Therefore, ln (D_{in}/D_{out}) should be linearly related to ln ($[H^+]_{in}/[H^+]_{out}$) with a slope of n.

In preliminary experiments, we found that the dependence of epinephrine uptake on $\Delta\psi$ suggests a stoichiometry of two H^+/epinephrine cation (Knoth et al., 1980). Because dopamine is transported more quickly than epinephrine (DaPrada et al., 1975), it is a better substrate for investigating the stoichiometry of uptake. We show here that the stoichiometry of dopamine uptake is two H^+/dopamine cation whether uptake is driven by the membrane potential or the pH gradient.

Materials and Methods

Chromaffin granule ghost membranes were prepared from bovine adrenal medullas as described previously (Njus & Radda, 1979). These ghosts were formed in 0.2 M Tris–phosphate, pH 7.0, to make the internal space well buffered. All experiments were completed within 12 h of slaughter.

[^{3}H]Dopamine uptake (Figures 1–3) was determined by collecting ghosts on cellulose acetate filters (25-mm diameter, 0.45-μm pore size). A total of 100 μL of [^{3}H]dopamine (5 mM, 50 μCi/mL) was added to 10 mL of a ghost suspension; 1-mL samples were periodically filtered with gentle suction and washed with ~2 mL of cold buffer. Filters were placed in 10 mL of scintillation fluid, and radioactivity was counted on a Beckman LS100C scintillation counter. A known amount of [^{3}H]dopamine was also counted to convert counts per minute to nanomoles of dopamine.

The dopamine concentration gradient (D_{in}/D_{out}) was determined from the distribution of [^{14}C]dopamine. The pH gradient was calculated from the [^{14}C]methylamine distribution (eq 2), and membrane potential was calculated from the

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{[CH_3NH_3^+]_{in}}{[CH_3NH_3^+]_{out}} \quad (2)$$

$$\Delta\psi = \frac{RT}{F} \ln \frac{[SCN^-]_{in}}{[SCN^-]_{out}} \quad (3)$$

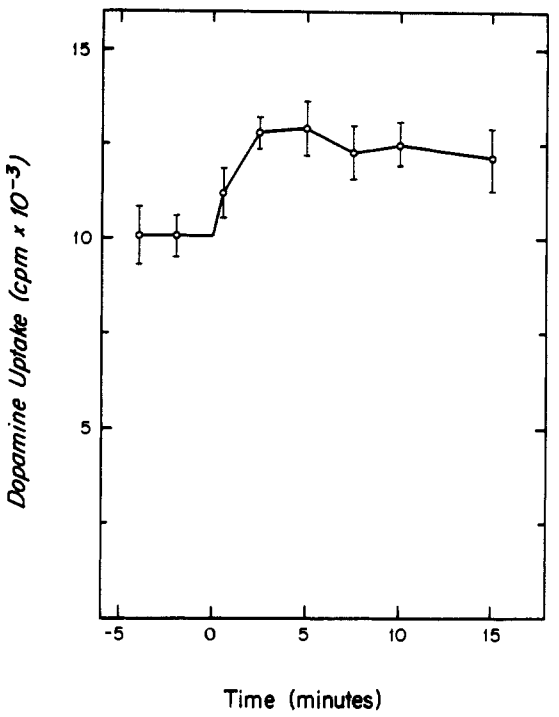

FIGURE 1: Dopamine uptake in response to a K^+ diffusion potential. A 1-mL sample of ghosts (3.76 mg of protein) was diluted into 9 mL of 0.4 M sucrose and 40 mM Hepes–Tris buffer, pH 7.4. At $t = -30$ min, 100 μL of [^{3}H]dopamine and 25 μL of 200 μM valinomycin in ethanol were added. The samples were incubated at 25 °C for 26 min. At $t = -4$ and -2 min, 1-mL samples were assayed for unstimulated dopamine uptake. At $t = 0$, 0.8 mL of 0.5 M K_2SO_4 was added. Periodically, 1-mL samples were assayed for dopamine uptake. Dopamine uptake was assayed as described under Materials and Methods. Points are each the average of six trials.

[^{14}C]thiocyanate distribution (eq 3) with RT/F taken as 25.7 mV. Internal and external concentrations of [^{14}C]dopamine, [^{14}C]methylamine, and [^{14}C]thiocyanate were determined as described before (Casey et al., 1977). Ghosts were incubated as specified with 1 μCi of ^3H_2O and the appropriate ^{14}C-labeled compound (0.72 μCi, 59 mCi/mmol of potassium [^{14}C]thiocyanate; 0.5 μCi, 49 mCi/mmol of [^{14}C]dopamine; or 0.67 μCi, 50 mCi/mmol of [^{14}C]methylamine). Following incubation for the specified time and temperature, the samples were centrifuged for 20 min at 25000g at the incubation temperature. Pellets and supernatants were processed as in Casey et al. (1977). Internal volume was calculated from the protein concentration by assuming 3 μL/mg of protein. This value is consistently obtained in measurements of [^{14}C]-sorbitol-excluding volume.

Protein concentration was assayed with biuret reagent as described by Casey et al. (1976). In all figures and tables, error limits are standard deviations calculated from multiple samples. In Figures 4 and 5, straight lines are least-squares fits to the data points.

Results

Adding K_2SO_4 to a chromaffin granule ghost suspension in the presence of the K^+ ionophore valinomycin creates a transient (half-life ~2 min) K^+ diffusion potential (Njus & Radda, 1979). This diffusion potential causes a period of rapid dopamine uptake followed by a gradual return to equilibrium (Figure 1). When a permeant anion (SCN^-) was added to neutralize the K^+ or when valinomycin was omitted, uptake did not occur. Moreover, when K^+ was replaced by Na^+, which valinomycin carries much less readily than K^+, no uptake was observed. Thus, dopamine uptake into chromaffin

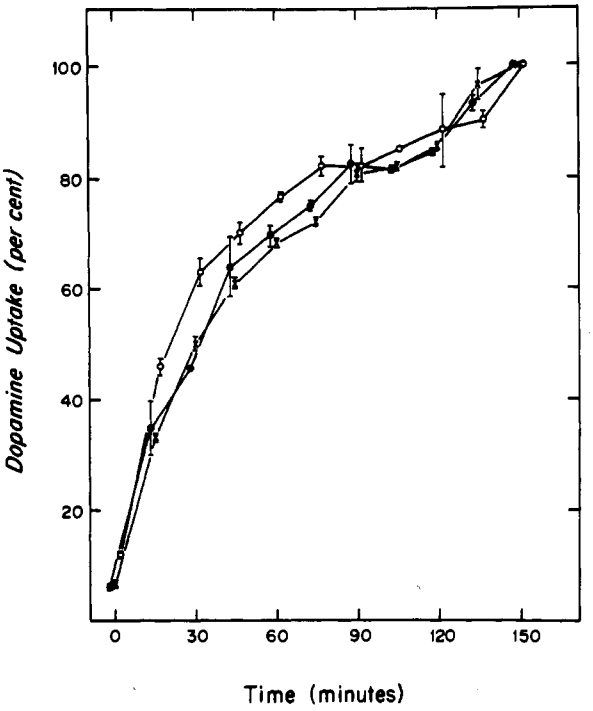

FIGURE 2: Effect of FCCP concentration on the rate of dopamine uptake. For each series of points, 0.25 mL of ghosts (4.28 mg of protein) was suspended in 5.75 mL of 0.4 M sucrose and 40 mM Hepes, pH 7.4. To this, 0.3 mL of 100 mM ATP, 100 mM $MgSO_4$, pH 7, 50 μL of [^{3}H]dopamine, and 5 μL of FCCP (× = 100 μM, ○ = 500 μM, or ● = 0 μM) were added, and the mixture was incubated at 20 °C. Aliquots (0.5 mL) were collected periodically and assayed as described under Materials and Methods. Dopamine uptake measurements were normalized to the value obtained after 150 min. These 100% values are 19.0 (●), 16.0 (×), and 7.7 (○) nmol/mg of protein.

granule ghosts occurs in response to a membrane potential (inside positive).

To measure the dependence of dopamine uptake on $\Delta\psi$ and ΔpH, it is necessary to have stable steady-state gradients. Steady-state membrane potentials and pH gradients can be generated by the proton-translocating ATPase. If ghosts containing a high concentration of buffer are suspended in sucrose medium, ATP addition generates a membrane potential (because proton translocation is electrogenic) but does not affect ΔpH (Knoth et al., 1980). If ghosts are suspended in KCl medium, ATP addition generates a pH gradient but has little effect on $\Delta\psi$. Because Cl^- is a permeant anion, it follows H^+ into the ghosts, lowering the internal pH and dissipating $\Delta\psi$. Thus, by suspending ghosts in the proper medium, we may observe dopamine uptake in reponse to either $\Delta\psi$ or ΔpH.

When ghosts are suspended in sucrose medium, dopamine uptake continues for at least 2 h (Figure 2). If the uncoupler FCCP is added to lower the steady-state membrane potential, the amount of dopamine uptake decreases. However, the rate of uptake appears to be proportional to the final equilibrium value. In particular, dopamine uptake after 60 min is ~70% of the final value at each $\Delta\psi$. Consequently, uptake after 60 min can be used as a measure of the equilibrium uptake level (see Discussion). The pH of the medium has a slight effect on the rate at which dopamine uptake equilibrates with $\Delta\psi$ but does not affect the final equilibrium level (Figure 3).

The relationship between the catecholamine gradient and $\Delta\psi$ was determined after a 60-min incubation (Figure 4). Dopamine gradients and $\Delta\psi$ were varied by adding different concentrations of FCCP. The natural logarithm of the dopamine gradient is linearly related to the natural logarithm of

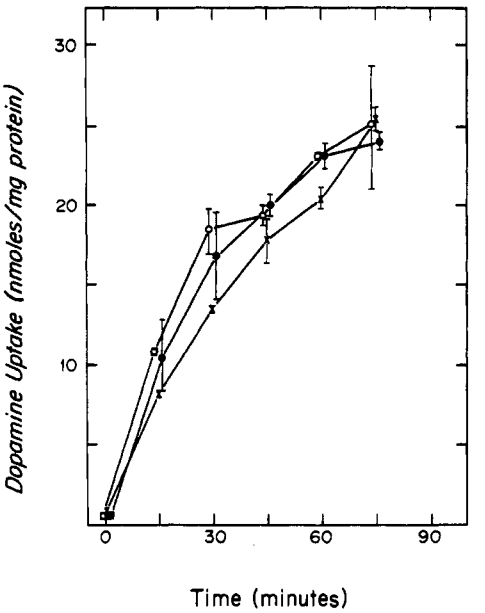

FIGURE 3: Effect of pH on dopamine uptake. For each series of points, 1 mL of ghosts (4.35 mg of protein) was suspended in 9 mL of 0.4 M sucrose and 40 mM Hepes at pH 6.8 (×), 7.4 (●), and 8.0 (○). To this, 0.5 mL of 100 mM ATP, 50 mM $MgSO_4$, pH 7, and 100 μL of [^{3}H]dopamine were added, and the mixture was incubated at 27 °C. Samples (1 mL) were collected periodically as described under Materials and Methods.

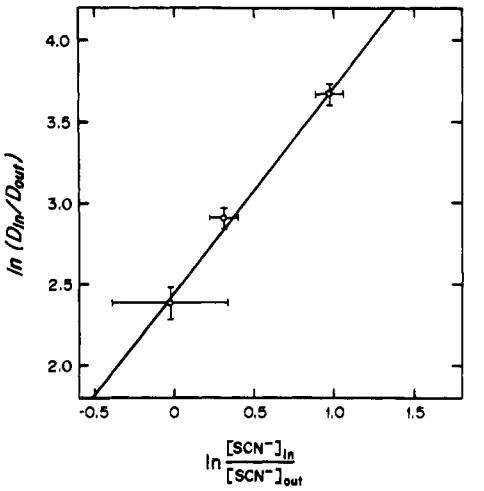

FIGURE 4: Dependence of dopamine uptake on $\Delta\psi$. D_{in}/D_{out} and $[SCN^-]_{in}/[SCN^-]_{out}$ were determined in the presence of 0.2, 0.05, or 0 nmol of FCCP. Each sample contained 0.25 mL of ghosts (1.65 mg of protein), 0.25 mL of 0.4 M sucrose, 100 μM KSCN, and 40 mM Hepes, pH 8.0. FCCP, 25 μL of 100 mM ATP, 100 mM $MgSO_4$, pH 7, and the appropriate radioactive tracers were added. Samples were incubated at 25 °C for 60 min and then processed as described under Materials and Methods. Plotted values are each the average of four determinations.

Table I: Dependence of Dopamine Uptake on ΔpH and $\Delta\psi$ [a]

variable	pH of medium	slope	no. of expt
$\Delta\psi$	6.8	1.10 ± 0.24	2
$\Delta\psi$	7.4	0.96 ± 0.34	2
$\Delta\psi$	8.0	1.14 ± 0.12	3
ΔpH	7.4	2.10 ± 0.04	2

[a] Slopes were obtained as in Figure 4 (variable = $\Delta\psi$) and Figure 5 (variable = ΔpH), and values obtained in replicate experiments were averaged.

the thiocyanate distribution. The slope is approximately 1.0 whether the pH of the medium is 6.8, 7.4, or 8.0 (Table I).

The relationship between the dopamine gradient and ΔpH was also determined after a 60-min incubation (Figure 5).

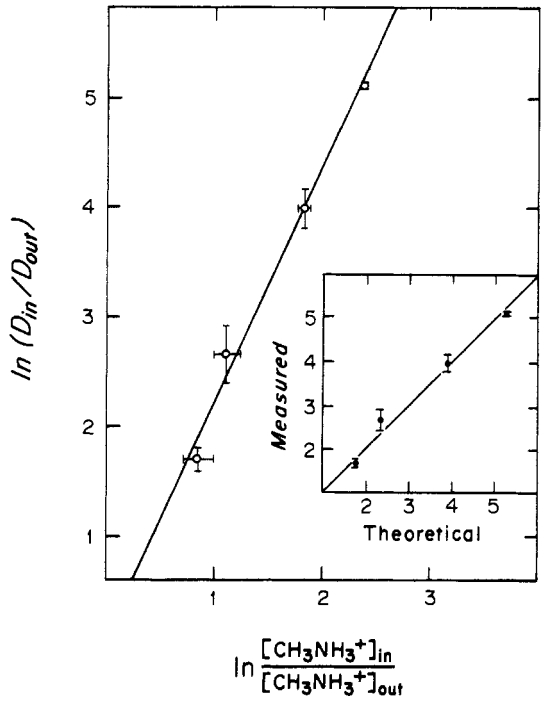

FIGURE 5: Dependence of dopamine uptake on ΔpH. D_{in}/D_{out}, $[CH_3NH_3^+]_{in}/[CH_3NH_3^+]_{out}$, and $[SCN^-]_{in}/[SCN^-]_{out}$ were determined in the presence of 0.15, 0.5, 1.5, or 0 nmol of FCCP. Ghosts (1.35 mg of protein in 0.25 mL) were added to 0.25 mL of 250 mM KCl, 150 mM sucrose, and 40 mM Hepes, pH 7.4. FCCP, 25 μL of 100 mM ATP, 100 mM $MgSO_4$, pH 7, and appropriate radioactive tracers were added. Samples were incubated for 60 min at 25 °C and then processed as described under Materials and Methods. Plotted values are each the average of two determinations. Values of Δψ varied from 2 mV in the presence of 1.5 nmol of FCCP to 12 mV in the absence of FCCP. Inset: Values of ln (D_{in}/D_{out}) measured above are plotted against theoretical values calculated from the methylamine and thiocyanate distributions by using eq 1 with $n = 2$.

Ghosts were suspended in KCl medium so ATP hydrolysis would generate a steady-state ΔpH rather than a membrane potential. Dopamine gradients and ΔpH were varied by adding FCCP. ln (D_{in}/D_{out}) and ln ($[CH_3NH_3^+]_{in}/[CH_3NH_3^+]_{out}$) are linearly related with a slope of approximately 2.0 (Table I). Measured ΔpH-dependent dopamine gradients correlate well with the theoretically predicted gradients (Figure 5, inset). The theoretical gradients were determined by substituting measured proton gradients and membrane potentials into eq 1 with $n = 2$.

Discussion

After it was recognized that catecholamine uptake into chromaffin granules is driven by a proton-translocating ATPase, Bashford et al. (1976) and Johnson & Scarpa (1976) proposed an electroneutral transport mechanism: the catecholamine, acting as a weak base, crosses the membrane in its unprotonated form and is reprotonated in the granule interior. However, a number of investigators (Holz, 1978; Njus & Radda, 1979; Johnson et al., 1979; Knoth et al., 1980; Kanner et al., 1980; Apps et al., 1980; Scherman & Henry, 1980) have found a correlation between catecholamine uptake and transmembrane potential. K^+ diffusion potentials (inside positive) drive uptake of epinephrine (Njus & Radda, 1979) and serotonin (Apps et al., 1980) into chromaffin granule ghosts. We have shown that a K^+ diffusion potential also drives dopamine uptake (Figure 1). When chromaffin granule ghosts are suspended in a medium devoid of permeant anions, the addition of ATP leads to the generation of a transmembrane potential (inside positive) but not to the creation of a pH gradient (Johnson et al., 1979; Knoth et al., 1980). Epinephrine and serotonin uptake occurs and therefore must be driven by the membrane potential. When increasing concentrations of the uncoupler FCCP are added, the membrane potential and epinephrine uptake decrease in parallel (Knoth et al., 1980). As shown in Figure 4, the same is true for dopamine uptake. From these observations, it is apparent that a transmembrane potential (positive inside) can drive dopamine uptake into ghosts. Consequently, dopamine, like epinephrine and serotonin, must be taken up via an electrogenic mechanism.

Since the uptake of the cationic dopamine is stimulated by a positive internal potential, uptake must be linked to the efflux of two or more other cations or to the influx of two or more anions. The fact that dopamine uptake is driven by the pH gradient (Figure 5) implies that the counterion is H^+ or OH^-. Since there is no thermodynamic distinction between OH^- symport and H^+ antiport, we have chosen to analyze dopamine transport as an exchange for H^+.

If n H^+ are exchanged for each dopamine cation, the equilibrium dopamine gradient is defined by eq 1. By substituting eq 2 and 3 into eq 1, we can express the dopamine gradient in terms of the measured $CH_3NH_3^+$ and SCN^- gradients:

$$\ln \frac{D_{in}}{D_{out}} = n \ln \frac{[CH_3NH_3^+]_{in}}{[CH_3NH_3^+]_{out}} + (n-1) \ln \frac{[SCN^-]_{in}}{[SCN^-]_{out}} \quad (4)$$

Equation 4 describes equilibrium. Because catecholamine transport is an extremely slow process, it is best to use dopamine, the catecholamine that is transported most rapidly (DaPrada et al., 1975). Even dopamine uptake is slow, however, so it is difficult to obtain reproducible equilibrium gradients. Fortunately, the requirement for equilibrium can be relaxed, since the amount of uptake after 60 min is proportional to the equilibrium dopamine gradient (Figure 2). This proportionality constant (~0.7) becomes an added constant (~−0.3) when the natural logarithm is taken in eq 4. Consequently, it does not affect the slopes of plots of ln (D_{in}/D_{out}) vs. ln ($[CH_3NH_3^+]_{in}/[CH_3NH_3^+]_{out}$) or ln ($[SCN^-]_{in}/[SCN^-]_{out}$). The slopes accurately reflect the stoichiometry n even if equilibrium has not been achieved. We have chosen to measure these slopes after 60 min because this period maximizes uptake while minimizing variability among replicate samples.

If ghosts are suspended in a medium devoid of permeant anions, ATP addition leads to uptake driven by Δψ, but the proton gradient remains constant (Knoth et al., 1980). Therefore, if the natural logarithm of the dopamine gradient is plotted against the natural logarithm of the SCN^- gradient, the slope will be $n - 1$ (eq 4). We have previously shown that this slope is 1 when epinephrine is used as the substrate (Knoth et al., 1980). Dopamine also gives a slope of 1 independent of the pH of the medium (Table I). This implies that the stoichiometry of Δψ-driven uptake is two H^+/dopamine cation.

If ghosts are suspended in a medium containing permeant anions, ATP addition leads to uptake driven by ΔpH while the membrane potential remains constant (Knoth et al., 1980). Therefore, if the natural logarithm of the dopamine gradient is plotted against the natural logarithm of the $CH_3NH_3^+$ gradient, the slope will be n. Under these conditions, the slope is approximately 2 (Figure 5). Thus, uptake depends more strongly on ΔpH than on Δψ. Moreover, as required theoretically, the Δψ dependence and ΔpH dependence of dopamine uptake both indicate an H^+/dopamine stoichiometry of 2.

When uptake is driven by ΔpH, the measured dopamine gradient is very close to the theoretical gradient calculated from eq 4 by using $n = 2$ (Figure 5, inset). Since uptake after 60 min is about 70% of the equilibrium value, the measured value of ln (D_{in}/D_{out}) should be within ~0.3 of the theoretical value. Phillips & Apps (1980) have also reported good agreement between measured dopamine gradients and gradients predicted by an H^+ exchange stoichiometry of 2.

The H^+/dopamine antiport stoichiometry remains constant over the pH range from 6.8 to 8.0 (Table I). By contrast, Ramos & Kaback (1977) found that the stoichiometry of H^+/proline symport in *Escherichia coli* membrane vesicles was 1 at pH 5.5 and 2 at pH 7.5. They interpreted this in terms of a translocator with an ionizable group having a p*K* of 6.8. Unlike the *E. coli* proline translocator, the catecholamine translocator does not appear to have an ionizable group with a p*K* in this range. Since the *E. coli* membrane must function in a variable environment, it is advantageous for transport to use only one H^+ when ΔpH is large and two H^+ when the driving force is smaller. Chromaffin granules, however, function in a stable cytoplasmic environment with a large and fairly constant pH gradient. Since a variable stoichiometry is not advantageous for chromaffin granules, it is not surprising that the stoichiometry is pH independent.

In our thermodynamic analysis of the equilibria between dopamine gradients and $\Delta\psi$ and ΔpH, we have not had to specify the translocation mechanism. We have analyzed transport as an exchange of two H^+/dopamine cation, but it should be recognized that the translocation stoichiometry could also be achieved by exchanging one H^+ for one neutral or zwitterionic dopamine molecule. Johnson & Scarpa (1979) have suggested that the translocator actually catalyzes the latter exchange. Because only a small fraction of the amine is in the neutral or zwitterionic form at physiological pH, this mechanism would require a translocator with an improbably low K_m (below 200 nM). Nevertheless, that scheme is consistent with our measured stoichiometry.

Finally, we should note that a stoichiometry of two H^+/dopamine accounts well for the high catecholamine concentration maintained in chromaffin granules. The intragranular catecholamine concentration is 550 nM (Njus et al., 1981). Although the cytoplasmic catecholamine concentration is unknown, a reasonable estimate is the apparent K_m of the translocator (20 μM). Therefore, the concentration gradient in vivo is roughly 25 000. Since the pH of the granular matrix is 5.7 (Johnson & Scarpa, 1976; Casey et al., 1977; Pollard et al., 1979), the pH gradient across the membrane is about 1.5 units. This ΔpH alone accounts for a catecholamine concentration gradient of 1000. Binding of catecholamine to the intragranular matrix adds a factor of about 3. Although $\Delta\psi$ in vivo is unknown, a potential of only 50 mV would bring the total catecholamine concentration gradient to the estimated value of 25 000.

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