Participation of a transmembrane proton gradient in 5-hydroxytryptamine transport by platelet dense granules and dense-granule ghosts

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Dense granules, the storage organelles for 5-hydroxytryptamine in blood platelets, have been isolated from porcine platelets and are shown to transport 5-hydroxytryptamine in response to a transmembrane proton gradient (ΔpH). Transport in the absence of ΔpH is minimal, and it is shown that a rapid increase in transport takes place as ΔpH increases. Direct measurements with [^{14}C]methylamine show a ΔpH of 1.1 units (acid inside) for intact granules. Osmotically active ghosts of dense granules from which 95% of the endogenous 5-hydroxytryptamine content has been released have also been prepared. Ghosts swell in the presence of ATP and Mg^{2+} , and this swelling is shown to be due to the entry of protons via a process linked to ATP hydrolysis. Proton entry is also apparently linked to anion penetration in ghosts. Steady-state 5-hydroxytryptamine transport in ghosts is stimulated approx. 3-fold on the addition of ATP to the incubation medium, and the stimulation of 5-hydroxytryptamine transport in ghosts correlates with the formation of a transmembrane ΔpH . Ghosts generate a ΔpH of 1.1–1.3 pH units (acid inside) in the presence of 5 mM-ATP/2.5 mM- MgSO_4 . ΔpH is generated within 3 min at 37°C and is dissipated by the ionophore nigericin and by NH_4Cl . It is shown that an Mg^{2+} -stimulated ATPase activity is present on the ghost membrane, and inhibition of the ATPase leads to a corresponding decrease in 5-hydroxytryptamine transport. The results presented support the idea that 5-hydroxytryptamine transport into platelet dense granules is dependent on the presence of a transmembrane ΔpH and, together with previous findings by others, suggest a generalized mechanism for biogenic amine transport into subcellular storage organelles.

Transport of biogenic amines by intracellular storage organelles has received a great deal of attention in recent years (Njus & Radda, 1978; Slotkin *et al.*, 1978; Phillips, 1974; Johnson *et al.*, 1978a; Maron *et al.*, 1979). Most of the work has involved chromaffin granules from adrenal medulla, which may be isolated in comparatively large quantities in homogeneous form. Studies by Bashford and colleagues (Bashford *et al.*, 1975, 1976) and

Johnson and his co-workers (Johnson *et al.*, 1978a; Johnson & Scarpa, 1976), who used intact granules, and studies by Phillips and colleagues (Phillips, 1978; Phillips & Allison, 1978), who used chromaffin-granule ghosts, have dealt with the determination of the energy source for amine transport in these organelles. These investigators have found that the intact chromaffin granule maintains an internal pH of approx. 5.5 as measured by distribution of [^{14}C]methylamine (Johnson & Scarpa, 1976) or by ^{31}P n.m.r. (Njus *et al.*, 1978), and that chromaffin-granule ghosts demonstrate a low internal pH after incubation with ATP (Phillips & Allison, 1978). This low internal pH may be raised by addition of appropriate ionophores and appears to be maintained by a membrane-bound ATPase that acts as an inwardly directed proton pump. In addition to these findings, Johnson *et al.*

Abbreviations used: ΔpH , (transmembrane) proton gradient; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid; butyl-PBD, 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole; dopamine, 3,4-dihydroxyphenethylamine.

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(1978a) have provided convincing evidence that adrenaline (epinephrine) and dopamine transport in intact chromaffin granules are closely correlated with the magnitude of the transmembrane ΔpH . Phillips (1978) has also shown that 5-hydroxytryptamine transport in chromaffin-granule ghosts is dependent on the transmembrane ΔpH .

We showed previously that the transport of 5-hydroxytryptamine into porcine platelet dense granules occurs predominantly via a carrier-mediated process at low substrate concentrations (Wilkins *et al.*, 1978). Recently, Johnson *et al.* (1978b) reported the existence of a transmembrane ΔpH (acid inside) in dense granules of porcine platelets of approx. 1 unit at an external pH of 6.9. Here we confirm and extend this observation and its implications. 5-Hydroxytryptamine transport into dense granules was examined in relation to the transmembrane ΔpH . In addition, a method for the preparation of osmotically sensitive sealed ghosts from dense granules is described. This preparation allows the study of the generation of ΔpH and of the relationship of the proton gradient with 5-hydroxytryptamine transport. The ghost preparation also provides the advantage that the bulk of intragranular components (e.g., 5-hydroxytryptamine) has been removed, thus decreasing the contribution of these components to transport and other assays. In addition, the role of ΔpH with regard to a generalized mechanism for amine accumulation by subcellular biogenic-amine-storage organelles is discussed.

Experimental procedures

Methods

Preparation of granules and their ghosts. Dense granules were isolated by a method described previously (Salganicoff *et al.*, 1975) by using the modifications introduced by Wilkins *et al.* (1978). Ghosts were prepared from dense granules that had been isolated as described above, except for the omission of the 2.5 M-sucrose layer from the discontinuous sucrose gradients (see Fig. 1 of Wilkins *et al.*, 1978). The amounts of the other four layers (i.e. 2.0 M-, 1.8 M-, 1.6 M- and 0.8 M-sucrose) were increased to 6.0 ml each in these gradients. We deleted the 2.5 M-layer, since by doing so, a better recovery was obtained with little sacrifice of purity. In previous work we found that 5-hydroxytryptamine was present in significant quantity in a fraction (designated F5) sedimenting immediately above 2.5 M-sucrose (Wilkins *et al.*, 1978). However, only 0.2 and 1.1% of the marker enzymes cytochrome oxidase and acid phosphatase respectively were present in the latter fraction. Also, examination of electron micrographs indicated very little increase in contamination of the dense granules by other

organelles when the F5 and pellet fractions were combined by deleting the 2.5 M-sucrose layer.

After centrifugation, the pellets from the modified gradients were resuspended immediately in 10 ml of a solution containing 10 mM-Hepes/1 mM-MgSO₄/0.1 mM-dithiothreitol, pH 7.0 ('lysis buffer') at 37°C. After gentle homogenization, the suspension was centrifuged at 27000g for 15 min at 4°C. The supernatant (SI) was removed and saved for determination of 5-hydroxytryptamine, and the pellet was resuspended again with gentle homogenization in lysis buffer at 4°C. The latter suspension was left overnight on ice. The following day the suspension was centrifuged at 27000g for 15 min. The supernatant (SII) was removed and saved. The pellet was resuspended with gentle homogenization in lysis buffer containing 0.25 M-sucrose to yield the dense-granule ghost preparation. The release of endogenous 5-hydroxytryptamine was monitored by using the fluorimetric assay of Udenfriend & Weissbach (1963). Release of endogenous 5-hydroxytryptamine was as follows: SI contained 50.2 ± 3.4 and SII contained $44.3 \pm 5.7\%$ respectively of endogenous 5-hydroxytryptamine, for a total released of $94.5 \pm 4.1\%$. The latter values represent means $\pm$ S.E.M. for three separate ghost preparations. A typical preparation of intact granules contained 534 nmol of 5-hydroxytryptamine/mg of protein. The corresponding ghost preparation contained 15 nmol of 5-hydroxytryptamine/mg of protein. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Determination of ΔpH . The transmembrane ΔpH in intact granules or granule ghosts was determined by measuring the distribution of [¹⁴C]-methylamine. This method has been used extensively to determine ΔpH in organelles in which the pH of the internal space is lower than that of the external medium (e.g. Reijngoud & Tager, 1973). The principles underlying this method have been discussed in detail elsewhere (e.g. Rottenberg, 1975). Samples of intact granules (0.3 mg/ml) or granule ghosts (0.6 mg/ml) were incubated with ³HOH (1 mCi/ml) and [¹⁴C]methylamine (58 Ci/mol) at a fixed temperature for the desired period of time (see the Tables). In routine experiments, ³HOH was added at a final concentration of 900 c.p.m./ μl , and [¹⁴C]methylamine was added at a final concentration of 8 μM (500 c.p.m./ μl). Either halving or doubling the methylamine concentration in the incubations had a negligible effect on the ΔpH values measured in control experiments, thus ruling out a significant effect of binding on the measurements. After incubation, samples were centrifuged in an Eppendorf model-3200 centrifuge at 12800g for 2 min. The supernatants were carefully and completely aspirated with a Pasteur pipette. Pellets were resuspended in

0.2 ml of 2% (v/v) Triton X-100. Samples of supernatants (10 μ l) and pellets (100 μ l) were then added to 5 ml of a scintillant consisting of 0.4% butyl-PBD and 33% (v/v) Triton X-100 in toluene and counted for radioactivity in a Beckman model-LS-330 liquid-scintillation counter. Protein in the pellets of granules and ghosts was measured by the method of Lowry *et al.* (1951).

Since pellets contained both intra- and extra-vesicular spaces, the size of the extravascular space (i.e. trapped volume) was determined by incubating samples of intact granules or granule ghosts with ^3HOH (900 c.p.m./ μ l) and either [^{14}C]polydextran (600 c.p.m./ μ l final concn.) or [^{14}C]sucrose (800 c.p.m./ μ l, final concn.). These samples were then centrifuged and treated exactly as samples in which methylamine distribution was measured. All values were corrected for cross-over by counting samples containing ^{14}C or ^3H alone. Δ pH was then calculated by determining the ratio of the methylamine concentration inside divided by the methylamine concentration outside the organelles (i.e. $C_{\text{methylamine in}}/C_{\text{methylamine out}}$). From this ratio, one may calculate the Δ pH. For amines of high pK_a :

$$\Delta\text{pH} = \log (C_{\text{methylamine in}}/C_{\text{methylamine out}})$$

Light scattering. Light scattering was measured at 37°C (540 nm at 90° from the incident light). A rectangular cuvette (1 cm $\times$ 1 cm, four sides clear) was used. The contents were stirred continuously by means of a small magnetic bar coupled to a motor and magnet located below the cuvette assembly. Scattering changes were amplified differentially by using a variable voltage to cancel the background signal and then expanding the scale. Protein concentration was selected for a linear region of response (see under 'Osmotic behaviour of ghosts: light-scattering measurements' below).

ATPase activity. ATPase activity was determined by measuring the amount of P_i released from ATP, the Malachite Green method (Hess & Derr, 1975) being used. This method depends on the formation of a complex between phosphomolybdate and Malachite Green at low pH and is sensitive to values of less than 1 nmol of P_i . The standard incubation buffer for ATPase assays contained 50 mM-Hepes, 2.5 mM- MgSO_4 and 5 mM- Na_2ATP at pH 7.0. Ghosts were incubated in this buffer at a final protein concentration of 100 $\mu\text{g}/\text{ml}$ at 37°C. Samples (1.0 ml) were withdrawn after the desired time intervals and added to 1.0 ml of ice-cold 5% (v/v) trichloroacetic acid. Trichloroacetic acid-treated samples were incubated for 5 min on ice and then centrifuged in an Eppendorf model-3200 centrifuge at 12 800 g for 2 min to remove precipitated material. Samples of ghosts that had been boiled or treated

with 5% trichloroacetic acid before ATP addition served as blanks and were treated exactly as stated for experimental samples. All measurements were made in duplicate or triplicate.

Transport of 5-hydroxytryptamine. Transport of 5-hydroxytryptamine by intact granules or by granule ghosts was assessed by Millipore-filtration assay essentially as previously described (Wilkins *et al.*, 1978). Intact granules or granule ghosts were incubated at a final protein concentration of 200 $\mu\text{g}/\text{ml}$ in the desired buffer at 37°C for various times. All assays contained 5-hydroxy-[2- ^{14}C]tryptamine (50 Ci/mol) at a final concentration of 5.7 μM . After the desired incubation time, 0.1 ml portions were removed and filtered through 0.1 μm -pore-size Millipore filters, followed by washing with 2.0 ml of ice-cold incubation buffer. Samples containing no protein were filtered and washed in the same way as experimental samples in order to correct for binding of 5-hydroxytryptamine to the filters. Filters were dissolved in 3 ml of 2-methoxy-ethanol and counted for radioactivity as described previously (Wilkins *et al.*, 1978).

Electron microscopy. Pelleted samples of ghosts were fixed overnight at 0°C with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. This was followed by post-fixation with 1% OsO_4 for 5 min and staining with 7.5% uranyl acetate for 10 min. Samples were then dehydrated in a graded series of ethanol solutions, followed by dehydration in 100% propylene oxide. Samples were then embedded in Epon 812, followed by thin sectioning with an LKB III-8800 ultramicrotome. Thin sections were stained with uranyl acetate followed by lead citrate. All specimens were viewed in a Philips model-300 electron microscope.

Materials

5-Hydroxy[2- ^{14}C]tryptamine creatinine sulphate was obtained from Amersham/Searle, ^3HOH , [$\text{U}-^{14}\text{C}$]sucrose, [^{14}C]methylamine hydrochloride and [*carboxyl*- ^{14}C]polydextran were purchased from New England Nuclear. Nigericin was generously supplied by Dr. Robert Hosley, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone, *NN*-dicyclohexylcarbodi-imide, oligomycin and ouabain were purchased from Sigma. Reserpine was supplied by Ciba Pharmaceutical Co. All other materials used were of reagent grade and obtained from commercial sources. All ionophores were dissolved in ethanol. *NN*-Dicyclohexylcarbodi-imide, oligomycin and reserpine were dissolved in dimethyl-formamide. The amount of solvent added to assays did not exceed 1% of the total assay volume, and solvent controls were routinely run.

Results and discussion

Transport of 5-hydroxytryptamine and Δ pH in intact granules

It has been suggested that a transmembrane Δ pH (acid inside) may play a role in 5-hydroxytryptamine accumulation by dense granules (Johnson *et al.*, 1978b). Initial experiments therefore focused on determination of 5-hydroxytryptamine transport in intact granules as a function of the pH of the external medium. As shown in Fig. 1, a dramatic increase in the initial velocity of 5-hydroxytryptamine transport into intact dense granules took place as the external pH was increased from 6.0 to 10.0. This increase in 5-hydroxytryptamine transport was completely abolished by the addition of nigericin, an ionophore that has been shown to mediate the electroneutral exchange of K^+ or Na^+ for H^+ and, as a consequence, to disrupt Δ pH (Estrada-O *et al.*, 1967; Gómez-Puyou & Gómez-Lojero, 1977) (results not shown). In order to measure directly the transmembrane Δ pH, the distribution of $[^{14}C]$ -methylamine was monitored. This technique has been used to measure pH gradients in dense granules (Johnson *et al.*, 1978b) and in chromaffin granules

(Johnson & Scarpa, 1976). Fig. 1 also shows the behaviour of Δ pH as the pH of the medium is changed. Clearly, an increase in 5-hydroxytryptamine transport correlated with a rise in the transmembrane Δ pH from values near zero at an external pH of 6.0 to more than 1.5 units at an external pH of 8.0. As shown in Table 1, dense granules isolated on sucrose gradients exhibited a pH gradient of 1.14 pH units (inside acid) at pH 7.0 (Expt. A). Raising or lowering the external pH produced a concomitant increase or decrease in the Δ pH (Expt. B). Also tested were the effects of various media on Δ pH. It is shown that Δ pH remained at approx. 1 unit regardless of medium composition (as long as iso-osmoticity was maintained). These latter observations indicate that Δ pH in the granules did not arise from the establishment of a Donnan equilibrium. Again, addition of either nigericin (1 μ g/ml) or NH_4Cl (20 mM) greatly decreased the magnitude of the observed pH gradients (Table 1).

Preparation and characterization of dense-granule ghosts

In order to study further the transport of 5-hydroxytryptamine by dense granules and its energy requirements, we decided to prepare a resealed-ghost preparation from dense granules. Such a preparation allows the study of 5-hydroxytryptamine transport under conditions where the concentration of endogenous amine has been greatly decreased. Further, the ghost preparation allows the study of the conditions necessary for the generation of a transmembrane Δ pH in the dense granule. Lysis of the dense granules was accomplished by suspension of pellets from sucrose gradients in a hypo-osmotic medium as described under 'Preparation of granules and their ghosts' above. It was found that two lysis steps, the first at 37°C and the second at 0°C, were required to release the bulk of endogenous 5-hydroxytryptamine. Approx. 95% of the endogenous 5-hydroxytryptamine was released by the procedure outlined and as measured fluorimetrically (see the Experimental procedures section). The remainder of the amine was apparently tightly bound to the vesicles. Fig. 2 shows an electron micrograph of a typical ghost preparation. Most of the structures in these preparations appeared to be dense-granule membranes and some contained what appeared to be a small amount of residual core material.

Dense-granule ghosts were assayed for 5-hydroxytryptamine transport activity by the Millipore-filtration assay described in the Experimental procedures section. Fig. 3 shows the time course of 5-hydroxytryptamine transport in the presence and absence of 5 mM-ATP and 2.5 mM- $MgSO_4$. Transport in the presence of ATP and Mg^{2+} , which was similar to that shown previously for intact granules (Wilkins *et al.*, 1978), remained linear for approx.

Fig. 1. *Transport of 5-hydroxytryptamine and Δ pH in intact granules as a function of external pH*

Initial rates of 5-hydroxytryptamine transport were assayed after 4 min incubation at 37°C as described in the Experimental procedures section. Δ pH was measured by using the distribution of $[^{14}C]$ -methylamine (also described in the Experimental procedures section). C_{in}/C_{out} represents the methylamine concentration gradients measured at the pH values indicated. All assays were done in 0.175 M-KCl containing 50 mM of one of the following buffers: pH 5.0 and 5.5, citrate; pH 6.0 and 6.5, Mes; pH 7.0, 7.5 and 8.0, Hepes; pH 9.0, 9.5 and 10.0, glycine. All solutions were adjusted to the desired pH with 5-M-KOH. ●, 5-Hydroxytryptamine transport; ○, C_{in}/C_{out} .

Table 1. ΔpH in intact dense granules

Intact dense granules, prepared as described in the Experimental procedures section, were incubated with ³HOH and either [¹⁴C]methylamine or [¹⁴C]polydextran in order to determine ΔpH. Incubations were for 5 min and were initiated by adding the intact dense-granule suspension to the complete incubation mixture, which contained nigericin or NH₄⁺ where indicated. Samples were then centrifuged and treated as described in the Experimental procedures section. Values given represent means of three determinations. Data in Expts. A and B represent two separate preparations of granules respectively.

Medium	[¹⁴ C]Methylamine (in)/ [¹⁴ C]methylamine (out)	ΔpH {= log [(in)/(out)]}
Expt. A		
0.4 M-Sucrose/10 mM-Hepes, pH 7.0	13.9	1.14
0.175 M-KCl/50 mM-Hepes, pH 7.0	9.7	0.99
0.4 M-Sucrose/10 mM-Hepes (pH 7.0)/20 mM-K ₃ PO ₄ + nigericin (1 μg/ml)	2.6	0.41
0.4 M-Sucrose/10 mM-Hepes, pH 7.0, +20 mM-NH ₄ Cl	1.4	0.15
Expt. B		
0.175 M-KCl/50 mM-Mes, pH 6.0	0.56	-0.25
0.175 M-KCl/50 mM-Hepes, pH 7.0	14.1	1.14
0.175 M-KCl/50 mM-Hepes, pH 8.0	50.5	1.70
0.175 M-KCl/50 mM-Hepes, pH 7.0, + 1 μg/ml Nigericin	0.50	-0.30

Fig. 2. Electron micrograph of ghost preparation
Samples were prepared by the method outlined in the Experimental procedures section. Final magnification ×28 500.

Fig. 3. Time course of 5-hydroxytryptamine transport by dense-granule ghosts
The assay mixtures consisted of the following: 0.25 M-sucrose, 50 mM-K₃PO₄, 2.5 mM-MgSO₄, 5.7 μM-[¹⁴C]5-hydroxytryptamine, plus (○) or minus (●) 5.0 mM-Na₂ATP at pH 7.0. The solutions were equilibrated for 5 min at 37°C. After equilibration, ghosts were added to a final concentration of 200 μg of protein/ml and portions were taken at the appropriate times as described in the Experimental procedures section. In addition, one sample was incubated for 5 min with [¹⁴C]5-hydroxytryptamine and ATP at 37°C as described above and after 5 min (arrow), nigericin was added at a final concentration of 1 μg/ml (▲).

4 min and reached a near-steady-state value after 10 min of incubation. 5-Hydroxytryptamine transport in the absence of ATP, however, was low and typically reached only about 30% of the steady-state

value attained in the presence of ATP. It is possible that residual internal 'storage complexes' may be responsible in part for transport in the absence of ATP. Also shown in Fig. 3 is the effect of nigericin addition on the steady-state amount of 5-hydroxytryptamine transport. Nigericin, which disrupted the transmembrane ΔpH in intact granules, also caused release of approx. 80% of 5-hydroxytryptamine accumulated by ghosts in response to ATP addition.

Osmotic behaviour of ghosts: light-scattering measurements

In order to study the osmotic behaviour of the ghosts, light-scattering experiments were performed. Ghosts were found to behave in a predictable way when suspended in media of various osmotic pressures, as shown in Fig. 4. The latter finding indicates that the ghosts are osmotically active and obey the Boyle–Van't Hoff law for osmometers over a range of osmolarities (50–260 mosm).

ATP-induced ghost swelling

A well-known property of chromaffin granules from adrenal medulla is that they swell on addition of ATP to their medium (Phillips & Allison, 1978; Casey *et al.*, 1976; Trifaró & Poisner, 1967; Lishajko, 1969). This swelling is time-dependent and is thought to be due to entry of protons via a proton-translocating ATPase in the chromaffin-

Fig. 4. Linearity of relationship between light-scattering and osmotic pressure

Ghosts were suspended in sucrose solutions containing 1 mM-MgSO₄ buffered with 10 mM-Hepes, pH 7.0, and these suspensions were allowed to equilibrate for 10 min at 37°C. After this, light scattering of the suspensions was measured as described in the Experimental procedures section. '% Scatter' represents the percentage of scattering obtained at an osmotic pressure of 262 mosm. Results shown are the means of duplicate determinations.

granule membrane. In order to determine whether dense-granule ghosts behave in a similar manner, ghosts were suspended in 0.25 M-sucrose/10 mM-Hepes, pH 7.0, containing 1 mM-MgSO₄ at a final protein concentration of 100 µg/ml. After stabilization of the trace, Na₂ATP was added to a final concentration of 3 mM. As shown in Fig. 5(a), rapid swelling of the ghosts was followed by a slower phase of swelling. Swelling of ghosts was dependent on the concentration of ATP added, being half-maximal at 2 mM-ATP (results not shown). The time-dependent swelling of the ghosts on the addition of ATP is apparently due to inwardly-directed proton translocation. In support of this view, inhibition of the Mg²⁺-dependent ATPase of the membrane by the addition of 2 mM-EDTA or

Fig. 5. Swelling of ghosts induced by ATP and nigericin. Ghosts were added to a solution (final protein concn. 100 µg/ml) containing 0.25 M-sucrose, 10 mM-Hepes, 1 mM-MgSO₄ [neutralized to pH 7.0 with 5 M-NaOH (a and b) or 1 M-Tris (c)] in a final volume of 2.5 ml. The immediate increase in light-scattering on addition of ghosts is not shown. The suspensions were equilibrated at 37°C. Reagents were added (arrows) at the following final concentrations: Na₂ATP, 3.0 mM; nigericin (Nig), 1 µg/ml; Tris/ATP, 2.0 mM; NaCl, 20 mM. The top scale represents calibration for (a) and (b); the bottom scale represents that for (c). ΔS is the change in scattering.

0.5 mM-*NN*-dicyclohexylcarbodi-imide leads to a corresponding inhibition (>70%) of ATP-induced swelling (see below). Slow swelling of the ghosts in the presence of ATP continued for long periods (>30 min) in the absence of other additions. However, if nigericin (1 $\mu\text{g}/\text{ml}$) was added after ATP, an immediate increase in the swelling rate occurred, which lasted for several minutes. The swelling then returned to the slower rate obtained before nigericin addition (Fig. 5*a*). This effect of nigericin was absolutely dependent on the presence of ATP. As shown in Fig. 5(*b*), if nigericin was added first, there was no effect on the scattering of the ghost suspension. After ATP addition, however, a rapid burst of swelling ensued. Since nigericin catalyses electroneutral exchange between H^+ and K^+ or Na^+ , it was reasoned that Na^+ of the buffering medium rapidly exchanged for protons that had been pumped into the ghosts. This possibility was tested by replacing Na^+ /Hepes with Tris/Hepes in the buffering medium. As shown in Fig. 5(*c*), addition of Tris/ATP to a final concentration of 2 mM caused an immediate swelling similar to the one seen with Na^+ /ATP, followed by a slower phase of swelling. However, the subsequent addition of nigericin did not elicit an increase in the rate of swelling. If nigericin addition was then followed by the addition of 20 mM- NaCl , a rapid phase of swelling was seen. This phenomenon was also seen when KCl was substituted for NaCl under these circumstances. This result demonstrates that rapid swelling induced by nigericin in the presence of ATP is due in part to an electroneutral exchange of Na^+ for H^+ catalysed by nigericin.

Another aspect of ion movements in the presence of ATP is shown in Fig. 6. Fig. 6(*a*) shows that addition of carbonyl cyanide *m*-chlorophenylhydrazide, an ionophore that has been shown to allow electrogenic H^+ movement across biological membranes, has little effect on the rate of ATP-induced swelling. Establishment of a K^+ diffusion potential (inside positive) by valinomycin and potassium phosphate addition before ATP addition also caused no discernible change in ATP-induced swelling (Fig. 6*b*). In Fig. 6(*c*), a combination of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazide in the presence of K^+ causes rapid swelling in the presence of ATP. This effect is analogous to adding nigericin in the presence of K^+ , since the combination of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazide allows exchange between K^+ and H^+ . The above results suggest that ATP-induced H^+ movement is at least partially non-electrogenic in nature. The identity of the anion(s) that accompany proton translocation into the ghosts is unknown at present; however, isolated chromaffin granules appear to transport both sulphate (Phillips & Allison, 1978) and ATP (Kostron *et al.*, 1977).

Fig. 6. Effect of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazide (CCCP) on ATP-induced swelling

Ghosts were equilibrated at 37°C exactly as described in Fig. 5. Reagents were then added (arrows) at the following final concentrations: Na_2ATP , 3 mM; CCCP, 2 μM ; valinomycin (VAL), 10 μM ; potassium phosphate, pH 7.0, 10 mM. The top scale represents calibration for (a) and (b); the bottom scale represents that for (c). ΔS is the change in scattering.

Ghost ATPase activity

The occurrence of an ATP-stimulated nigericin-sensitive 5-hydroxytryptamine transport and of ATP-dependent ghost swelling suggested the presence of a proton-translocating ATPase activity in the dense-granule-ghost membranes. A Mg^{2+} -dependent ATPase activity has been reported in rabbit platelet dense granules (Heinrich *et al.*, 1972). In order to characterize this activity further, the ghost ATPase was assayed as described in the Experimental procedures section. The time course of ATPase activity is shown in Fig. 7. An initial burst of ATP hydrolysis in the first minute was followed by a slower phase of activity during the rest of the 30 min assay period. We also detected an initial burst of ATPase activity by using the coupled-assay system of Schwartz *et al.* (1969) (results not shown). This rapid burst of activity may be important, since it correlates with the rapid burst of swelling seen on the addition of ATP to ghosts in light-scattering assays and also with the rapid

formation of ΔpH (see below). The slower phase of activity was significantly inhibited in the presence of either EDTA (2 mM) or *NN*-dicyclohexylcarbodi-imide (0.5 mM), both of which are known to inhibit ATPase activities of other amine-storage organelles (Bashford *et al.*, 1976; Toll *et al.*, 1977) (Table 2). The inhibition of the ATPase by EDTA and *NN*-dicyclohexylcarbodi-imide correlate well with their effects on ATP-induced swelling. Ca^{2+} (2.5 mM) did

Fig. 7. ATPase activity of ghosts
ATPase activity was monitored at 37°C as described in the Experimental procedures section. Results shown represent data from two separate experiments.

not substitute for Mg^{2+} and caused almost complete inhibition of the ATPase activity. On the other hand, three other agents, namely reserpine, which inhibits 5-hydroxytryptamine transport by dense granules, oligomycin and ouabain, were without significant effect on the ghost ATPase. The lack of inhibition by oligomycin and ouabain indicates that the ATPase activity measured here is not due to contaminating ATPases from mitochondria or plasma membranes respectively. ATPase activity was found to be low at 0°C. Also shown in Table 2 are the effects of ATPase inhibitors on ATP-stimulated steady-state 5-hydroxytryptamine transport. Addition of ATPase inhibitors such as *NN*-dicyclohexylcarbodi-imide, *N*-ethylmaleimide or EDTA cause a concomitant decrease in 5-hydroxytryptamine transport. On the other hand, reserpine, which actually stimulates ATPase activity slightly, strongly inhibits 5-hydroxytryptamine transport and ouabain has little effect on either ATPase or 5-hydroxytryptamine-transport activities.

Generation of a transmembrane ΔpH by ghosts

In order to compare the pH gradients achieved in the ghost preparation after incubation with ATP with those in intact granules directly, the distribution of [^{14}C]methylamine was measured after incubation

Table 2. Inhibition of steady-state ATPase and 5-hydroxytryptamine-transport activities

(a) ATPase assays: dense-granule ghosts were preincubated for 10 min at 37°C with the desired inhibitor in 50 mM-Hepes buffer, pH 7.0, at a final protein concentration of 100 $\mu\text{g}/\text{ml}$. (Control preincubations contained no inhibitors). All preincubations with the exception of those indicated (*) contained 2.5 mM- MgSO_4 . After preincubation, Na_2ATP was added to a final concentration of 5 mM, and the samples were incubated for another 10 min at 37°C. Samples were then assayed for P_i by the Malachite Green method as described in the Experimental procedures section. Control ATPase activity (mean $\pm$ S.E.M.) was $3.27 \pm 0.2 \mu\text{mol}$ of $\text{P}_i/10 \text{ min}$ per mg of protein (three expts.). (b) 5-Hydroxytryptamine-transport assays: dense-granule ghosts were preincubated for 10 min at 37°C with the desired inhibitor in 0.25 M-sucrose/50 mM- K_3PO_4 /2.5 mM- MgSO_4 , pH 7.0, at a final protein concentration of 200 $\mu\text{g}/\text{ml}$. (Control preincubations contained no inhibitors). After preincubation, Na_2ATP and 5-hydroxy[^{14}C]tryptamine were added simultaneously at final concentrations of 5 mM and 5.7 μM respectively. The samples were allowed to incubate for another 10 min in order to attain a steady-state value for 5-hydroxytryptamine transport. Portions were then withdrawn and filtered as described in the Experimental procedures section. Control 5-hydroxytryptamine-transport activity (mean $\pm$ S.E.M.) was $2.5 \pm 0.3 \text{ nmol}$ of 5-hydroxytryptamine/10 min per mg of protein (three expts.). Shown are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. Abbreviations used: DCCD, *NN*-dicyclohexylcarbodi-imide; N.D., not determined.

Inhibitor	Concentration	(a)	(b)
		ATPase activity (% of control)	5-Hydroxytryptamine transport (% of control)
DCCD	250 μM	50 ± 3 (2)	N.D.
	500 μM	22 ± 3 (3)	9 ± 2 (3)
<i>N</i> -Ethylmaleimide	1 mM	53 ± 9 (3)	37 ± 5 (3)
EDTA*	2 mM	48 ± 5 (3)	42 ± 3 (3)
Reserpine	5 μM	115 ± 3 (3)	16 ± 2 (3)
Oligomycin	60 $\mu\text{g}/\text{mg}$ of protein	77 ± 10 (3)	N.D.
Ouabain	200 μM	98 ± 7 (3)	74 ± 8 (3)
CaCl_2^*	2.5 mM	2 ± 1 (2)	N.D.
0°C†		11 ± 3 (2)	0

† Preincubated for 10 min at 37°C, then cooled to 0°C and incubated with 5 mM-ATP for 10 min.

of ghosts with ATP under various conditions. A Δ pH of approx. 1.3 units (acid inside) was generated after incubation of ghosts with 5 mM-ATP/2.5 mM-MgSO₄ for 1 min at 37°C (Table 3, Expt. A). The Δ pH value declined somewhat on further incubation. Incubation of ghosts at 0°C with ATP resulted in much lower pH gradients than those measured on incubation at 37°C. The Δ pH value measured in the presence of nigericin was somewhat higher in ghosts than in intact granules, indicating that slightly more methylamine binding occurred in ghosts (cf. Δ pH values with nigericin added; Tables 1 and 3). The reason for this difference is unclear at present; however, it may relate to the slightly different methods of preparation used. Since the relationship between Δ pH and the H⁺ concentration gradient is a logarithmic one, Δ pH values at low H⁺ concentration gradients are magnified greatly and introduce a small error into all measurements (cf. Δ pH are methylamine concentration gradient values). The problem has been considered previously (Phillips & Allison, 1978). An attempt to minimize methylamine binding was made by the addition of salts (i.e. 20 mM-K₂SO₄ or 20 mM-K₃PO₄) (Table 3, Expt. B). However, a small methylamine space was always found in the presence of nigericin or NH₄⁺ ions.

We found it difficult to measure the initial rate of Δ pH formation at 37°C by using the present methodology, since the time required for centrifugation introduced uncertainty into the initial time-course measurements. It may be said with certainty, however, that maximal pH gradients are generated within 3 min at 37°C.

Stimulation of 5-hydroxytryptamine transport by ATP was further studied. Samples of ghosts were incubated in the standard manner with 5.7 μ M-5-hydroxytryptamine (Fig. 8). After 5 min, various concentrations of ATP were added such that the final concentrations shown were attained. Stimulation of 5-hydroxytryptamine transport was maximal at 10 mM-ATP and was almost negligible at the lowest ATP concentration used (0.5 mM). Again, addition of nigericin to a final concentration of 1 μ g/ml caused almost complete release of accumulated 5-hydroxytryptamine in the presence of 5 mM-ATP.

Since a pH gradient is generated in the presence of ATP and Mg²⁺ in the ghosts, and this Δ pH can apparently supply at least part of the driving force for 5-hydroxytryptamine transport, an artificially imposed Δ pH should also drive increased 5-hydroxytryptamine transport in the absence of ATP. The results of such an experiment are shown

Table 3. *Generation of Δ pH in dense-granule ghosts*

Dense-granule ghosts were incubated at the times and temperatures indicated with 5 mM-ATP, 2.5 mM-MgSO₄ and ³HOH. After the incubation times indicated, [¹⁴C]methylamine was added to a final concentration of 8.0 μ M and the samples were centrifuged and processed as described in the Experimental procedures section. Samples for determination of trapped volumes were treated exactly as above, except that [¹⁴C]sucrose was added after incubation. Ionophores and/or salts were added before the incubation periods. Results shown for experiments A and B represent data collected from four different preparations. Values shown represent means of duplicate determinations, since values obtained agreed closely.

Medium	Incubation time (min)	Temp. (°C)	[¹⁴ C]Methylamine (in)/ [¹⁴ C]methylamine (out)	Δ pH {= log [(in)/(out)]}
Expt. A				
0.25 M-Sucrose/10 mM-Hepes, pH 7.0	1	37	20.4	1.30
	5	37	20.2	1.30
	10	37	17.6	1.25
	10	0	6.0	0.78
0.25 M-Sucrose/10 mM-Hepes, pH 7.0, + 1 μ g/ml Nigericin	10	37	3.4	0.53
Expt. B				
0.25 M-Sucrose/10 mM-Hepes, pH 7.0, + 20 mM-K ₃ PO ₄	5	37	24.6	1.39
0.25 M-Sucrose/10 mM-Hepes, pH 7.0, + 20 mM-K ₂ SO ₄	5	37	29.7	1.47
0.25 M-Sucrose/10 mM-Hepes/ 20 mM-K ₃ PO ₄ , pH 7.0, + 20 mM NH ₄ Cl	5	37	5.1	0.70
0.25 M-Sucrose/10 mM-Hepes/ 20 mM-K ₃ PO ₄ , pH 7.0, + nigericin (1 μ g/ml)	5	37	5.7	0.76

Fig. 8. Stimulation of 5-hydroxytryptamine transport by ATP

Transport of 5-hydroxytryptamine was assayed as described in the Experimental procedures section. Samples of ghosts (100 μ g of protein/ml) were incubated at 37°C with 5.7 μ M-5-hydroxy[14 C]tryptamine in 0.25 M-sucrose/50 mM- K_2PO_4 , pH 7.0. At the time shown (arrow), Na_2ATP was added at the final concentrations shown on the right: Δ , 10 mM-ATP; $\circ$, 2 mM-ATP; $\square$, 0.5 mM-ATP; $\bullet$, 0 mM-ATP; $\blacktriangle$, 5 mM-ATP + nigericin (1 μ g/ml).

in Fig. 9. Two portions of ghosts were incubated separately, each at pH 6.7, and 5-hydroxytryptamine transport was assayed at the times indicated as described in the Experimental procedures section. At the time shown (arrow), one assay mixture received 10 μ l of 0.5 M-NaOH such that its pH was raised to 7.7. The second assay mixture received 10 μ l of distilled water and thus remained at pH 6.7. The shift in pH from 6.7 to 7.7 in the first incubation resulted in an immediate increase in 5-hydroxytryptamine transport. In contrast, 5-hydroxytryptamine transport at a constant pH of 6.7 remained low and reached less than one-third of the steady-state values attained with the pH jump (Fig. 9).

In summary, the present work has examined the role of the transmembrane Δ pH in amine accumulation by the subcellular storage organelles for 5-hydroxytryptamine in the blood platelet, the dense granules. These findings support the idea that 5-hydroxytryptamine transport into platelet dense granules is driven by a gradient of protons across the membrane. This Δ pH is apparently generated *in vivo* by an inwardly directed proton-translocating ATPase in the granule membrane, and the results presented here strongly suggest its participation in the formation of Δ pH and thus in 5-hydroxytryptamine transport. The present results also correlate well with other findings in chromaffin granules and synaptic vesicles and, as stated above, support the idea that common mechanisms exist

Fig. 9. Accumulation of 5-hydroxytryptamine in response to an artificially imposed Δ pH

Ghosts (100 μ g of protein/ml) were incubated with 5.7 μ M-5-hydroxy[14 C]tryptamine at 37°C in a buffer containing 0.25 M-sucrose, 10 mM-Hepes, 10 mM- K_2SO_4 and 2.5 mM- $MgSO_4$ at pH 6.7. After 5 min of incubation (arrow), 10 μ l of 0.5 M-NaOH was added such that the pH of the incubation medium was raised to 7.7 ($\circ$). Alternatively, 10 μ l of deionized water was added ($\bullet$). 5-Hydroxytryptamine transport was assayed as described in the Experimental procedures section.

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References

- Bashford, C. L., Radda, G. K. & Ritchie, G. A. (1975) *FEBS Lett.* **50**, 21–24
- Bashford, C. L., Casey, R. P., Radda, G. K. & Ritchie, G. A. (1976) *Neuroscience* **1**, 399–412
- Casey, R. P., Njus, D., Radda, G. K. & Sehr, P. A. (1976) *Biochem. J.* **158**, 583–588
- Estrada-O, S., Graven, S. N. & Lardy, H. A. (1967) *J. Biol. Chem.* **242**, 2925–2932

- Gómez-Puyou, A. & Gómez-Lojero, C. (1977) *Curr. Top. Bioenerg.* **6**, 222–257
- Heinrich, P., DePrada, M. & Pletscher, A. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1769–1775
- Hess, H. H. & Derr, J. E. (1975) *Anal. Biochem.* **63**, 607–613
- Johnson, R. G. & Scarpa, A. (1976) *J. Gen. Physiol.* **68**, 601–631
- Johnson, R. G., Carlson, N. J. & Scarpa, A. (1978a) *J. Biol. Chem.* **253**, 1512–1521
- Johnson, R. G., Scarpa, A. & Salganicoff, L. (1978b) *J. Biol. Chem.* **253**, 7061–7068
- Kostron, H., Winkler, H., Peer, L. J. & König, P. (1977) *Neuroscience* **2**, 159–166
- Lishajko, F. (1969) *Acta Physiol. Scand.* **75**, 255–256
- Lowry, D. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maron, R., Kanner, B. I. & Schuldiner, S. (1979) *FEBS Lett.* **98**, 237–240
- Njus, D. & Radda, G. K. (1978) *Biochim. et Biophys. Acta* **463**, 219–244
- Njus, D., Sehr, P. A., Radda, G. K., Ritchie, G. A. & Seely, P. J. (1978) *Biochemistry* **17**, 4337–4343
- Phillips, J. H. (1974) *Biochem. J.* **144**, 311–318
- Phillips, J. H. (1978) *Biochem. J.* **170**, 673–679
- Phillips, J. H. & Allison, Y. P. (1978) *Biochem. J.* **170**, 661–672
- Reijngoud, D. J. & Tager, J. M. (1973) *Biochim. Biophys. Acta* **297**, 174–178
- Rottenberg, H. (1975) *Bioenergetics* **7**, 61–74
- Salganicoff, L., Hebda, P. A., Yandrasitz, J. & Fukami, M. (1975) *Biochim. Biophys. Acta* **385**, 394–411
- Schwartz, A., Allen, J. C. & Harigaya, S. (1969) *J. Pharmacol. Exp. Ther.* **168**, 31–41
- Slotkin, T. A., Seidler, F. J., Whitmore, W. L., Lau, C., Salvaggio, M. & Kirksey, D. F. (1978) *J. Neurochem.* **31**, 961–968
- Toll, L., Gunderson, C. B. & Howard, B. D. (1977) *Brain Res.* **136**, 59–66
- Trifaró, J. M. & Poisner, A. M. (1967) *Mol. Pharmacol.* **3**, 572–580
- Udenfriend, S. & Weissbach, H. (1963) *Methods Enzymol.* **6**, 604–605
- Wilkins, J. A., Greenawalt, J. W. & Huang, L. (1978) *J. Biol. Chem.* **253**, 6260–6265