A pH difference (acid inside) across the platelet plasma membrane increases both the rate and extent of serotonin accumulation inside plasma membrane vesicles. Even in the absence of other transmembrane ion gradients, this pH difference (ΔpH) serves as the sole driving force for serotonin accumulation, leading to a serotonin concentration 18-fold higher inside the vesicle. This process requires Na⁺ and is blocked by imipramine, indicating that it is mediated by the serotonin transporter. At physiological pH, internal K⁺ is counter-transported with serotonin, and high internal K⁺ stimulates transport maximally. Internal K⁺ also blocks the ΔpH stimulation of serotonin transport. Conversely, low internal pH (5.6) inhibits the ability of internal K⁺ to stimulate transport. This apparent competition between K⁺ and protons suggests that ΔpH drives serotonin accumulation through counter-transport with protons, and that serotonin is transported in its cationic form.

Serotonin is transported across the plasma membrane of platelets, nerve endings, and mast cells by a Na⁺-dependent, imipramine-sensitive transporter (Sneddon, 1973; Grippenberg, 1976). Plasma membrane vesicles isolated from platelets provide an ideal system for studying this prototype of a neurotransmitter re-uptake system (Rudnick, 1977). Serotonin is co-transported with Na⁺ and probably Cl⁻. Both external ions are required for activity (Sneddon, 1971; Lingjaerde, 1969). Internal K⁺, although not required, stimulates transport by accelerating the rate-limiting step of the transport cycle. This is the step in which the transporter is converted from the form which releases internal substrates to the form which binds external substrates (Nelson and Rudnick, 1979). One unanswered question has been: Do other ions substitute for K⁺?

In this paper, we present evidence that internal protons can replace internal K⁺. Thus, a pH difference (acid inside) across the plasma membrane acts as a driving force for serotonin accumulation by platelet plasma membrane vesicles. Transport of at least one other neurotransmitter, glutamate, is reported either to require, or to be stimulated by internal K⁺ (Kanner and Sharon, 1978; Schneider and Sacktor, 1980). Moreover, transport of norepinephrine and dopamine into synaptosomes is stimulated by K⁺, although this might represent activation of the Na⁺, K⁺-ATPase (Paton, 1976; Horn, 1976). Our results suggest that possibly also in these cases, neurotransmitter re-uptake is coupled to proton counter-transport.

**EXPERIMENTAL PROCEDURES**

**Methods**

Preparation and Equilibration of Membrane Vesicles—The isolation of platelet plasma membrane vesicles from fresh porcine blood was performed as described previously (Barber and Jamieson, 1970; Rudnick and Nelson, 1978). Prior to assay, vesicles were diluted 20-fold into a medium of 86 mM lithium or potassium gluconate, containing 1 mM MgSO₄, approximately 10 mM N-(2-acetamido)iminodiacetic acid adjusted to pH 5.6, 6.5, or 7.5 with either KOH or LiOH, and 1 to 8 mM mannitol to keep the osmolarity constant at 200 mosm. Buffer concentrations were adjusted so that in every case, the alkali cation concentration was 0.1 eq/liter. Lithium ion does not substitute for, or compete with, internal K⁺ or external Na⁺ (Rudnick, 1977; Nelson and Rudnick, 1979) and is used here as an inert cation. The suspensions were incubated at 37 °C for 15 min, sedimented at 48,000 × g for 20 min at 4 °C, and resuspended to the original volume in the incubation buffer.

Transport Assay—Serotonin transport was assayed at 25 °C by 40-fold dilution of a pre-equilibrated vesicle suspension (20 μg of membrane protein) into 92 mM NaCl, containing 1 mM MgSO₄, 0.06 μM 5-hydroxy[1,2-³H]tryptamine (17,000 cpm/pmol), approximately 5 mM ADA' adjusted to pH 5.6, 6.5, or 7.5 with NaOH, and adjusted to 200 mosm with 0 to 4 mM mannitol. Again, the Na⁺ concentration was always 0.1 eq/liter. Initial rates of transport were measured only during the first 15 s. Reactions were stopped by filtering the vesicle suspensions on nitrocellulose filters (Millipore HAWP), washing with cold, 0.1 M NaCl, and counting as described previously (Rudnick, 1977).

Efflux Measurements—Vesicles (80 μg of membrane protein) equilibrated as described above with lithium gluconate buffer at pH 5.6 or 7.5, were diluted 10-fold into NaCl buffer at the same pH, containing 30 to 160 mM [³H]serotonin. After 2 min at 25 °C, the reaction mixture was diluted 40-fold with NaCl medium of the pH indicated in the figure legend, containing 1 μM imipramine. The incubation was continued at 25 °C and 0.5-ml aliquots were taken at 5 s, and 1, 2, 3 min. Efflux was terminated by filtration as described previously (Nelson and Rudnick, 1979). The loss of accumulated 5-hydroxytryptamine was linear with time under these conditions.

Protein Determinations—Protein was determined by the method of Lowry et al. (1951).

**Materials**

5-Hydroxy[1,2-³H]tryptamine (64 Ci/mmol) was obtained from New England Nuclear. Imipramine was donated by Dr. Charles A. Brownley, Geigy Pharmaceuticals, and monensin was a gift of Dr. Robert J. Hoeley, Lilly Research Laboratories. All other reagents were reagent grade, purchased from commercial sources.

**RESULTS**

Serotonin Accumulation Is Driven by ΔpH—In the absence of other sources of energy, a transmembrane pH difference

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The abbreviations used are: ADA, N-(2-acetamido)iminodiacetic acid; ΔpH, the transmembrane pH difference.
serves as a driving force for serotonin accumulation by platelet plasma membrane vesicles. The experimental results shown in Fig. 1 demonstrate that in the absence of a transmembrane Na⁺ gradient, ΔpH drives the carrier-mediated transport of serotonin. Membrane vesicles equilibrated with NaCl medium buffered at pH 5.6 and diluted into the same medium buffered at pH 7.5 accumulate serotonin to a significantly higher level than if no ΔpH is imposed. From the internal volume of the vesicles (12 µl/mg of protein, Rudnick, 1977), we calculated that the internal serotonin concentration was 18-fold higher than that of the medium. In this case, the only possible driving force for transport is the imposed ΔpH. The data in Fig. 1 represent that portion of the serotonin accumulation which is sensitive to the ionophore monensin and therefore dependent on ΔpH. This ionophore (which catalyzes electrically neutral exchange of protons and alkali cations, and is expected to dissipate both ΔpH and any residual Na⁺ gradient) has no effect in the absence of a ΔpH at pH 5.6, 6.5, or 7.5, but dramatically inhibits ΔpH-driven transport.

Serotonin transport may be driven by ΔpH via many possible mechanisms. One possibility is that serotonin, a weakly basic amine, equilibrates across the membrane by nonionic diffusion of the more lipophilic free base, and is trapped inside by the high proton concentration (Nichols and Deamer, 1976). This possibility is unlikely to account for our results, however, since imipramine, a specific inhibitor of the serotonin transporter, completely blocks ΔpH-driven transport (Fig. 1). Moreover, little ΔpH-driven accumulation was observed when Na⁺ was replaced by Li⁺ and no transport in the absence of Na⁺ was imipramine-sensitive (data not shown). Another possible explanation for ΔpH-driven transport is that the plasma membrane vesicle preparation is contaminated with small amounts of membrane vesicles derived from platelet storage granules, which are believed to accumulate serotonin in response to an ATP-generated ΔpH (Rudnick et al., 1980). This possibility is unlikely for two reasons. First, the granular serotonin transport system is not Na⁺-dependent, and second, the serotonin accumulation shown in Fig. 1 is insensitive to reserpine, a specific inhibitor of the granule system (data not shown).

pH Rate Profile for Serotonin Transport—To define further the mechanism by which a ΔpH drives the serotonin transport system, we examined the effect of pH on the rate of transport. Fig. 2 shows the pH rate profile for serotonin accumulation by vesicles equilibrated with either K⁺ or Li⁺. We have previously shown that internal K⁺ increases the rate and extent of serotonin accumulation (Rudnick, 1977; Rudnick and Nelson, 1978; Nelson and Rudnick, 1979). Therefore, although we expected faster transport into K⁺-loaded vesicles, we were surprised by the different shape of the pH rate profiles. While transport into K⁺-containing vesicles rises to a maximum rate between pH 6.5 and 7.5, and does not decrease in rate at high pH, vesicles lacking K⁺ show a distinct pH optimum at approximately pH 6.5, and transport more slowly at high pH. Furthermore, the K⁺ stimulation (relative to Li⁺) varies over the pH range tested, from a 5- to 6-fold stimulation at pH 7.5 to essentially no difference at pH 5.6.

Time Course of Serotonin Transport—The time course of serotonin transport reflects not only the influence of both ΔpH and external pH, but also additional factors. Fig. 3 demonstrates that when plasma membrane vesicles isolated from platelets are equilibrated in Na⁺- and K⁺-free buffer at pH 5.6 and then diluted into NaCl medium buffered at pH 7.5, serotonin accumulates within the vesicles to a much higher extent than if no ΔpH is imposed at either pH 5.6 or 7.5. Immediately after dilution into Na⁺ medium, the vesicles accumulate serotonin rapidly and reach a peak serotonin content at 5 to 10 min after dilution. As the ion gradients which drive serotonin transport decay with time, the accumulated serotonin gradient also decays, until little intravesicular serotonin remains 2 h after dilution. This result suggests that ΔpH acts as an additional driving force for serotonin transport even when superimposed over pre-existing transmembrane gradients of Na⁺ and Cl⁻. The data presented in Fig. 4 indicate that the maximum extent of serotonin accumulation by K⁺-loaded vesicles is also much higher when a ΔpH (5.6 in, 7.5 out) is imposed across the membrane than at pH 5.6 or 7.5 in the absence of ΔpH.

However, Figs. 3 and 4 also show the time course of transport into vesicles equilibrated and diluted with media at an intermediate pH, 6.5. In the absence of internal K⁺ (Fig. 3), serotonin influx is slower at pH 6.5 (in = out) than with a ΔpH, in contrast to the results in Fig. 2 which indicate faster transport at pH 6.5 than at 5.6 or 7.5. The only difference is that in the experiment shown in Fig. 3, a ΔpH was imposed (the internal pH was 5.6 instead of 7.5). The effect of internal...
pH on transport rate will be described below. In the absence of a ΔpH, at pH 6.5, the vesicles transport serotonin to almost the same level (Fig. 3) or even higher levels (Fig. 4) than when ΔpH is imposed, in contrast to the results shown in Fig. 1, in the absence of an Na+ gradient.

It is clear from Figs. 3 and 4 that the peak level of serotonin accumulated within the vesicles reflects not only the forces driving serotonin across the membrane, and the rate at which serotonin equilibrates with these driving forces but also the rate at which these forces (the transmembrane ion gradients) decay. For example, at pH 7.5, with no ΔpH, serotonin enters the vesicle rapidly, but fails to reach high internal concentrations, possibly because the ion gradients decay too fast. As another example, in the presence of internal K+ at pH 6.5, the serotonin gradient is maintained much longer than when a ΔpH is imposed, suggesting that the ion gradients are more stable under these conditions. It is clear, therefore, that imposition of a ΔpH on top of pre-existing ion gradients affects many aspects of transport, some of which may obscure any coupling between proton and serotonin transport. Although it is customary to use transport time courses such as shown in Figs. 3 and 4 as evidence for coupling between substrate and proton transport, our experience suggests that this approach may give misleading results.

Stimulation of Transport Rate by Low Internal pH—Internal K+ is thought to stimulate serotonin transport by facilitating interconversion of the transporter from the form which releases internal serotonin to a form which can bind external serotonin. Potassium ion leaves the vesicle in the process (Nelson and Rudnick, 1979). To test the possibility that a low internal pH acts in the same way, we measured the effect of ΔpH on the initial rate of serotonin transport into vesicles equilibrated with either K+ or Li+. The results, shown in Table I, demonstrate that at high external pH (7.5) either a low internal pH or high internal K+ stimulates the rate of transport. With K+-loaded vesicles at pH 5.6 (in = out), the rate is slow, as also shown in Fig. 4. If the internal pH is raised to 7.5, the rate is also low, suggesting that low external pH (5.6) is sufficient to down regulate transport. If the external pH is raised to 7.5 (pH 5.6 inside), however, the rate increases dramatically. At pH 7.5 outside, the rate is still controlled primarily by the external pH, since raising the internal pH from 5.6 to 7.5 has no effect.

In contrast, when internal K+ is replaced with Li+, both internal and external pH determine transport rate. As with K+-loaded vesicles, increasing internal pH has little effect (external pH = 5.6), and increasing external pH dramatically increases the rate (internal pH = 5.6). In the absence of internal K+, however, the maximal transport rate requires an acidic interior. If the internal pH is raised to 7.5, transport slows down to the rate in acid media. Moreover, the transport rate in the presence of a ΔpH (interior acid) is faster than at the pH optimum (pH 6.5 in = out), and approximately two-thirds of the rate with K+-loaded vesicles under the same conditions.

One possible mechanism by which an acidic interior might stimulate transport is by decreasing the fraction of internal serotonin in the neutral (free base) form, thereby decreasing passive efflux. To test this possibility, we measured rates of passive efflux under various conditions. Vesicles were actively loaded to various internal serotonin concentrations at various pH, and diluted into NaCl medium containing imipramine at a concentration which completely blocks efflux via the serotonin transport system (Talvenheimo et al., 1979). The results in Fig. 5 show the dependence of passive efflux upon internal and external pH. As expected, efflux is slowest at low pH and fastest at high pH (in = out), and does not appear to saturate as the internal serotonin concentration is increased. However, the stimulation of transport rate by low internal pH (Table I) is much faster than this passive efflux. Even under conditions where passive efflux is fastest, it accounts for less than 7% of the increase in net influx due to a ΔpH. Thus, stimulation by low internal pH is due to an increase in influx, and not a decrease in passive efflux.

Competition between H+ and K+—If internal protons substitute for internal K+, stimulation by K+ should vary with internal pH. This phenomenon is demonstrated by the data shown in Fig. 6. In this experiment, we measured the initial rate of serotonin transport into K+-loaded and K+-free vesicles as a function of internal pH, and plotted the ratio of the two rates. In one set of points (filled circles) the external pH varied with internal pH, but the open circles represent rates where the external pH was held constant at 7.5. In both cases,
H⁺-driven Serotonin Transport

There are many possible mechanisms by which a pH difference (ΔpH) across the platelet plasma membrane might stimulate the rate and extent of serotonin accumulation. The simplest possibility is that by influencing the distribution between the protonated, cationic form of serotonin and the more permeant, neutral form, the ΔpH traps serotonin on the acidic side of the vesicle membrane. For example, in liposomes, serotonin and dopamine are concentrated in response to ΔpH in the absence of other driving forces or transporters (Nichols and Deamer, 1976). Such a simple mechanism cannot account for our observation that ΔpH-driven transport in platelet membrane vesicles is blocked by imipramine and requires Na⁺. Obviously, the ΔpH acts through the serotonin transporter.

A more interesting possibility is that the neutral form of serotonin is the true substrate, rather than the cationic form which predominates at physiological pH. Thus, a ΔpH might stimulate the rate and extent of transport by increasing the concentration of substrate on the outside and decreasing it on the inside. In most cases it is impossible to distinguish this type of stimulation from one where protons are counter-transported out of the vesicle as amine molecules are transported in. For example, in the chromaffin granule it has been impossible to distinguish between exchange of 1 proton with 1 neutral amine molecule on one hand and exchange of 2 protons for a protonated amine molecule on the other hand (Kanner et al., 1980; Knoth et al., 1980; Johnson et al., 1981). Knoth et al. (1981) recently proposed that in chromaffin granules only the cationic form of the substrate is transported, but their conclusions are based on many assumptions which have not yet been tested.

Fortunately, in the case of the serotonin transporter, the unique antagonism of ΔpH by internal K⁺ provides a way to distinguish between these two mechanisms. If ΔpH acted merely to change the substrate concentration, there would be no interaction between internal protons and internal K⁺. Yet, it is clear that high internal K⁺ blocks the stimulation by internal protons while high internal proton concentrations inhibit stimulation by internal K⁺ (Table I). In light of the proposal that K⁺ and serotonin are counter-transported, the simplest interpretation of our data suggests that when K⁺ is not present internally, protons replace K⁺ and are counter-transported with serotonin. A second argument in favor of counter transport is that internal protons stimulate the initial rate of transport under conditions where serotonin efflux is negligible.

Fig. 7 presents this interpretation in schematic form. After the transporter releases serotonin, Na⁺, and Cl⁻ on the vesicle interior (k₀), it binds either K⁺ or H⁺, and returns to an

**Discussion**

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![Proposed mechanism for stimulation of serotonin transport by K⁺ or H⁺](attachment:proposed-mechanism.png)
“external” form (either \(T_K\) or \(T_H^+\)), which releases \(K^+\) or \(H^+\) to the external medium, forming \(T_\text{e}\). This form of the transporter binds external \(Na^+\), serotonin, and \(Cl^-\) \(\left(k_o\right)\), and transports them to the interior \(\left(k_i\right)\). In the absence of internal \(K^+\), the rate of this cycle is determined by the internal pH. At physiological pH or higher, where \(T_H^+\) is low, internal \(K^+\) stimulates transport markedly by providing another pathway \(\left(k_{\text{out}(K^+)}\right)\). At low internal pH, where protons are not as limiting, \(K^+\) only slightly increases the rate. This mechanism easily explains the pH rate profiles shown in Fig. 2. Just as external \(K^+\) inhibits transport (Nelson and Rudnick, 1979), low external pH limits transport, regardless of internal \(K^+\). Up to pH 6.5, the rate of transport increases with pH. At this point, the rate becomes limited by internal pH \(\left(T_H^+\right)\), and decreases as the internal proton concentration drops. When internal \(K^+\) is present, however, it can fulfill the internal cation requirement and the rate remains high at high pH.

The substitution of \(H^+\) for \(K^+\) dictates that the same number of charges will cross the membrane in \(K^+\)-loaded or \(K^+\)-free vesicles. Thus, if the transport system is electroneutral when internal \(K^+\) is present (Rudnick and Nelson, 1978; Nelson and Rudnick, 1979), it should also be electroneutral in the absence of \(K^+\). The glutamate transport system of mammalian kidney also is stimulated by, but does not require, internal \(K^+\) (Kanner and Sharon, 1978). An intriguing possibility is that the neutral pH. The system would therefore appear like the microenvironment in the brain, the transporter pK, decreases to the point where internal protons cannot replace \(K^+\) at neutral pH. The system would therefore appear like the serotonin system at high pH, where internal \(K^+\) is almost negligible. However, stimulation by \(\Delta p\text{H}\) represents another distinctive feature of this system which can be tested with other neurotransmitter transporters, such as those for glutamate, norepinephrine, and dopamine. Furthermore, our results indicating that \(\Delta p\text{H}\) does not stimulate in the presence of internal \(K^+\) render unlikely the possibility that serotonin is transported in its neutral form, and further define the stoichiometry of the transporter.

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