Proc. Natl. Acad. Sci. USA
Vol. 78, No. 3, pp. 1638–1642, March 1981
Biophysics

NMR studies of 5-hydroxytryptamine transport through large unilamellar vesicle membranes

(platelet granule model/proton transport/pH gradients)

D. B. VISCIO AND J. H. PRESTEGARD*

Department of Chemistry, Yale University, New Haven, Connecticut 06511

Communicated by Kenneth B. Wiberg, November 21, 1980

ABSTRACT **Nuclear magnetic resonance techniques developed to study membrane permeability in closed membrane systems have been used to investigate transport of 5-hydroxytryptamine across the phospholipid membranes of large unilamellar vesicles. The vesicles, modeling the 5-hydroxytryptamine storage organelles of blood platelets, contained a high internal level of ATP buffered at a pH low relative to the external solution. The resultant pH gradient drove accumulation of 5-hydroxytryptamine to a level consistent with selective transport of the neutral amine. The upfield shifts of the 5-hydroxytryptamine resonances resulting from complexation with internally confined ATP were utilized to resolve and, simultaneously, to observe the internal and external amine. Simulation of the time evolution of the 5-hydroxytryptamine concentration allowed measurement of a permeability coefficient of $1.4 \pm 0.5 \times 10^{-5}$ cm/sec for the neutral amine.**

The kinetics of membrane transport are generally investigated by measuring the time evolution of solute concentrations on one or both sides of a biological membrane. Concentrations of cellular contents most often have been measured by using microelectrodes, osmotic response, or quantitative analysis after physical separation of internal and external contents (1, 2). However, the small size of many cellular organelles precludes the use of microelectrodes, osmotic response is nonselective, and physical separation of contents often places limitations on the range of permeability coefficients that can be measured. Clearly, improved techniques are needed to study transport *in vivo*.

NMR spectroscopy can be used to investigate membrane transport and has several properties that make it uniquely suited to the study of biological systems. For example, most biomolecules have easily observable magnetic nuclei (^1H, ^{13}C, ^{31}P). Because the chemical shifts of these nuclei are environment dependent, internal and external contents often can be resolved and observed simultaneously without physical separation. The area of an NMR signal is a measure of the number of molecules in that particular environment. Also NMR is a nonperturbing technique and can be used at physiological conditions. Until recently, low sensitivity was a severe problem for NMR. However, the present availability of high-field high-sensitivity Fourier-transform NMR spectrometers has facilitated the observation of even species at low concentration.

The biogenic amine 5-hydroxytryptamine (5-HT) is stored at amazingly high concentrations (as high as 1 M) in specialized bilayer membrane vesicles within blood platelets. These 1000- to 2000-Å organelles also contain 0.5 M ATP and 0.5 M alkaline earth cations (Mg^{2+}, Ca^{2+}) and have a low internal pH (ca. 5.7) (3). It has been suggested that ATP forms an efficient trap for 5-HT by formation of an impermeable low osmolarity aggregate (4). Aggregates of molecules such as ATP which have high ring current anisotropic magnetic susceptibilities are known to exhibit NMR resonances shifted well upfield from their monomeric positions (5). It is conceivable that this shift could form the basis for a natural differentiation of internal and external species in a transport study.

At the low levels of 5-HT found in platelet cytoplasm, the accumulation of 5-HT in organelles is proposed to occur by a carrier-mediated process (6). Experiments on platelet granules and related systems suggest that transport either is directly coupled to a membrane bound ATPase or is driven by an electrochemical pH gradient produced by the ATPase (3). At higher 5-HT levels, or in the absence of the carrier-mediated processes, 5-HT transport still occurs, presumably via a diffusive process. Investigations of both mediated and diffusive processes are necessary for a complete understanding of storage granule function. A number of investigations of transport in platelet granules and related systems have therefore appeared (7, 8).

We present here an investigation of 5-HT transport in a model vesicle system by using NMR methods. It contributes to our understanding of diffusive transport across bilayer membranes. It also illustrates the potential of NMR spectroscopy for membrane permeability studies in closed biomembranes.

Fig. 1 illustrates the vesicles used in the studies. The unilamellar bilayer vesicles are 2000–3000 Å in diameter and contain an ATP/phosphate buffer in the pH range 5–6. The external buffer was a phosphate buffer in the pH range 7–8. Upon addition of 5-HT to the outside, the pH gradient should lead to accumulation of 5-HT on the inside. The resulting ring current shifts on stacking of 5-HT with internal ATP should resolve the internal and external 5-HT resonances and allow continuous monitoring of both internal and external 5-HT concentrations.

MATERIALS AND METHODS

Large unilamellar vesicles were prepared by the ether-injection procedure of Deamer and Bangham (9). Because samples were to be used for proton NMR studies, all buffers were deuterated and p^2H is reported as pH meter reading plus 0.4. Into 5 ml of hot (60–65°C) 0.025 M ATP/0.1 M sodium phosphate buffer in the p^2H range 5–6 was injected, at 13 ml/hr, 35–40 ml of diethyl ether containing 70 mg of egg yolk phosphatidylcholine, 7 mg of phosphatidic acid, and 1 ml of methanol. After completion of the ether injection, the vesicles were concentrated ≈2-fold by ultrafiltration (Millipore ultrafiltration apparatus and Pellicon PSED molecular filters). Exchange of external

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: 5-HT, 5-hydroxytryptamine.

* To whom reprint requests should be addressed.

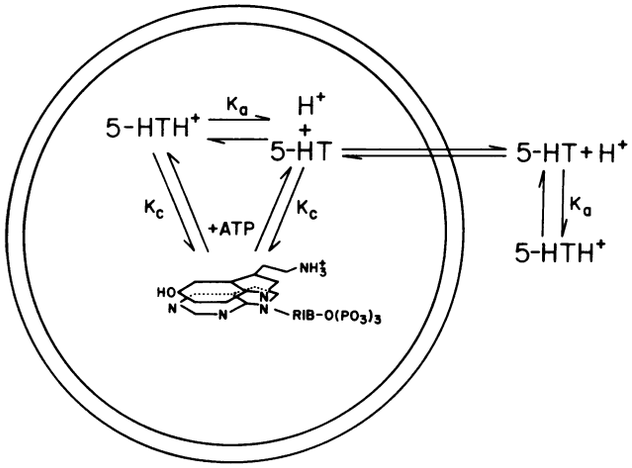

FIG. 1. Diagram of a vesicle system containing ATP and 5-HT. Typical initial concentrations are 0.025 M ATP in 0.06 M phosphate buffer at pH 5.8 on the inside and 0.08 M phosphate buffer at pH 8.0 on the outside of the vesicle.

buffer for an ATP-free solution was accomplished by dialyzing 2.5 ml of the vesicle concentrate for 24 hr against an osmotically matched sodium phosphate buffer in the p^2H range 7–8 (four 15-ml changes). Because both ATP and phosphate are membrane impermeable under these conditions, the fractional entrapped volume was determined by measuring the apparent ATP concentrations of (*i*) the vesicle preparation before ultrafiltration, (*ii*) the final vesicle suspension after dialysis, and (*iii*) the final dialysate. The ATP concentrations were determined from the absorbance at 257 nm in a Bausch and Lomb Shimadzu Spectronic 200 UV spectrophotometer. Entrapped volumes ranged from 25% to 40% of the total sample volume. Internal and external p^2H values were checked after preparation by using the well-established relationship between ^{31}P chemical shifts and pH (10). In all cases they were within 0.1 pH unit of preparation conditions.

Total lipid in the final sample was determined by using phosphate analysis after rupture of vesicles and removal of buffers by dialysis. The method used was that of Yang *et al.* (11). Molar quantities of lipids and an area of 70 $Å^2$ per lipid were used to estimate the bilayer area in the sample. Bilayer areas along with 25–40% entrapped volume suggest that the vesicles would vary in size from 2100 to 2900 Å if membranes were unilamellar. This size range is consistent with electron microscopy and light scattering measurements on similar preparations (2400 ± 1000 Å) (unpublished data).

5-HT oxalate, obtained from Sigma, was made oxalate free by precipitation and separation of the oxalate by addition of a stoichiometric amount of $CaCl_2$ at pH 7 followed by lyophilization of the solution. A 0.015 M stock solution of this oxalate free 5-HT was prepared in the final phosphate buffer dialysate.

In a typical experiment, 0.24 ml of the vesicle suspension and 0.24 ml of the 5-HT stock solution were injected into an NMR tube within the NMR spectrometer by using a stopped-flow apparatus at an ambient temperature of 20 ± 2°C (12). Successive NMR spectra were accumulated and stored on a magnetic disk in an automated fashion by using a Bruker HX270 NMR spectrometer operating in the pulsed Fourier transform mode with quadature detection at 270 MHz. Each spectrum resulted from 100 scans of 4096 data points, a 90° pulse, and a 3000-Hz sweep-width. Areas of the internal and external 5-HT resonances were analyzed by cutting and weighing the peaks. Usually the external and internal resonances for H_4 and H_6 could be resolved. Areas presented are the averages of these measurements and are reproducible to 6%. T_1 values for internal and external 5-HT were found to be approximately 0.6 sec; therefore no corrections for partial saturation were made.

RESULTS

The general features of the ^1H NMR spectrum of vesicle systems are illustrated in Fig. 2. The high-field region of the spectrum is dominated by the resonances of the phospholipids which effectively obscure any resonances of low-concentration solutes in the region. By contrast, the low-field spectral region is free of lipid resonances, and the aromatic resonances of ATP and 5-HT are easily observable.

Fig. 3 shows a set of ^1H NMR spectra of the 5-HT aromatic protons as a function of time after injection of the vesicles with

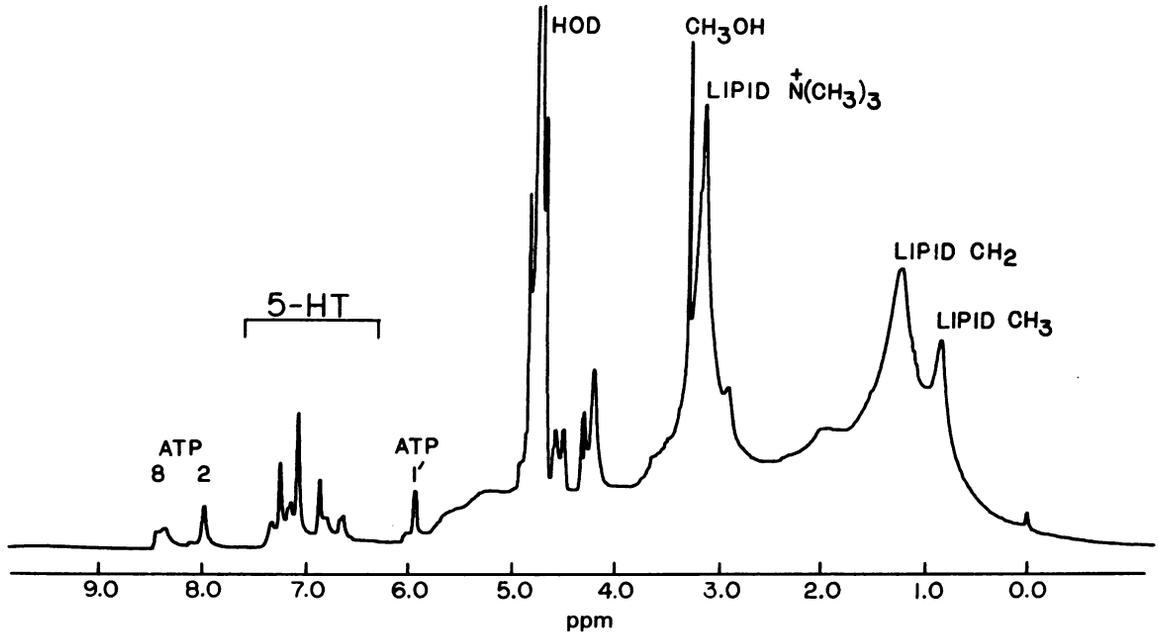

FIG. 2. Proton NMR spectrum (270 MHz) of a vesicle preparation containing ATP and 5-HT. The reference was sodium 3-(trimethylsilyl)-propanesulfonate (DSS).

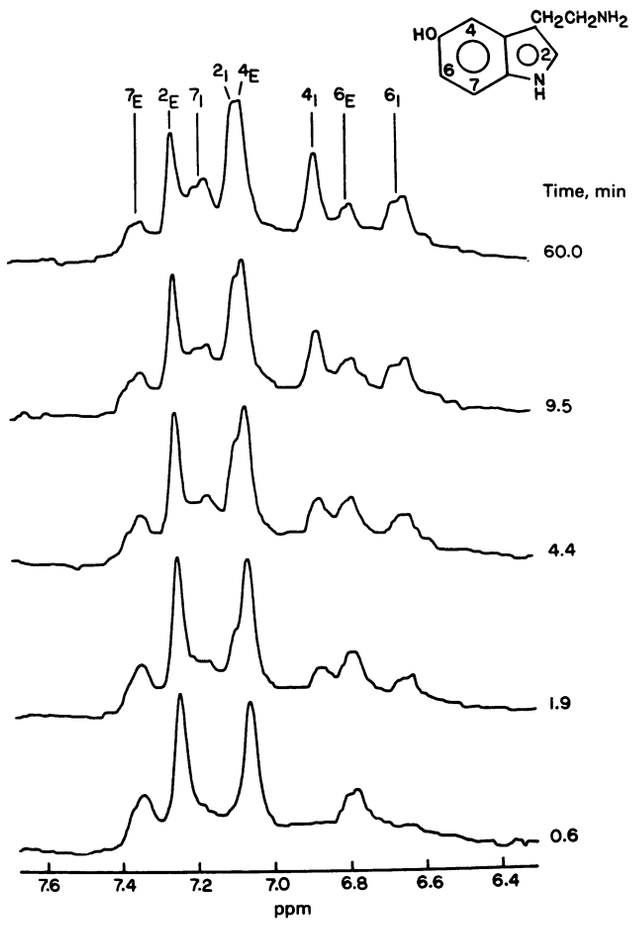

FIG. 3. Time evolution of the aromatic spectral region of a vesicle preparation with ATP initially inside the vesicles and 5-HT outside the vesicles. Resonances are assigned by using the numbering system indicated in the *Inset*. Subscripts: E, external to the vesicle membrane; I, internal to the vesicle membrane.

5-HT. The initial 5-HT concentration was 8.75 mM, the external p^2H was 8.1, and the internal p^2H was 5.8. At time 0, only one set of four resonances was observed with chemical shifts the same as those of 5-HT in the absence of ATP. These resonances are obviously from external 5-HT. They can be assigned to individual protons as indicated by using spin-spin coupling information apparent in higher-resolution spectra. As time progressed, a second set of four resonances appeared ≈0.2 ppm upfield of the initial external resonances. These grew in intensity as the intensities of the external resonances decreased. The upfield positions of the resonances are consistent with association of ATP and 5-HT in ring stacked molecular complexes (13). The aromatic adenine moiety of ATP has a high anisotropic susceptibility which shifts the 5-HT resonances upfield of their normal positions. Thus, the second set of resonances is due to internal 5-HT.

Integration of the internal 5-HT resonances for each spectrum results in a quantitative measure of the number of molecules transported. A plot of the data as a function of time along with theoretical predictions will be presented later.

In Fig. 3, transport is seen to occur with a half-time of approximately 3 min. It is also significant that accumulation plateaus after 12 min with an internal-to-external 5-HT ratio of 1.5:1. Given an internal volume % of 14%, this corresponds to a 9-fold concentration excess on the inside of the vesicle.

Because concentration of amine likely was driven by the pH gradient, it is reasonable to ask if the pH gradient is depleted

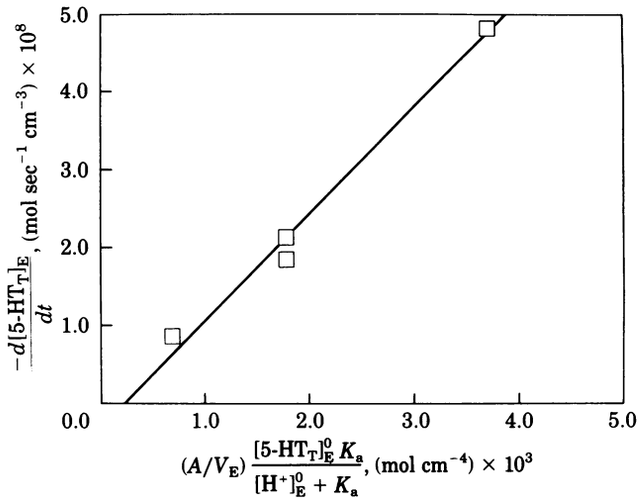

FIG. 4. Initial rates of 5-HT transport as a function of neutral amine concentration.

to any extent. External p^2H, easily measured by a glass electrode, was found to decrease slightly to 7.8. The internal pH can be measured by the relationships between ^{31}P chemical shifts of ATP or inorganic phosphate and p^2H. The final internal p^2H was found to have risen to 6.7.

DISCUSSION

The observation of two distinct sets of 5-HT resonances and their time evolution as shown in Fig. 3 are consistent with 5-HT diffusing through the intact membrane from the exterior to the interior of the vesicle. Concentration of an amine on the side of the membrane lowest in pH is characteristic of a system in which the neutral molecule is the only species transported (14). Under these circumstances, a pseudo-equilibrium should be reached in which the neutral species are at approximately equal concentrations on the two sides of the membrane. If the pH differential is known along with the pK_a of the amine, total amine concentrations on each side should reach specific values. Using a pK_a of 10 estimated from NMR titrations and measured pH differentials, one would predict a concentration ratio of 12. This is in reasonable agreement with the concentrations shown in the plateau region of Fig. 3 and supports selective transport of the neutral species.

Permeability coefficients are most easily extracted from initial rates. The selective transport of the neutral species leads to a specific dependence as indicated:

$$\frac{d[5\text{-HT}_T]_E}{dt} = -P\frac{A}{V_E}\frac{[5\text{-HT}_T]^0_E K_a}{[H^+]^0_E + K_a}. \quad [1]$$

In this equation, P is the neutral 5-HT permeability coefficient, A is the membrane area, V_E is the external volume, $[5\text{-HT}_T]^0_E$ is the initial total 5-HT concentration, $[H^+]^0_E$ is the initial $^2H^+$ concentration, and K_a is the amine dissociation constant. Fig. 4 is a plot of the initial rates of $[5\text{-HT}_T]_E$ change vs. $(A/V_E)[5\text{-HT}_T]^0_E K_a/([H^+]^0_E + K_a)$. The plot is linear and also supports the predominate transport of neutral 5-HT. The slope of the line gives the value of the neutral 5-HT permeability coefficient as $P = 1.4 \times 10^{-5}$ cm/sec.

The entire time evolution of the 5-HT concentrations from time 0 to equilibrium can also be described. The theoretical expressions to be derived describe the time evolution of four species: the internal and external total 5-HT concentrations and the internal and external $^2H^+$ concentrations. Under the as-

sumption that only neutral 5-HT diffuses through the membrane, a set of four coupled differential equations result:

$$\frac{d[5\text{-HT}_T]_E}{dt} = -P\frac{A}{V_E}([5\text{-HT}]_E - [5\text{-HT}]_I), \quad [2]$$

$$\frac{d[5\text{-HT}_T]_I}{dt} = P\frac{A}{V_E}([5\text{-HT}]_E - [5\text{-HT}]_I), \quad [3]$$

$$\frac{d[H^+]_E}{dt} = -\frac{d[5\text{-HTH}^+]_E}{dt} + \frac{d[HPO_4^{2-}]_E}{dt}, \text{ and} \quad [4]$$

$$\frac{d[H^+]_I}{dt} = -\frac{d[5\text{-HTH}^+]_I}{dt} + \frac{d[HPO_4^{2-}]_I}{dt} + \frac{d[ATP^{4-}]_I}{dt}. \quad [5]$$

Subscripts E and I denote external and internal quantities, respectively; [5-HT] is the neutral 5-HT concentration; and [5-HTH^+] is the protonated 5-HT concentration. These equations can be reduced to four variables by using equilibrium constant expressions for 5-HT, ATP, and phosphate.

The differential equations were solved numerically by using a Runga–Kutta integration routine with the initial 5-HT, $^2H^+$, ATP, and phosphate concentrations and the fraction internal volume. A least-squares best fit to the experimental data was obtained by using the neutral 5-HT permeability coefficient as the variable quantity. Fig. 5 shows the data for three different pH ranges with their respective best fit curves superimposed. In all cases the curves fit the data within experimental error. The neutral 5-HT permeability coefficient obtained from the best fit curves was $P = 1.3 \pm 0.4 \times 10^{-5}$ cm/sec and was the same as the value obtained from the initial rates.

Despite the rather high precision of the permeability coefficient for a given membrane preparation, we must point out that these are membrane-dependent quantities. The ether injection vesicles contain a low level of phosphatidic acid, they exhibit a small degree of multilamellarity, and the rather high permeability coefficients found for proton-hydroxyl and sodium for these vesicles have raised questions about the uniqueness of the preparation. Arguments in favor of the system as a model, however, can be made (15) and the permeability coefficient measured for 5-HT falls within the range expected for a molecule of this structure. Transport of the catecholamine epinephrine, measured recently by chemical means in small sonicated lipid vesicles, gives a value of $2.7 \pm 1.5 \times 10^{-6}$ cm/sec (J. A. Cramer, personal communication). This slightly smaller value can be rationalized on the basis of the greater hydrophobicity of 5-HT.

Perhaps more significant than the determination of a specific permeability coefficient is the illustration of the potential of NMR methods for the study of transport in closed membrane systems. The two major difficulties in studying vesicular systems, both artificial and natural, are (*i*) observing vesicular contents and (*ii*) distinguishing internal solutes from their external counterparts. As illustrated in Fig. 2, it has been clearly demonstrated that NMR can be used to observe the contents of

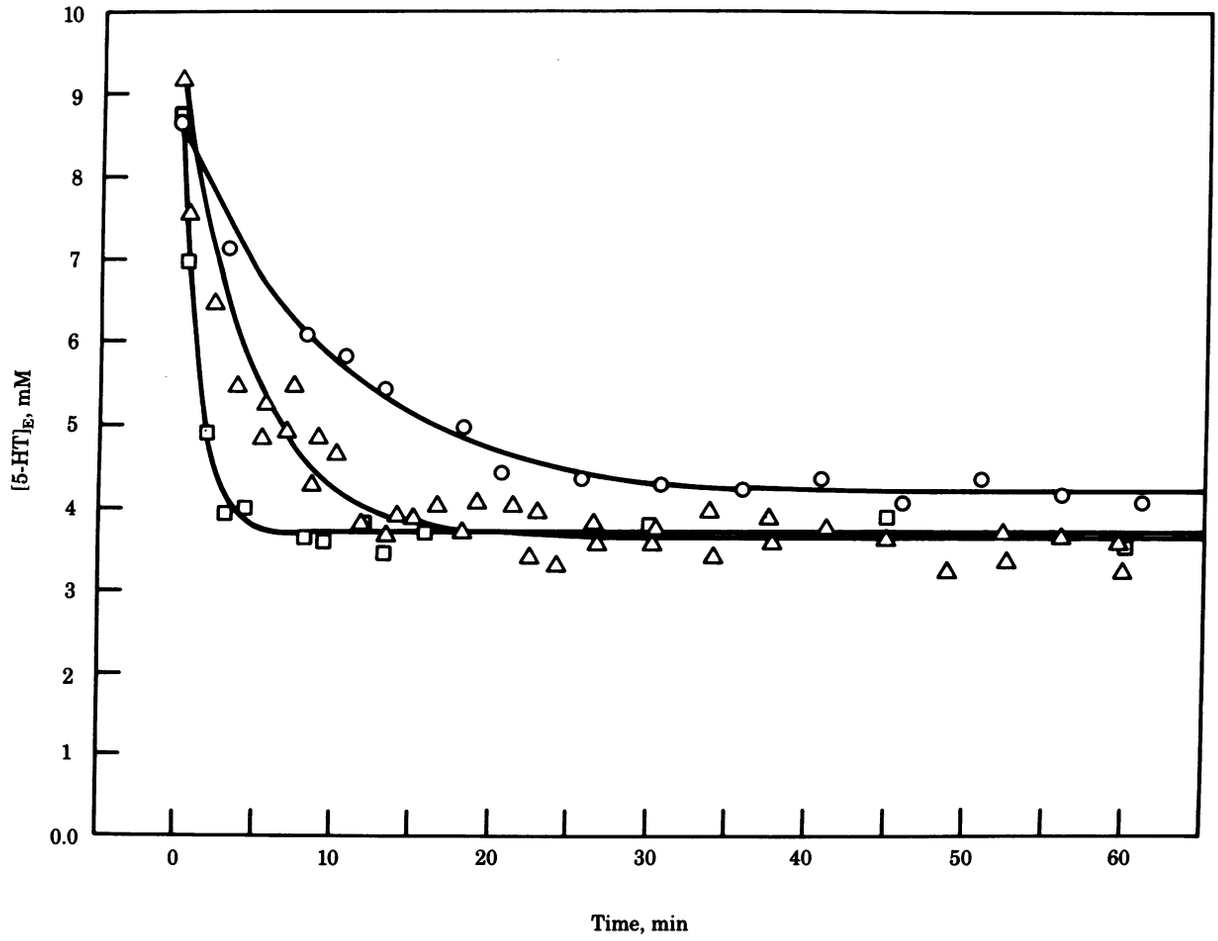

FIG. 5. Time evolution of external 5-HT concentration at various initial pH conditions. □, Internal pH 5.8 and external pH 8.1; △, internal pH 5.4 and external pH 7.6; ○, internal pH 5.2 and external pH 7.3. Solid lines are best fit theoretical curves.

vesicles if the appropriate spectral region or magnetic nuclei are examined. Likewise, as demonstrated in Fig. 3, resolution of internal solutes from external solutes can be achieved by utilizing the different environments on the two sides of the membrane to achieve differences in chemical shifts. In the experiments described here, the natural propensity of the internal ATP to associate with 5-HT and the accompanying ring current shifts have been used to resolve the internal 5-HT resonances from the external resonances. This resolution allowed continuous examination of both the internal and the external amine without physical separation of the contents or disruption of the membrane.

We thank M.-J. Liao for assistance with kinetic data and S. Sigal for assistance with light scattering data. This work was supported by Grant GM19035 from the National Institute of General Medical Sciences and benefited from light scattering apparatus made available by Grant CHE 78-02922 from the National Science Foundation.

1. Macey, R. I. (1979) in *Membrane Transport in Biology*, eds. Giebish, G., Tosteson, D. C. & Ussing, H. H. (Springer, New York), Vol. 2, pp. 1–54.
2. Lassen, U. V. & Rasmussen, B. E. (1978) in *Membrane Transport in Biology*, eds. Giebish, G., Tosteson, D. C. & Ussing, H. H. (Springer, New York), Vol. 1, pp. 169–202.
3. Johnson, R. G., Scarpa, A. & Salganicoff, L. (1978) *J. Biol. Chem.* **253,** 7061–7068.
4. Berneis, K. H., DaPrada, M. & Pletscher, A. (1969) *Science* **165,** 913–914.
5. Granot, J. (1978) *J. Am. Chem. Soc.* **100,** 1539–1548.
6. Wilkins, J. A., Greenwalt, J. W. & Haung, L. (1978) *J. Biol. Chem.* **253,** 6260–6265.
7. Pletscher, A., DaPrada, M., Berneis, K. H., Steffen, H., Tutold, B. & Weber, H. G. (1974) in *Advances in Cytopharmacology*, eds. Ceccaelli, B., Clementi, F. & Meldolesi, J. (Raven, New York), pp. 257–264.
8. Johnson, R. G., Pfister, D., Carty, S. E. & Scarpa, A. (1979) *J. Biol. Chem.* **254,** 10963–10972.
9. Deamer, D. & Bangham, A. D. (1976) *Biochim. Biophys. Acta* **433,** 629–634.
10. Casey, R. P., Njus, D., Radda, G. K. & Sehr, P. A. (1977) *Biochemistry* **16,** 972–976.
11. Yang, S. F., Freer, S. & Benson, A. A. (1967) *J. Biol. Chem.* **242,** 477–484.
12. Liao, M.-J. & Prestegard, J. H. (1980) *Biochim. Biophys. Acta* **599,** 81–94.
13. Nogrady, T., Hrdina, P. D. & Ling, G. M. (1972) *Mol. Pharmacol.* **8,** 565–574.
14. Prestegard, J. H., Cramer, J. A. & Viscio, D. B. (1979) *Biophys. J.* **26,** 575–584.
15. Nichols, J. W. & Deamer, D. W. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2038–2042.